MOLECULAR PATHOGENESIS OF RARE ANEMIAS – ERYTHROENZYMOPATHIES

Ph.D. Thesis

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DECLARATION/PROHLÁŠENÍ:

I hereby declare that this Ph.D. thesis entitled "MOLECULAR PATHOGENESIS OF RARE ANEMIAS – ERYTHROENZYMOPATHIES" was carried out by me under the guidance and supervision of Renáta Mojzíková, Ph.D., and all used literature is cited accordingly.

Tímto prohlašuji, že předloženou práci s názvem "MOLEKULÁRNÍ PATHOGENEZE VZÁCNÝCH ANÉMIÍ – ENZYMOPATIÍ" jsem vypracovala samostatně pod vedením školitelky Mgr. Renáty Mojzíkové, Ph.D., a s použitím citované literatury.

ABSTRACT:

The aim of this research is to make a contribution to the molecular pathogenesis of erythroenzymopathies, rare hereditary disorders causing nonspherocytic hemolytic anemia. This work represents an advanced and up-to-date knowledge in the field of red blood cells enzyme deficiencies in the Czech and Slovak populations since the nineteen-eighties when mutations in Czech and Slovak subjects resulting in glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiencies were described. Both deficiencies are the most common erythroenzymopathies worldwide. Since 2013, our laboratory has focused on the introduction of direct enzyme assays followed by genetic testing in patients with hemolytic anemia with suspected erythroenzymopathy. To date, several different enzyme defects were diagnosed in twenty-four patients. Except G6PD and PK deficiencies (9 and 12 cases), 2 families with glucose phosphate isomerase (GPI) defect and 1 family with very rare hexokinase (HK) deficiency have been identified. The last two deficiencies were diagnosed in the Czech and Slovak populations for the first time. Among the 22 identified mutations, 5 of them, namely G6PD p.(Phe216Tyr), PK p.(Arg518Leufs*12), p.(Asp293Val) and GPI p.(Ser160Pro), p.(Arg472Cys) were novel and have not been previously reported in literature.

Furthermore, this thesis describes a pathogenic mechanism of several novel mutations responsible for deficiencies of hexokinase (HK), phosphoglycerate kinase (PGK) and phosphofructokinase (PFK) in more detail. HK deficiency is a very rare disorder that has been rarely studied at molecular level. In collaboration with other research centers, the largest cohort of HK deficient patients was selected and extensively studied. Using molecular techniques and *in silico* analyses, potential pathogenic mechanisms of novel mutations p.(His867Tyr), p.(Thr600Met), c.873-2A>G and c. 493-1G>A have been elucidated. Further, we contributed to the identification and characterization of a novel intronic mutation c.756+3A>G in *PGK1* in two brothers with PGK deficiency. Although PGK deficiency is mostly associated with hemolytic anemia and neurological impairment, muscle weakness and myopathy were the only clinical features in these cases. Finally, we contributed to the detection of a novel mutation p.(Asp309Gly) in *PFKM* gene, the first case of a rare PFK deficiency diagnosed in a 65-year-old female of Spanish origin.

Characterization of erythroenzymopathies by biochemical and molecular analyses can contribute to a better understanding of pathophysiology of this rare disorder. Furthermore, the direct enzyme assays may have a wide variety of scientific/diagnostic applications; it may improve diagnostic testing of other disorders or it may be useful to study the mechanism of drug's actions.

ABSTRAKT:

Tato práce se zabývá studiem molekulární patogeneze enzymopatií - vzácného onemocnění způsobující vrozenou nesférocytární hemolytickou anémii. Deficit glukosa-6-fosfátdehydrogenasy (GPD) a pyruvátkinasy (PK) byl v české a slovenské populaci popsán již v 80. letech. Nicméně až tato studie představuje zatím chybějící ucelený přehled o výskytu tohoto onemocnění v české a slovenské populaci, zvláště pak jeho vzácnější formy. Od roku 2013 se naše laboratoř zabývá metodou přímého stanovení enzymů s následným genetickým vyšetřením u pacientů se suspektní enzymopatií. V současné době jsme jedinou specializovanou laboratoří zabývající se touto diagnostikou v České republice resp. ve střední Evropě. Doposud byl enzymový deficit diagnostikován u dvaceti čtyř pacientů. Vedle nejčastějších deficitů G6PD a PK (9 a 12 případů), byly identifikovány 2 rodiny s deficitem glukosafosfátisomerasy (GPI) a 1 rodina s velmi vzácným deficitem hexokinasy (HK). Deficit GPI a HK byl v české a slovenské populací diagnostikován poprvé. Celkově bylo identifikováno dvacet dva kauzálních mutací, z nichž pět nebylo doposud v literatuře popsáno - G6PD p.(Phe216Tyr), PK p.(Arg518Leufs * 12), p.(Asp293Val), GPI p.(Ser160Pro) a p.(Arg472Cys).

Současně tato práce studuje patogenní mechanismy vybraných mutací vedoucí k deficitu HK, fosfoglycerátkinasy (PGK) a fosfofruktokinasy (PFK). Deficit HK byl pouze výjimečně studován na molekulární úrovni. Na základě zahraniční spolupráce jsme vytvořili a detailněji prostudovali největší soubor pacientů s tímto deficitem. Patogenní mechanismus kauzálních mutací p.(His867Tyr), p.(Thr600Met), c.873-2A>G a c.493-1G>A byl objasněn pomocí molekulárních technik a *in silico* analýz. Dále jsme identifikovali a charakterizovali novou intronovou mutaci c.756+3A>G v genu *PGK1* vedoucí k PGK deficitu. Ve většině případů je deficit PGK spojen s hemolytickou anémií a neurologickými poruchami. Avšak v tomto případě byla svalová slabost a myopatie jediným klinickým příznakem. Dále jsme u pacientky španělského původu identifikovali a charakterizovali novou mutaci p.(Asp309Gly) v genu kódující PFK. Jedná se o první případ PFK deficitu ve Španělsku.

Charakterizace erytroenzymopatií pomocí biochemických a molekulárních analýz může přispět k porozumění patofyziologie tohoto vzácného onemocnění. Navíc metoda přímého stanovení erytrocytárních enzymů může vést ke zlepšení diagnostiky jiných onemocnění či je možné ji využít při studiu farmakologických sloučenin.

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1 INTRODUCTION

A major part of the theoretical background related to erythroenzymopathies was quoted verbatim from my first-authored publication **“Rare hereditary red blood cell enzymopathies associated with hemolytic anemia – pathophysiology, clinical aspects, and laboratory diagnosis”** (Koralkova et al., 2014). Several sections were expanded and updated to complete current knowledge. All references were cited accordingly.

1.1 ERYTHROPOIESIS

Erythropoiesis is a highly regulated process characterized by proliferation and progressive maturation of hematopoietic stem cells through lineage–committed progenitors and erythroblast precursors to mature enucleated red blood cells (RBCs). Erythroid progenitors emerge from two distinct sources during the mammalian development. The early erythropoiesis, primitive erythropoiesis, occurs in “blood islands” within the yolk sack. Primitive erythroblasts arise from mesodermal cells and provide a transient pool of unique erythroid progenitors (EryP–CFC) (Palis, 2014; Palis et al., 2010). Definitive erythropoiesis takes place in fetal liver and postnatal bone marrow. Burst-forming unit erythroid (BFU–E) and colony-forming unit erythroid (CFU–E) are the most immature erythroid progenitors. They further progress to nucleated pro-erythroblast, basophilic, polychromatophilic and orthochromatic erythroblast. The erythroid maturation results in enucleation and reticulocytes formation (Figure 1). Whereas reticulocytes undergo rapid maturation, the extruded nuclei in form of pyrenocytes are engulfed by macrophages (Migliaccio, 2010; Palis, 2014). The erythroid differentiation is regulated at multiple levels by many hormones/cytokines, transcription factors and nutritional supply. The most important cytokine of erythropoiesis is erythropoetin (EPO). At early stages of erythroid differentiation (CFU–E stage to basophilic pro-erythroblasts), EPO induces erythropoiesis by promoting proliferation and inhibiting apoptosis. Several transcription factors, such as GATA–1 (GATA–binding factor 1), KLF1 (Krüppel like factor 1) or NF–E2 (nuclear factor-erythroid-derived 2), are implicated in erythroid specific gene expression (Chateauvieux et al., 2011; Tsiftsoglou et al., 2009). Furthermore, many nutritions, such as folate, vitamin B_{12} and iron, are important for hemoglobin synthesis and normal RBC maturation (Koury and Ponka, 2004).

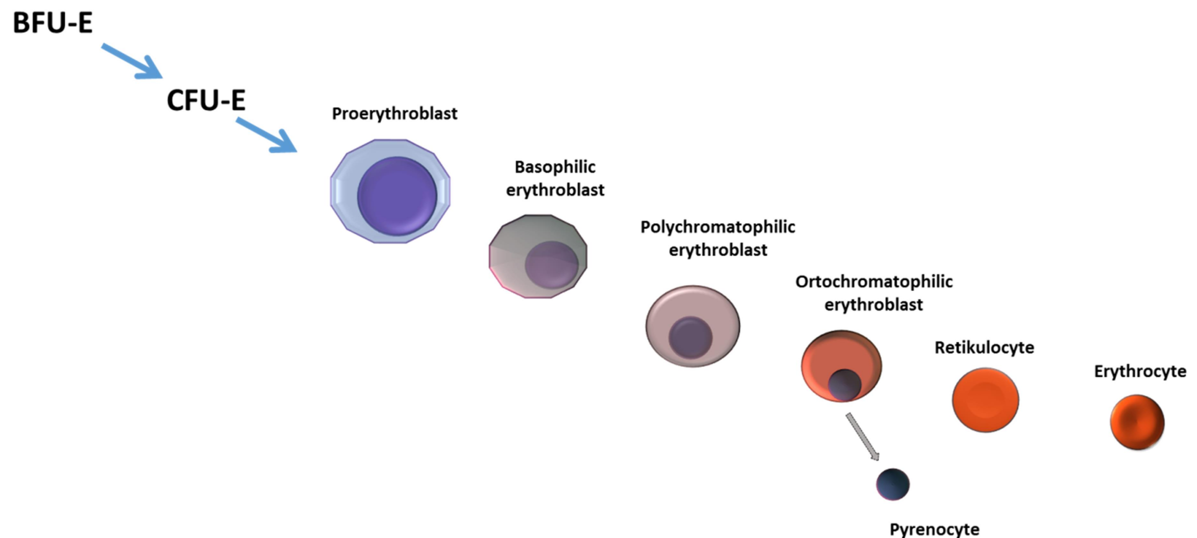

Figure 1: A schematic overview of different erythroid differentiation stages. *Differentiation of the most immature progenitors - burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) to mature erythrocyte (Koury and Haase, 2015).*

1.2 THE RED BLOOD CELL METABOLISM

During maturation RBCs lose their nucleus, mitochondria and other organelles. Thus, they are unable to conduct oxidative phosphorylation and protein/lipid synthesis. Despite this limited metabolic machinery, RBCs are able to fully perform their functional role which mainly involves the transport and delivery of oxygen to tissue. Metabolic pathways involved in cellular function and survival comprise anaerobic glycolysis, hexose monophosphate shunt, glutathione metabolism and nucleotide salvage pathway (Arya et al., 1995; Jacobasch and Rapoport, 1996; Prchal and Gregg, 2005; van Wijk and van Solinge, 2010). These pathways serve to maintain (1) the integrity and flexibility of the RBC membrane, (2) hemoglobin in its reduced state, (3) the redox balance of the glutathione pool, and (4) the adenine pool. The simplified RBC metabolism is shown in a schematic overview (Figure 2).

Anaerobic glycolysis, also known as the Emden-Mayerhof pathway, is the sole source of energy for the RBC. Glucose is metabolized in a multi-step process resulting in the production of pyruvate/lactate, ATP and NADH. ATP drives proper function of K^+/Na^+ pumps which are involved in maintenance of flexibility and integrity of the red blood cell membrane. NADH is a cofactor of NADH-cytochrome b5 reductase, playing a key role in the reduction of oxidized hemoglobin. The Rapoport–Luebering shunt is unique to the red cell. This bypass step of glycolysis serves mainly the production of 2,3-bisphosphoglycerate which together with ATP regulates the affinity of oxygen to hemoglobin (Rapoport and Luebering, 1950). Under standard physiological conditions 90% of glucose is metabolized through anaerobic glycolysis to produce ATP. The remaining amount of glucose is metabolized through the hexose monophosphate shunt whose

main function is the production of redox potential in the form of NADPH. It is the only way of NADPH formation in RBC. NADPH acts as a cofactor for glutathione reductase which maintains glutathione in its reduced state, thereby provides protection against oxidative stress (van Zwieten et al., 2014). RBC nucleotide metabolism contributes to maintaining the energy balance in erythrocytes. Nucleotide content in mature erythrocytes is predominantly regulated by the intracellular purine metabolic cycle (Dudzinska et al., 2006). Pyrimidines are degraded by pyrimidine–5'–nucleotidase during reticulocyte maturation and are present only as trace amounts in the mature RBC. In contrast, adenine derivatives (AMP, ADP, ATP) represent about 97% of the total nucleotide pool (Valentine and Paglia, 1980a). Because mature RBCs lack phosphoribosyl–1–pyrophosphate, an essential enzyme for *de novo* synthesis of purines, purine derivatives are reconstituted from intermediates of purine catabolism. This mechanism is known as a salvage pathway (Dudzinska et al., 2006).

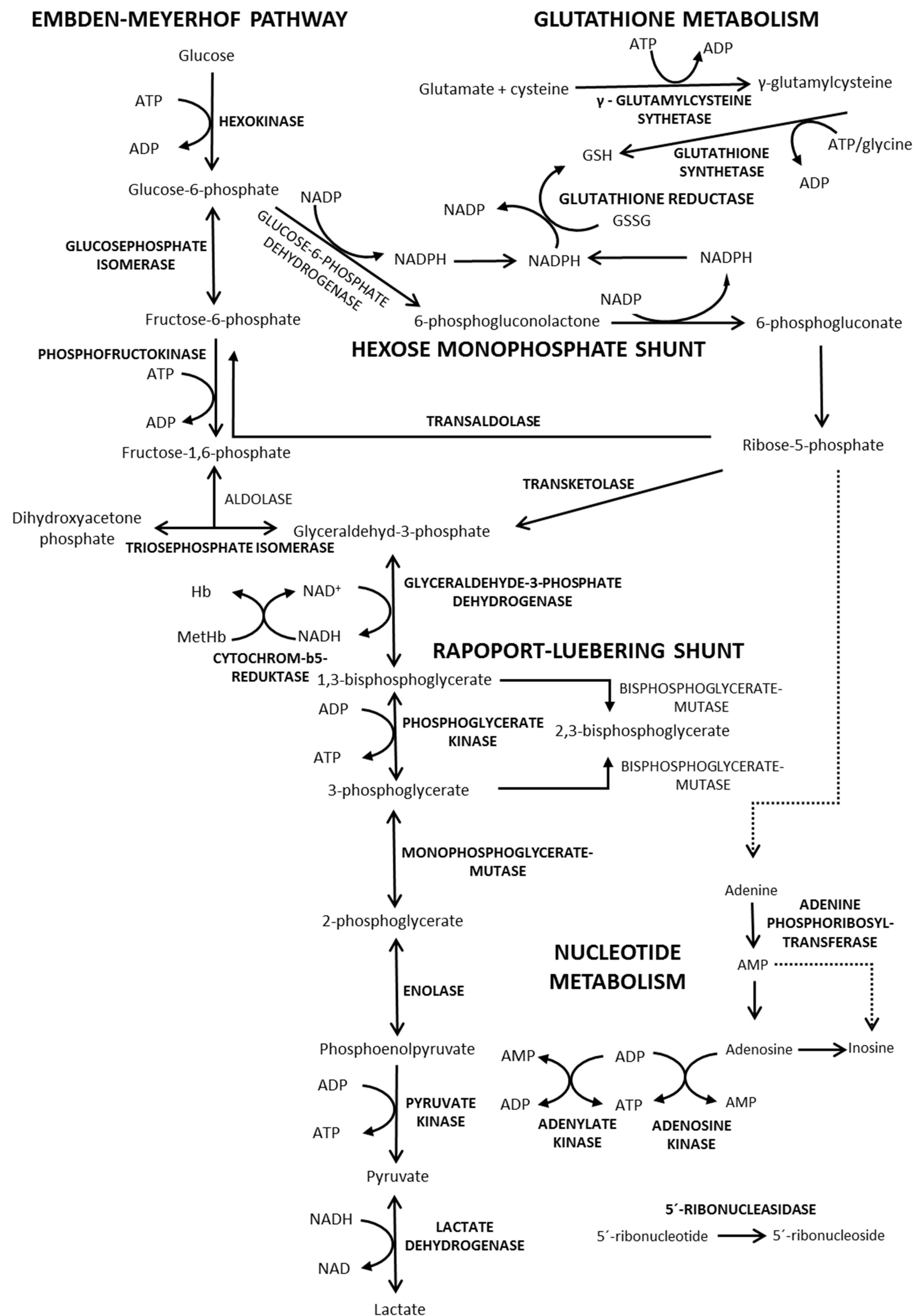

Figure 2: A schematic overview of red blood cell metabolic pathways. *Hb – hemoglobin, MetHb – methemoglobin, GSH – reduced glutathione, GSSG – oxidized glutathione*

1.3 ANEMIA

Anemia, the most common blood disorder, is the major source of morbidity and mortality worldwide (Sankaran and Weiss, 2015). Anemia is characterized by a reduced red blood cell count mostly resulting in a decrease of hemoglobin (Hb) or hematocrit (Htc) under the control range depending on the age and the human race (white population: men – Hb: 135 g/L, Htc: 38.8; women – Hb: 120 g/L, Htc – 34.9) (Brugnara, 2014). Anemia can be classified according to multiple attributes – red blood cell mass, red blood cell morphology, pathophysiology etc. Based on the red blood cell mass, anemia is classified as absolute and relative. Absolute anemia may result from (1) decreased red cell production, (2) increased red cell destruction or (3) blood loss and blood redistribution. Relative anemia reflects increased plasma volume which is typically observed during pregnancy or macroglobulinemia. The detailed classification of anemia is listed in Table 1 (Prchal, 2015).

Table 1: Classification of anemia *(based on (Prchal, 2015))*

ABSOLUTE ANEMIA	
A. Decreased red cell production	
1. Acquired	2. Hereditary
a) Pluripotential hematopoietic stem cell failure	a) Pluripotential hematopoietic stem cell failure
b) Erythroid progenitor cell failure	b) Erythroid progenitor cell failure
c) Functional progenitor impairment due to nutritional and other causes	c) Functional progenitor impairment due to nutritional and other causes
B. Increased red cell destruction (Hemolytic anemias)	
1. Acquired	2. Hereditary
a) Mechanical	a) Hemoglobinopathies
b) Antibody mediated	b) Red cell membrane disorders
c) Hypersplenism	c) Red cell enzyme defects
d) Red cell membrane disorders	
C. Blood loss and blood redistribution	
RELATIVE ANEMIA	
a) Macroglobulinemia	
b) Pregnancy	
c) Athletes	
d) Postflight astronauts	

1.3.1 HEMOLYTIC ANEMIA

The survival of RBCs in the circulation is approximately 115 days (Franco, 2012). During microcirculation and aging, RBCs decrease cell volume, surface charge and metabolic activity and increase cell density. These changes affect cell deformability (phosphatidylserine externalization, CD47 and sialic acid decrease etc.), which further leads to the destruction of RBCs by macrophages (erythrophagocytosis) mainly in the spleen and also in the liver. Under physiological conditions, the rate between erythropoiesis and hemolysis is maintained in the balance (Huang et al., 2011).

Hemolytic anemia (HA) is a group of heterogeneous disorders characterized by a premature destruction and removal of RBCs from bloodstream. RBCs can be destroyed by two distinct mechanisms. Intravascular hemolysis, the less common mechanism, is associated with the direct destruction of RBCs in the circulation releasing hemoglobin into the plasma. In case of extravascular hemolysis, RBCs are phagocytized by macrophages in the spleen and liver. The causes of HA can be extrinsic (mechanical stress, complement system activation or various infections) or intrinsic (defects of Hb synthesis, abnormal membrane composition or defected RBC metabolism). While the extrinsic causes are mostly acquired, the intrinsic factors are mainly associated with inherited defects (Dhaliwal et al., 2004).

The increased RBC destruction leads to an extensive breakdown of hemoglobin resulting in unconjugated hyperbilirubinemia clinically apparent as jaundice, increased lactate dehydrogenase (cellular destruction), reticulocytosis or cholelithiasis. Beside this, decreased levels of plasma haptoglobin, a marker of RBC destruction, are evidenced regardless the site of hemolysis (intravascular or extravascular) (Barcellini and Fattizzo, 2015; Ruiz and Cervantes, 2015). HA can be present as chronic or acute; hemolytic crisis is observed in several cases. Clinical severity of HA greatly differs among patients. Some patients may exhibit well–compensated HA with no clinical signs of hemolysis; others show life-threatening HA hardly controlled by RBC transfusions. The topic of this thesis focuses on the inherited causes of HA. Thus, only these disorders will be further discussed according to the interest – especially the red blood cells enzymopathies as the main subject of the thesis and the membrane defects on which I focused during my doctoral study as well.

1.3.1.1 HEMOGLOBINOPATHIES

Hemoglobin, the most abundant protein inside the RBC, has multiple biological functions. The major task of hemoglobin is to provide oxygen transport from lungs to tissues and cells (Giardina et al., 1995). A hemoglobin molecule is a tetramer composed of a prosthetic heme group with a central iron (Fe^{2+}) and two distinct pairs of globin chains (α, β, γ, δ, ζ, ε). The synthesis of globin chains is controlled by separate α–like and β–like globin gene clusters located on human chromosomes 16

and 11, respectively (Thom et al., 2013). The proportion of individual globin chains in a hemoglobin molecule differs with prenatal gestation time and postnatal age (Forget and Bunn, 2013). The overall composition of hemoglobin in adults is Hb A_1 (95–98%), Hb A_2 (2–3%) and Hb F (<1%) (Brugnara, 2014).

Up to date, over 1000 globin mutations associated with aberrant hemoglobin synthesis and/or structure/function have been reported. Hemoglobin disorders can be divided into two major groups 1) thalassemia syndromes and 2) structural hemoglobin variants. Thalassemia syndromes are characterized by an imbalance in production of globin chains, thus, are referred as quantitative defects. The most common thalassemia syndromes are α- and β-thalassemias, however, defects in delta (δ)– or gamma (γ)–globin chains have been described. Although majority of these defects cause inherited autosomal recessive disorders, several patients exhibit acquired form of α-thalassemia in association with myelodysplastic syndrome (Forget and Bunn, 2013). Several families with thalassemia syndromes have been previously reported in the Czech and Slovak populations (Indrak et al., 1992). Recent molecular–genetic screening of patients from the Czech Republic and the Slovak Republic revealed 29 β–thalassemia mutations in 356 heterozygotes from 218 unrelated families. Most of the mutations were of Mediterranean origin (82% of cases); five mutations have not been previously reported (Divoka et al., 2016a). In most cases the phenotype corresponds to β-thalassemia minor; rarely to β–thalassemia intermedia or major. In addition, deletions of variable size were identified in alpha-globin gene cluster of eighty Czech patients or immigrants living in the Czech Republic. Besides common Mediterranean and Asian deletions (especially -α3.7 and –SEA), large deletions encompassing entire alpha globin locus or only the HS-40 regulatory region were detected in several cases (Divoka et al., 2016b).

Structural hemoglobin variants are qualitative defects in which mutations predominantly alter hemoglobin structure and/or its biochemical properties, further affecting oxygen affinity and/or hemoglobin stability (Thom et al., 2013). The endemic hemoglobin variants HbS, HbC and HbE are among the most studied and frequent pathologies. In the **HbS variant** (β7 Glu–Val), deoxygenated HbS polymerizes inside the RBCs and decreases RBC deformability. It damages a RBC membrane cytoskeleton which accounts for the irreversibly sickled RBCs seen in the peripheral blood. Furthermore, sickle RBCs enable adhesive interactions with endothelial cells and together with other factors may result in vasoocclusions. The homozygous form of HbS is responsible for the most common and most severe variant of the sickle cell disease. On the other hand, heterozygosity for HbS has a protective effect to malaria parasitization of RBCs (Frenette and Atweh, 2007; Steinberg, 2008). The **HbE variant** (β26 Glu–Lys) is extremely frequent in Asian population (Fucharoen and Weatherall, 2012; Orkin et al., 1982). The mutation slightly affects oxygen affinity, thermal stability and sensitivity to oxidation stress. In addition, G to A substitution activates a cryptic splice site that further affects mRNA processing (Orkin et al., 1982). A homozygous form of HbE is associated with

mild anemia and marked morphological abnormalities. Both HbS and HbE variants have been detected in Czech population, but mainly in immigrants living in the Czech Republic (Divoky, 2005). The **HbC** (β7 Glu-Lys) variant forms insoluble crystals which in RBCs, however, do not polymerize as seen in HbS. Thus, while HbC crystals reduce red cell deformability, HbC itself cannot induce vasoocclusion. There are many other unstable Hb variants (Hb Boras, Hb Bristol, Hb Brockton, etc.) or hemoglobin M variants (HbM Boston, Hb Milwaukee-I). However, they are less prevalent in the populations (http://globin.cse.psu.edu/hbvar/menu.html) (Patrinos et al., 2004).

Although hemoglobin disorders predominantly occur in malaria areas, several families with structural hemoglobin variants have been detected in the Czech and Slovak populations. The presence of endemic variants is likely a consequence of migration (similarly to thalassemias). However, unstable variants or variants with altered oxygen affinity result from *de novo* mutations. Previously reported unstable hemoglobin variants – Hb Sydney, Hb Köln, Hb Nottingham, Hb Santa Ana, HbM-Saskatoon and Hb Saint Louis – have been identified among these families. Moreover, three unique Hb variants were characterized in three Czech families and were detaily studied (Divoky, 2005; Indrak et al., 1998). The first variant, unstable Hb Hradec Kralové, manifests as severe transfusion dependent HA. It is caused by an Ala-Asp substitution at position 115 in the β-globin chain resulting in high instability of the β-globin chain (Divoky et al., 1993). The second unstable Hb variant, Hb Haná, is a result of a His to Asn substitution at residue 63 in the β-globin chain. The clinical manifestation of this unstable Hb variant was worsened by partial glutathione reductase deficiency (Mojzikova et al., 2010). The third variant is caused by an Ala to Asp substitution in the β-globin chain leading to Hb Olomouc. Unlike the previous variants, the mutation does not affect the stability but it increases oxygen affinity resulting in erythrocytosis (Indrak et al., 1998; Indrak et al., 1987).

Affected individuals with mild anemia or compensated hemolysis do not require any treatment. In case of chronic hemolysis, supplementation of folic acid is recommended. Supportive therapy (blood transfusions) and chelation therapy to avoid the adverse effects of iron overload are required in severe hemolytic crisis.

High-performance liquid chromatographic methods and hemoglobin electrophoresis are commonly used as screening methods for diagnosis of thalassemias and hemoglobin variants (Ou and Rognerud, 2001). Direct sequencing is considered the golden standard to screen for all mutations including common, rare, and yet unknown mutations (Harteveld, 2014). However, many other molecular techniques have been developed to identify a pathological mutation causing hemoglobinopathies at DNA level. Multiplex Ligation-dependent Probe Amplification (MLPA) assays enable to detect large deletions or duplications (known or unknown) within α- and β-globin gene clusters (Gallienne et al., 2009). In endemic hemoglobinopathy regions or in isolated ethnic groups, detection of the most prevalent mutations may be performed using restriction enzyme digestion of PCR products or other

PCR-based techniques, such as amplification refractory mutation system (ARMS) or reverse dot-blotting (Harteveld, 2014).

1.3.1.2 RED BLOOD CELL MEMBRANE DISORDERS

RED BLOOD CELL MEMBRANE STRUCTURE

The biconcave shape, cytoplasmic viscosity and large membrane deformability enable RBCs to provide an adequate gas transport between blood and tissues (Mohandas and Gallagher, 2008). The majority of critical features of their biological function are mainly determined by the structural organization of the RBC membrane (Da Costa et al., 2013). The RBC membrane is composed of a lipid bilayer and a spectrin-based cytoskeleton. The lipid bilayer consists of two asymmetrical phospholipid leaflets and various membrane proteins which together act as a selective barrier for certain molecules and ions. In addition to the transport function, the membrane proteins are responsible for the mechanical capacity and antigenic properties of the RBC membrane. The inner cytoskeleton network, mainly composed of spectrin and actin, is connected to the lipid bilayer via the ankyrin and 4.1R complexes (King et al., 2015) (Figure 3). The precise transport regulations as well as structural organization of membrane proteins are essential for the maintenance of volume, biconcave shape, integrity and deformability of RBCs. Any imbalance or pathological alterations associated with a decrease or increase of a surface area and/or abnormal deformability may lead to premature removal of the RBC from bloodstream (Mohandas and Gallagher, 2008).

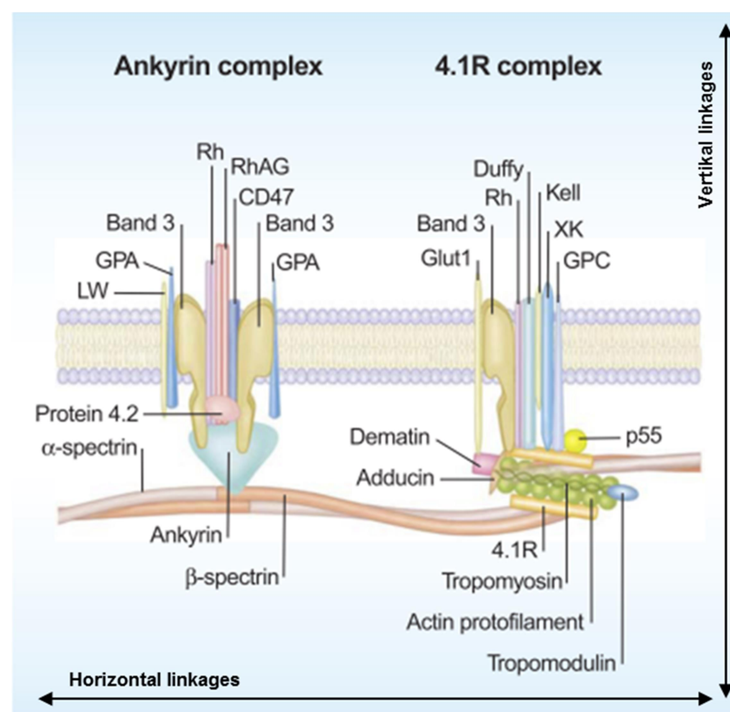

Figure 3: Structural organization of the RBC membrane. *The RBC membrane is composed of a lipid bilayer and a spectrin-based cytoskeleton which are interconnected via the ankyrin and 4.1R complexes (Mohandas and Gallagher, 2008)*

HEREDITARY SPHEROCYTOSIS

Hereditary spherocytosis (HS) is one of the most common RBC membrane disorders in the world with a prevalence of 1 out of 2000–3000 affected cases in North America and Northern European countries (Da Costa et al., 2013). In 75% of cases, HS is inherited in an autosomal dominant manner (An and Mohandas, 2008). The remaining cases are associated with a recessive pattern of inheritance and *de novo* mutations (Barcellini et al., 2011). HS is caused by mutations in genes encoding for various red cell membrane proteins including ankyrin, band 3, protein 4.2, α or β-spectrin and Rh-associated glycoprotein (RhAG) (Da Costa et al., 2013). The defective protein destabilizes the vertical linkages between the phospholipid bilayer and the membrane cytoskeleton, which results in a loss of surface area and an associated decreased surface area–to–volume ratio. These changes account for the spheroidal shape of RBCs, the common characteristic of HS (Da Costa et al., 2013; Mohandas and Gallagher, 2008). Patients with HS exhibit a broad clinical phenotype and variable disease severity ranging from mild to very severe according to the decrease of RBC surface area. However, "typical" HS consists of evidence of hemolysis with anemia, jaundice, reticulocytosis, gallstones, splenomegaly as well as spherocytes on peripheral blood smear (An and Mohandas, 2008).

HEREDITARY ELLIPTOCYTOSIS

Hereditary ellipctocytosis (HE) is an autosomal dominant disorder resulting from a protein 4.1R deficiency or spectrin malformations, affecting the horizontal interaction of the red cell cytoskeleton (King et al., 2015).The world-wide prevalence has been estimated to be 3 to 5 in 10 000 individuals but a higher prevalence occurs in malaria endemic regions (Da Costa et al., 2013). A majority of HE patients are asymptomatic (no anemia or hemolysis) and the only laboratory finding is the presence of elliptocytes on peripheral blood smear. Approximately 10% of HE patients exhibit mild to moderate HA with reticulocytosis. The laboratory findings include the presence of elliptocytes and fragmented cells on peripheral smear, positive markers of hemolysis and abnormal EMA binding test and osmotic fragility test (Gallagher, 2013).

HEREDITARY STOMATOCYTOSIS

Hereditary stomatocytosis (HSt) is an autosomal dominant disorder characterized by the presence of variable numbers of stomatocytes on peripheral blood smear (Barcellini et al., 2011). Affected individuals usually exhibit mild to moderate HA with reticulocytosis and marked macrocytosis. The HSt results from an increase of red cell membrane cation permeability and the cell's inability to regulate its cation homeostasis (Barcellini et al., 2011; Delaunay et al., 1999; Flatt and Bruce, 2009). The changes in cation content affect water balance and, thus, red cell volume homeostasis. According to the red cell hydration, HSt is classified into two major types: A) overhydrated stomatocytosis and B) dehydrated stomatocytosis.

Overhydrated stomatocytosis (OhSt) is characterized by increased intracellular sodium and a slight decrease in intracellular potassium. In addition to increased cation permeability, RBCs of many affected individuals lack the membrane protein stomatin (Fricke et al., 2003; Glogowska and Gallagher, 2015). Nevertheless, no mutation in gene encoding stomatin (*EPB72* or *STOM*) has been identified. Thus, whether the stomatin loss is a consequence or a cause of the red cell membrane defect have not been clearly established yet (Badens and Guizouarn, 2016). Recently, mutations in RhAG have been considered the cause of this sydrome. Other two proteins, Band 3 and Glut 1, are associated with OhSt with cation leak at a low temperature.

Dehydrated stomatocytosis (DhSt) (also known as hereditary xerocytosis) is the second type of HSt. RBCs of affected individuals exhibit reduced intracellular potassium and increased sodium content (Badens and Guizouarn, 2016). Variable numbers of stomatocytes are seen on peripheral blood and in mild cases can be easily overlooked. The definitive diagnosis of DHSt is ascertained by osmotic gradient ektacytometry, which shows a leftward shift of the bell–shaped curve (Andolfo et al., 2013). To date, a total of 2 membrane proteins have been reported to be responsible for DhSt. Several mutations have been reported in *FAM38A* gene encoding mechanosensitive channel PIEZO1 (Albuisson et al., 2013; Andolfo et al., 2013; Bae et al., 2013; Beneteau et al., 2014; Shmukler et al., 2014; Zarychanski et al., 2012). More recently, defects in the Gardos channel have been linked to DhSt (Andolfo et al., 2015; Rapetti-Mauss et al., 2015).

Treatment of anemia (transfusion and chelation therapy) depends on the cause and severity. Splenectomy is useful.

In 2015, International Council for Standardization in Hematology released a new guideline for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. In addition to "classical" blood smear examination, several screening methods are currently available for identification of membrane disorders. Typical HS cases with positive family history can be confirmed by osmotic fragility test (OF), acid glycerol lysis time test (GLT) or eosin–5'–maleimide binding test (EMA). Quantitative membrane defects, mainly associated with typical HS and HE, can be identified using SDS–polyacrylamide gel electrophoresis (SDS-PAGE). All three defects (typical HS, typical HE and HSt) can be detected using ektacytometry (or Laser-assisted optical rotational red cell analyzer), the only simple and reliable screening test for the diagnosis of HSt. Molecular genetic analysis is usually applied in recessive mode of inheritance, and in cases of suspected *de novo* mutation or compound heterozygosity (King et al., 2015).

Several novel mutations in band 3 or spectrin genes associated with HS have been described in patients of Czech origin (Hassoun et al., 1997; Jarolim et al., 1996; Jarolim et al., 1995). Currently, only a couple of laboratories in the Czech Republic offer screening tests (EMA test, OF test, GLT,

cryohemolysis test and Pink test) for membrane defects. Nevertheless, lack of molecular analysis hampers the diagnosis of membrane defects in atypical cases.

1.3.1.3 RED BLOOD CELL ENZYMOPATHIES

Hereditary red blood cell enzymopathies are disorders arising from mutations (point mutations, insertions, deletions, splice defects, etc.) in genes coding for red cell metabolic enzymes (Figure 2). Deficiencies or malfunctions of these enzymes generally impair cellular energy balance and/or increase the levels of oxidative stress. A lack of energy and reduced reductive power of red blood cells ultimately affects cellular integrity, leading to premature removal in the spleen and, consequently, decreased red blood cell survival (Arya et al., 1995; van Wijk and van Solinge, 2010). Most enzyme disorders are inherited in an autosomal recessive form with hemolysis occurring only in homozygous or compound heterozygous individuals. Some enzyme deficiencies are X-linked; adenosine deaminase deficiency is autosomal dominant. A summary of clinically relevant erythroenzymopathies and their main characteristics is presented in Table 2.

The degree of hemolysis depends on the affected metabolic pathway, the role of the mutated enzyme in this pathway, the functional abnormalities introduced by the underlying mutation, and the possibility for the cell to compensate for the loss of enzymatic activity (Jacobasch and Rapoport, 1996). The severity of hemolysis is variable, ranging from mild or fully compensated hemolysis without apparent anemia to fatal anemia at birth, severe transfusion–dependent hemolysis, and even death in early childhood. Typical clinical symptoms further include splenomegaly, jaundice, gallstones, and iron overload (also in non-transfused patients). Notably, some enzyme deficiencies are associated with systemic (non-hematological) manifestations, such as neurological dysfunction, mental retardation, myopathy and susceptibility to infection (van Wijk and van Solinge, 2010).

Table 2: A summary of clinically relevant enzyme disorders

Enzyme	Clinical manifestation	Neurological symptoms	Myopathy	Genetic transmission	No. of reported cases, mutations
Embden-Meyerhof pathway					
Hexokinase	HNSHA; chronic	-	-	AR	30 cases, 10 mutations
Glucose phosphate isomerase	HNSHA; chronic	+/-	-	AR	> 55 families, 31 mutations
Phosphofructokinase	HNSHA; chronic (mild)	-	+	AR	100 families, 23 mutations
Aldolase	HNSHA; chronic	+/-	+/-	AR	8 cases, 8 mutations
Triosephosphate isomerase	HNSHA; chronic (severe)	+	-	AR	40 cases, 19 mutations
Phosphoglycerate kinase	HNSHA; chronic	+	+	X–linked	40 cases, 23 mutations
Bisphosphoglyceratemutase	erythrocytosis	-	-	AR	4 cases, 4 mutations
Pyruvate kinase	HNSHA; chronic	-	-	AR	> 500 families, > 260 mutations
Hexose monophosphate shunt					
Glucose-6-phosphate dehydrogenase	HNSHA; induce by oxidant drugs/infections, favism	-	-	X–linked	400 million cases, 186 mutations
Glutathione metabolism					
Glutathione synthetase	HNSHA; chronic	+	-	AR	>50 families, 33 mutations
γ–glutamylcysteine synthetase	HNSHA; chronic	+	-	AR	12 families, 5 mutations
Glutathione reductase	HNSHA; induce by oxidant drugs/infections, favism	-	-	AR	2 families, 3 mutations
Nucleotide metabolism					
Adenosine deaminase (hyperactivity)	HNSHA; chronic	-	-	AD	3 families, no mutations
Adenylate kinase	HNSHA; chronic	-	-	AR	12 families, 7 mutations
Pyrimidine–5'–nucleotidase	HNSHA; chronic	-	-	AR	> 60 families, 26 mutations

HNSHA - chronic nonspherocytic hemolytic anemia; AR - autosomal recessive, AD - autosomal dominant.

ENZYME DISORDERS OF THE EMBDEN - MEYERHOF PATHWAY

The Embden-Meyerhof pathway (Figure 2) transforms glucose to pyruvate or lactate, and generates ATP. This metabolic pathway is regulated by three rate-limiting steps, involving the catalytic action of hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK). Cellular NADH and ATP levels also control glycolytic flux. Most enzyme disorders of this pathway are associated with chronic nonspherocytic hemolytic anemia (HNSHA). Except for phosphoglycerate kinase (PGK) deficiency, which is X-linked, all enzyme disorders of anaerobic glycolysis are transmitted in the autosomal recessive manner (van Wijk and van Solinge, 2005).

Pyruvate kinase deficiency. The most prevalent enzyme disorder of anaerobic glycolysis is PK deficiency. The estimated frequency of PK deficiency is 1:20,000 in general white population (Beutler and Gelbart, 2000). PK is an allosteric enzyme which catalyzes the irreversible transfer of high-energy phosphate group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. The enzyme is allosterically regulated by fructose–1,6–bisphosphate (positive regulation) and inhibited by its product ATP. There are four mammalian isozymes of PK: M1, M2, L and R, encoded by 2 different genes. *PKLR*, localized on chromosome 1 (1q21) (Kanno et al., 1992), directs the expression of PK-R and PK–L in red blood cells and liver, respectively. The deficiency of PK–R has two major metabolic consequences: depletion of ATP, and accumulation of 2,3-bisphosphoglycerate (Valentine and Paglia, 1980b). The lack of ATP eventually leads to premature removal of red blood cells from the circulation whereas increased levels of 2,3–bisphosphoglycerate decrease the affinity of hemoglobin for oxygen. The latter partly compensates for tissue hypoxia due to anemia (Oski et al., 1971).

More than 260 different mutations in *PKLR* gene associated with PK deficiency are known (http://www.hgmd.org/) (Canu et al., 2016). Most of the mutations are single nucleotide substitutions (72%) leading to mainly amino acid substitutions; in twelve cases a stop codon formation. The clinical features of PK deficiency are highly variable, ranging from a fully compensated hemolysis to severe transfusion–dependent hemolytic anemia (Canu et al., 2016; Zanella et al., 2005). A few cases of hydrops fetalis and death in neonatal period due to severe PK deficiency have been reported (Ferreira et al., 2000). The severity of anemia is usually stable in adulthood but may worsen during infections or other forms of physiological stress (Zanella et al., 2007). Iron overload, occurring in the most PK deficient patients, is considered to be multifactorial (Zanella et al., 1993). There is no link among the residual PK activity, degree of hemolysis and clinical severity of PK deficiency. A high rate of reported mutations (60%) are distributed in Europe (Canu et al., 2016). Among these, the 1456T mutation and the 1529A mutation are the most prevalent (Lenzner et al., 1997; Zanella et al., 2005). Several mutations p.(Gly332Ser), 1060delAAG, p.(Asn361Asp), p.(Arg486Trp), p.(Arg498His), p.(Arg532Trp) and 101-1G>A have been previously identified in Czech population (Lenzner et al., 1997). Distribution of other identified mutations in the Czech and Slovak populations will be discussed in section 4.2.

Glucose-6-phosphate isomerase (GPI) deficiency. The second most frequent glycolytic enzyme disorder is GPI deficiency. To date, 34 mutations in more than 55 families with GPI deficiency have been identified (Adama van Scheltema et al., 2015; Manco et al., 2016). In the most affected individuals, residual GPI activity is less than 25%. Low enzyme activity results from impaired kinetic properties, reduced thermostability of the mutant enzyme, or defective protein folding (Lin et al., 2009). Clinical features in GPI activity range from mild to severe hemolytic anemia. Hydrops fetalis appears to occur more often in GPI deficiency than in other erythroenzymopathies (Matthay and Mentzer, 1981). Most affected individuals are diagnosed during the neonatal and childhood period (Adama van Scheltema et al., 2015). GPI deficiency may also be associated with non-hematologic symptoms, in particular neurologic impairment or mental retardation (Schroter et al., 1985; van Wijk and van Solinge, 2005).

Phosphofructokinase (PFK) deficiency, aldolase deficiency (ALD) and triosephosphate (TPI) deficiency. PFK, ALD and TPI deficiencies are more rare enzyme disorders. Erythrocyte PFK is composed of two types of subunits: PFKL (liver) and PFKM (muscle) that may form 5 different isozymes (M_4, M_3L_1, M_2L_2, M_1L_3, L_4). In case of a mutated PFKM subunit, only a functional PFKL subunit is expressed in red blood cells, causing a partial deficiency of PFK. Thus, patients with PFK deficiency usually exhibit mild or fully compensated hemolytic anemia. Since muscle cells contain only PFKM, the deficiency is more pronounced in muscle cells, leading to myopathy (Nakajima et al., 2002). TPI deficiency is the most severe enzyme defect of anaerobic glycolysis, frequently leading to death in early childhood. TPI catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde–3–phosphate (GAP). In red blood cells the deficiency leads to hemolytic anemia which may occur due to accumulation of DHAP (Ahmed et al., 2003). However, the deficiency is associated with more severe consequences, such as mental retardation and other neurological dysfunctions. These may occur because of the formation of toxic protein aggregates, induced by misfolded TPI (Ahmed et al., 2003; Orosz et al., 2009). Moderate to severe hemolytic anemia is observed in patients with aldolase deficiency. As in TPI deficiency, defects of aldolase are associated with neurological disorders (Beutler et al., 1973). A few affected individuals have been reported, showing recurrent episodes of rhabdomyolysis and muscle weakness (Kreuder et al., 1996; Yao et al., 2004). Severe defects of aldolase probably lead to death in embryonic state (Esposito et al., 2004).

Hexokinase (HK) deficiency. HK catalyzes the initial step of the Embden-Meyerhof pathway, the transfer of a phosphoryl group from ATP to glucose. Hexokinase is the enzyme with the lowest *in vitro* activity of all glycolytic enzymes (van Wijk and van Solinge, 2005). Two different HK isozymes, HK–1 and HK–R, are present in human RBCs. Both HK isozymes originate from the same gene *HK1,* however, their transcriptions are controlled by alternative promoters. While HK-R isozyme is present only in RBCs, HK–1 isozyme is expressed in various mammalian tissues (Murakami et al.,

2002). Deficiency of this key regulatory enzyme is generally associated with severe hemolytic anemia, and may lead to death in the neonatal period (Kanno et al., 2002). To date, 30 cases of hexokinase deficiency have been described (de Vooght et al., 2009; Koralkova et al., 2016; van Wijk et al., 2003).

Phosphoglycerate kinase (PGK) deficiency. PGK is the only enzyme of glycolysis whose gene is X-linked. PGK deficiency is associated with chronic hemolysis, neurologic impairment (including mental retardation and ataxia) and myopathy (exercise intolerance or muscle weakness) (Fermo et al., 2012). There are only a few affected individuals who suffer the full spectrum of clinical features. Thus, PGK deficiency shows a very wide clinical phenotype. Some cases of PGK deficiency with myopathy but without hemolysis have been described (Beutler, 2007; Cohen-Solal et al., 1994; Fermo et al., 2012; Sotiriou et al., 2010; Spiegel et al., 2009).

Deficiencies of other glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, monophosphoglycerate mutase, and enolase have been described but they appear not to be associated with hemolysis (Beutler, 1979).

ENZYME DISORDERS OF THE HEXOSE MONOPHOSPHATE SHUNT AND GLUTATHIONE METABOLISM

The red blood cell is continuously exposed to various forms of oxidative stress. To protect cellular hemoglobin and other macromolecules against oxidative damage, reductive power is required. The reduced form of glutathione (GSH) constitutes the cell’s main source of reductive power. NADPH, generated by the hexose monophosphate shunt, is required to maintain glutathione in its reduced form. The key enzymes of this defense mechanism are glucose-6-phoshate dehydrogenase, glutamate cysteine ligase, glutathione synthetase, and glutathione reductase (van Zwieten et al., 2014). Generally, disorders of these enzymes cause hemolysis only under conditions of increased levels of oxidative stress (acute hemolytic anemia).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency. G6PD catalyzes the first step of the hexose monophosphate shunt thereby producing NADPH. The human G6PD monomer (515 amino acids) consists of two domains: the coenzyme (N-terminal) domain, and the other, larger, $\alpha+\beta$ domain. Both domains are connected by an α helix consisting in fully conserved region, which serves as a substrate binding site. The enzyme is active as a tetramer or dimer, in a pH-dependent equilibrium (Cappellini and Fiorelli, 2008). G6PD deficiency is the most common enzyme defect with estimated 400 million individuals affected worldwide (Nkhoma et al., 2009). The highest prevalence is observed in Asia and sub-Saharan Africa (Howes et al., 2012). G6PD deficiency is an X-linked disorder associated with mutations in *G6PD* gene consisting of 13 exons. This gene is highly polymorphic; more than 400 G6PD variants have been described, among which 186 mutations are associated with G6PD deficiency. Majority of the mutations are single nucleotide substitutions, however, deletions

and intronic mutations (G6PD Varnsdorf, G6PD Zurich) have also been identified (Efferth et al., 2004; Minucci et al., 2012; Xu et al., 1995). G6PD-deficient variants are divided into five classes according to the level of residual activity and severity of phenotype. Most of the variants belong to classes II, III, or IV and are associated only with acute hemolysis. In contrast, the rare class I variants, characterized by a very low activity of G6PD (<1%), are associated with chronic HNSHA. The class I mutations are usually located in exons6, 10 and 13 encoding the regions that bind the enzyme substrate, dimer interface and $NADP^+$ structural site, respectively (Minucci et al., 2012). Class II and III mutations are highly distributed in malaria-endemic regions, among which the G6PD variant A^- (c.376A>G and c.202G>A) has the highest frequency (90%) in Africa. The second most frequent G6PD variant, G6PD Mediterranean (c.563C>T), is present in the Mediterranean countries. However, it is also widespread in the Middle East, including Israel, where it accounts for almost all G6PD deficiency in Kurdish Jews, India, and Indonesia (Cappellini and Fiorelli, 2008). Most G6PD-deficient individuals are asymptomatic but may develop acute hemolytic crises upon conditions that increase the levels of oxidative stress, such as the intake of specific drugs, fava beans, or the occurrence of infections (Cappellini and Fiorelli, 2008; Minucci et al., 2009). Although, in general, no morphological abnormalities are associated with enzyme disorders, Heinz bodies (precipitated hemoglobin) are typically found in G6PD-deficient individuals. Two single nucleotide substitutions (G6PD Praha, G6PD Olomouc) and one intronic mutation (G6PD Varnsdorf) have been previously described in Czech population (Xu et al., 1995). Distribution of other mutations in both the Czech Republic and Slovak Republic will be discussed in section 4.2.

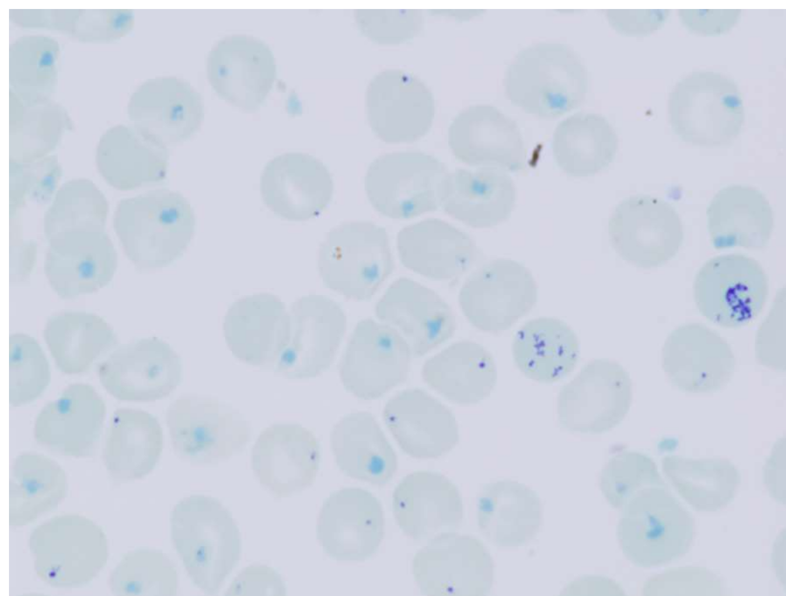

Figure 4: A peripheral blood smear of a G6PD deficient patient showing a presence of Heinz bodies

Glutathione reductase (GR) deficiency. GR is important in the regeneration of GSH from oxidized glutathione (GSSG). Hereditary GR deficiency is a very rare autosomal recessive disorder characterized by increased susceptibility to oxidative stress. The clinical phenotype resembles a deficiency of G6PD with affected individuals being generally asymptomatic and with the induction of acute hemolytic crisis by the intake of drugs or fava beans (Kamerbeek et al., 2007; van Zwieten et al., 2014). Importantly, acquired deficiency of GR may also occur as a result of insufficient flavin intake. GR requires flavonoids for enzymatic activity. Such acquired deficiencies do not themselves lead to a clinical phenotype (Beutler, 1979). A partial GR deficiency has been described in two Czech patients who were concomitantly carriers of the unstable hemoglobin variant Haná. The combination of both defects resulted in methemoglobinemia and Heinz body hemolytic anemia (Mojzikova et al., 2010).

γ–glutamylcysteine synthetase (γ–GCS) and glutathione synthetase (GS) deficiencies. The tripeptide glutathione is synthesized in red blood cells from cysteine, glutamine, and glycine in a 2–step process catalyzed by γ–GCS and GS, respectively. Defects of both enzymes decrease cellular GSH content and, hence, increase cellular susceptibility to oxidative stress. Disorders caused by deficiencies of bothof these enzymes are rare: >50 families with GS deficiency have been described whereas only 12 families with γ–GCS deficiency have been reported. Apart from acute hemolytic episodes, the deficiency may display additional non-hematological symptoms, such as mental retardation (γ–GCS deficiency) and 5-oxyprolinuria (GS deficiency) (Ristoff and Larsson, 2007).

Deficiencies of glutathione peroxidase and other enzymes involved in red blood cell anti-oxidant defense, such as catalase, have been reported. However, they are not associated with hemolysis (Beutler, 1979).

ENZYME DISORDERS OF NUCLEOTIDE METABOLISM

Purine metabolism maintains the red blood cell ATP and GTP levels. Ninety-seven per cent of total nucleotides are adenine derivatives (AMP, ADP, ATP). Guanine derivatives constitute 1–3%. Pyrimidines are removed during the maturation process and are therefore only present in trace amounts (Valentine et al., 1974; Valentine and Paglia, 1980a). There is a number of enzymes involved in nucleotide metabolism. The most important ones are pyrimidine-5'-nucleotidase (pyrimidine metabolism), and adenylate kinase and adenosine deaminase (purine metabolism) (Figure 1). Defects of all three enzymes are associated with HNSHA.

Pyrimidine-5'-nucleotidase (P5N) deficiency. Erythrocyte P5N is a cytosolic enzyme which catalyzes the hydrolysis of cytidine/uridine monophosphates, rendering diffusible cytidine/uridine and inorganic phosphates (Rees et al., 2003; Valentine et al., 1974). P5N deficiency is probably the third most prevalent enzyme disorder associated with HNSHA. P5N deficiency is an autosomal recessive disorder associated with mild to severe chronic hemolytic anemia, splenomegaly and jaundice.

P5N deficiency results in the accumulation of pyrimidine nucleotides. They form insoluble aggregates which are visible as basophilic stippling on a peripheral blood smear. Although not specific, prominent basophilic stippling is characteristic of P5N deficiency (Zanella et al., 2006). To date, it is not known how the accumulation of pyrimidine nucleotides contributes to the hemolytic process. However, extensive studies of P5N deficient patients revealed that other laboratory parameters, such as GSH levels, ribose-phosphate pyrophosphokinase activity and transketolase levels, are markedly altered (Barasa et al., 2016; Magni et al., 2013).

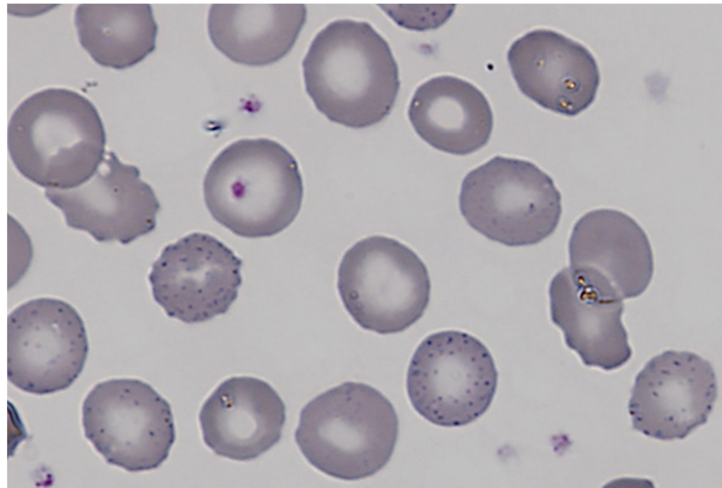

Figure 5: A pronounced basophilic stippling in P5N deficient RBCs (Koralkova et al., 2014)

Adenylate kinase (AK) deficiency and adenosine deaminase (ADA) hyperactivity. AK and ADA are both important in maintaining the adenine pool. Deficiency of both AK and hyperactivity of ADA probably deplete the cell from adenine nucleotides and, consequently, ATP, eventually resulting in chronic hemolytic anemia. AD deficiency is an autosomal recessive disorder which has been described only in 12 families. Affected individuals exhibit moderate or severe hemolytic anemia. Some patients with psychomotor impairment have been reported (Abrusci et al., 2007). Hyperactivity of ADA is a very rare autosomal dominant disorder for which no causative mutations have been described yet (Chen and Mitchell, 1994). The elevated ADA activity is also seen in Diamond-Blackfan anemia and in patients with AIDS or tuberculosis. On the other hand, adenosine deaminase deficiency is one cause of severe combined immunodeficiency.

DIAGNOSTIC APPROACH AND THERAPY

Red blood cell enzymopathies are associated with normocytic normochromic anemia and common signs of hemolysis, i.e. increased plasma levels of bilirubin and lactate dehydrogenase, low levels of haptoglobin and high reticulocyte count (Tefferi, 2003). The diagnosis of HNSHA due to a red blood cell enzymopathy is generally a diagnosis that is based on exclusion: a negative direct Coombs test, a normal osmotic fragility/cryohemolysis test, no specific morphological abnormalities, and no evidence for abnormal hemoglobin. A differential diagnosis of a red blood cell enzymopathy requires

a demonstration of reduced enzymatic activity and confirmation of the diagnosis on the DNA level. Importantly, there is no relationship between the degree of loss of enzymatic activity and clinical severity.

Nowadays, there are several quantitative or semi-quantitative screening assays for determination of the most prevalent erythroenzymopathies (deficiencies of G6PD and PK). G6PD deficiency screening tests, such as fluorescent spot tests or cytochemical assays, are based on the direct/indirect detection and/or quantification of NAPDH produced by G6PD (Cappellini and Fiorelli, 2008). Direct tests measure the production of NAPDH which has it absorption maximum at 340 nm and fluorescence emission at 460 nm. Indirect tests translate NADPH production into a colorimetric readout using chromophores like brilliant cresyl blue, resazurin, or formazan derivatives (Shah et al., 2012). PK deficiency screening tests are usually indirect colorimetric assays based on the detection of chromophore products. Although these assays are usually rapid and inexpensive, they are able to detect only the most common enzyme defects. Thus, the classical methods described by Beutler still represent the method of choice for the diagnosis of erythroenzymopathies (Beutler, 1984). The general basis comprises the determination of a specific enzyme activity using a spectrophotometric assay. Enzymatic activity is directly related to changes in absorbance over time. Specific reactions need to be prepared for each enzyme and changes in substrate (either NAD^+, $NADP^+$, NADH, or NADPH) are monitored at 340 nm for a set amount of time. The specific enzymatic activity of each particular enzyme is expressed as U/gHb (Beutler, 1984). In red blood cell enzymopathies, enzyme activity of affected individuals is usually reduced to 25% of the normal. As stated, there is no correlation between the residual activity of the enzyme and severity of the clinical picture. Despite the many benefits of these assays (high sensitivity and high specificity), there are several pitfalls which may influence results and their interpretation. Enzymatic activities are influenced by factors such as (1) the conditions during storage and shipping of blood samples (i.e. *in vitro* stability of enzymes), (2) the proper removal of leukocytes and platelets from the red blood cell sample (leukocytes and platelets may express isozymes, encoded by other genes, which interfere with red cell enzyme activity measurements), (3) blood transfusions (blood sample will contain donor erythrocytes), (4) the age of the patient (the expression of many enzymes is age-related), and (5) the presence of a high number of reticulocytes in the patient's blood sample. The activity of many enzymes (HK, PK, G6PD and P5N) is red blood cell age-related. Thus, the youngest cells have the highest enzymes activity. In the case of a high reticulocyte count, any deficient enzyme may show a normal or even increased *in vitro* activity (Arya et al., 1995; Beutler, 1984). This masking effect can be eliminated when activity of age-related enzymes (PK, G6PD, P5N) is normalized to activity of HK.

As stated, morphological abnormalities of red blood cells associated with red blood cell enzymopathies are generally nonspecific. Prominent basophilic stippling is present in P5N deficiency. In addition, Heinz bodies (precipitated hemoglobin) may occur in G6PD deficiency or enzyme

deficiencies of glutathione metabolism. However, the occurrence of both abnormalities is also related with other disorders. Pronounced basophilic stippling can also be seen in patients with sideroblastic anemia, thalassemia, or lead poisoning, whereas Heinz bodies may be present in red blood cells from patients with unstable hemoglobin or thalassemia. Besides both mentioned morphological abnormalities, other various abnormal types of red blood cells associated with erythroenzymopathies can be seen on blood smear. "Bite" cells, keratocytes or blister cells may occur in G6PD deficiency as the result of oxidative stress and acute hemolysis (Bain, 2005); anisocytosis, poikilocytosis and contracted echinocytes can be present in PK deficiency (Zanella et al., 2005).

A number of methods (radioactive, capillary electrophoresis or high-perform liquid chromatography) have been described for determination of red cell P5N activity (Zanella et al., 2006). However, the reproducibility of most of these methods does not meet the standards for use in clinical practice. A simple screening test of P5N deficiency, or any other enzyme involved in nucleotide metabolism, comprises the spectrophotometric measurement of pyrimidine and purine nucleotides in deproteinized hemolysate. This method uses the differences in absorption maxima of purines (260 nm) and pyrimidines (280 nm) (Beutler, 1984). The ratio between these reflects overall cellular purine and pyrimidine content. For instance, an accumulation of pyrimidine nucleotides, as seen in P5N deficiency, decreases the purine/pyrimidine nucleotide ratio.

In case of a suspected red blood cell enzyme deficiency the molecular characterization of the defect on the DNA level is essential for confirmation of the diagnosis. Mutations are usually unique, which makes it necessary to sequence the entire coding region, including flanking intronic splice site sequences. Importantly, *cis*-acting, often erythroid-specific, regulatory regions may harbour mutations interfering with gene expression and thereby causing a red cell enzyme disorder (de Vooght et al., 2009; van Wijk et al., 2003). The identification of a causative mutation enables proper genetic counseling and prenatal diagnosis. DNA investigations may be the only way to establish the diagnosis in neonatal patients or patients who are transfusion-dependent. Moreover, molecular characterization is required for a better understanding of the complex genotype-to-phenotype correlation in erythroenzymopathies.

No curative therapy is available for enzyme defects. Affected individuals with mild anemia or compensated hemolysis do not require any treatment. In more severe cases, treatment mainly consists of supportive therapy (blood transfusions) and chelation therapy to avoid the adverse effects of iron overload. In some cases, splenectomy may be considered. Generally, spleen removal can increase hemoglobin levels but the effect is variable and difficult to predict (Zanella et al., 2005). Patients with deficiency of enzymes involved in the protection against oxidative stress should avoid the intake of oxidative drugs or food.

Interestingly, novel drugs, AG–348 and AG–519, have recently been introduced as a potential therapy of PK deficiency. Both compounds act as activators which increase activity of mutated PK enzyme and restore ATP levels. Although the study is ongoing, the preliminary data show rapid and sustained Hb increase in treated patients (Hixon et al., 2016; Hixon et al., 2013; Chen et al., 2016).

2 AIMS OF THE THESIS

The presented work is focused on molecular pathogenesis of rare anemias – enzymopathies.

It was aimed **to identify genetic background, etiology and pathogenesis of enzyme disorders associated with HNSHA.**

Specific aims:

1. Critically review the literature concerning congenital hemolytic anemias.
2. Perform direct enzyme assays and genetic testing in selected Czech and Slovak patients with hemolytic anemia of unknown etiology.
3. Investigate the impact of selected HK mutations to the pathogenesis of HK deficiency.
4. Describe the molecular mechanism of a novel non-hemolytic anemia variant of PGK.
5. Characterize a novel mutation in *PFKM* gene using *in silico* modelling.

3 MATERIALS AND METHODS

Laboratory equipment and software

Thermocycler MJ Mini™ Personal Thermal Cycler (BioRAD); ThermoblockBio TDB-100 (Biosan); Vortex Bio V1 (Biosan); Centrifuge Hettich Mikro 200R (Hettich); Centrifuge 5415 D (Eppendorf); Centrifuge Jouan BR4i (Thermo Fisher Scientific); PowerPac™ Basic Power Supply (BioRAD); UV-transilluminator (UltraLum); Genetic analyzer ABI PRISM® 3100 (Applied Biosystems); ABI 3130 automated sequencer (PE Applied Biosystems); Spectrophotometer Infinite 200 Nanoquant (Tecan); Software i-control 1.6 (Tecan), pH meter 3510 (Jenway); Odyssey Infrared Imaging System (LI-COR Biosciences); ViiA™ 7 Real-Time PCR System (Life Technologies); Casy Cell Counter and Analyzer (Roche Life Science); NanoVueTM (GE Healthcare Life Sciences); The PyMOL Molecular Graphics System (Version 1.7.4 Schrödinger, LLC); PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/); GraphPad® Prism (GraphPad Software, Inc.); SIFT (http://sift.jcvi.org/); Human Splicing Finder (http://www.umd.be/HSF3/).

Biological material

Peripheral blood samples from patients and donors were collected into EDTA and/or heparin tubes. Each study was performed according to the Helsinki international standards. The informed consent was obtained from all patients, family members and donors.

Enzymes, substrates, cofactors, nucleotides

Glucose-6-phosphate dehydrogenase (S. cerevisiae, Sigma-Aldrich, USA); Lactate dehydrogenase (rabbit muscle, Sigma-Aldrich, USA); D-glucose-6-phosphate (Sigma-Aldrich); D-fructose-6-phosphate (Sigma-Aldrich); Phosphoenolpyruvate (Sigma-Aldrich); D-fructose-1,6-biphosphate (Sigma-Aldrich); 6-phosphogluconate (Sigma-Aldrich); D-(+)-glucose (Sigma-Aldrich); NADH (Serva); $NADP^{+}$(Serva); NADPH (Serva); NAD^{+}(Serva); ATP (Sigma-Aldrich); ADP (Sigma-Aldrich); AMP (Sigma-Aldrich).

Other chemicals

TRIzol Reagent (Thermo Fisher Scientific); Chloroform (Penta); Phenol(Serva); Hydrochloric acid (Penta); SDS (Serva); Agarose (Invitrogen); Microcrystalline cellulose (Sigma-Aldrich); α-cellulose (Sigma-Aldrich); Tris (Promega); Na_2EDTA (Serva); $MgCl_2$ (Chemos); KCl (Fischer Scientific); NaCl (LachNer); Ethanol pro UV (LachNer); 2-mercaptoethanol, 55 mM (Serva); Specific primers for *HK1, G6PD, PKLR*and *PGK1* (Eastport, Sigma-Aldrich); Proteinase K (Sigma-Aldrich, USA); 2-log DNA ladder (Qiagen); DNA loading buffer (Qiagen); Triton X-100 (Sigma-Aldrich); GelRed (Biotium); NuSieve Agarose 3:1 (Lonza); RIPA buffer (Teknova), Protease inhibitors (100 ×, Thermo Scientific); 8% precast SDS-PAGE gel (Bolt® Bis-Tris Plus gel, Life Technologies); PVDF membrane (Immobilion-FL, Millipore); Antibody anti-HK-1 (1:250; Abcam); Antibody Anti-actin

(Merck Millipore); Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit (1:7500; Invitrogen); Odyssey® Blocking Buffer (Li-COR); Ficoll-Paque (GE Healthcare); StemSpan SFEM (Stemcell Technology); Human stem cell factor (Amgen); Erythropoietin (Eprex); Interleukin-3 (Sigma-Aldrich); Dexamethasone (Sigma-Aldrich); SyntheChol™ NS0 Supplement (Sigma-Aldrich); Percoll (GE Healthcare).

Commercial kits

Geneaid Gel/PCR DNA Fragments Extraction Kit (Geneaid); QIAquick Gel Extraction Kit, MinElute PCR Purification Kit (Qiagen); NucleoSpin Gel and PCR Clean-up (Macherey-Nagel); HotStarTaq Master Mix Kit – HotStarTaq DNA Polymerase, PCR Buffer with 3 mM $MgCl_2$, and 400 µM of each dNTP (Qiagen); MasterAmp Taq DNA Polymerase (Eppicentre); PCR buffer (Applied Biosystems); dNTP (Applied Biosystems); BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA); (BigDye Xterminator purification kit, Applied Biosystems); GeneAmp® RNA PCR Core Kit (Applied Biosystems); SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen); FastStart SYBR Green Master (Roche); ROX Passive Reference (BioRad); TOPO® TA Cloning® Kit for Sequencing (Invitrogen); High-Speed Plasmid Mini Kit (Geneaid); CutSmart buffer and restriction endonuclease *MslI* (NEB).

General laboratory reagents and buffers

0.9% M sodium chloride (NaCl); 0.1 M $MgCl_2$; 1 M KCl; 10 mM Tris (pH=8.0); 10% SDS (w/v, pH=7.2); Tris – HCl buffer (pH=8.0) – 1 M Tris, 5 mM Na_2EDTA; 1% SDS (w/v); 2 mM EDTA (pH 8.0); RBC lysis solution – 1.5 M NH_4Cl, 100 mM NH_4HCO_3, 10 mM Na_2EDTA; 1xTBE buffer (pH=8.3) – 0.89 M Tris, 0.89 M boric acid, 0.2 mM Na_2EDTA; Stabilization solution – 2.7 mM Na_2EDTA, 0.7 mM 2-merkaptoethanol; 10 mM phosphate buffered saline(PBS, pH=7.4) – 0.137 M NaCl, 2.7 mM KCl, 8.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4; Blotting buffer – 25 mM Tris, 192 mM glycine; TBS – 10 mM Tris, 150 mM NaCl, pH=7.4; 1xTBS (pH= 7.5) – 25mM Tris, 140mM NaCl, and 3.0mM KCl;

3.1 ISOLATION OF RBCS FROM PERIPHERAL BLOOD AND HEMOLYSATE PREPARATION

A filtration column was prepared from a 5 mL syringe tube, filter paper and a mixture of equal weights of microcrystalline cellulose and α-cellulose. A small piece of filter paper was placed at the bottom of the syringe tube which was then fixed in a vertical position in 15 mL tube. The mixture of celluloses was mixed with 0.9% NaCl and poured to the 2 mL mark of the syringe tube. The column was washed with 2 mL of 0.9% NaCl and then 1 mL of whole blood was pipetted onto the column. The column was washed with additional 2-5 mL of 0.9% NaCl. Isolated RBCs were collected in the 15 mL tube, then spinned down at 590g for 5 min at 4°C. The leukocyte/platelet-free RBC pellet was washed with 5 mL of 0.9% NaCl, centrifuged at 590 g for 5 min at 4°C and aliquots were stored at -80°C(Beutler et al., 1977). Prior to the analyses, 50 µL of each RBC aliquot was mixed with 950 µL of stabilizing solution.

3.2 ENZYME ANALYSIS

All enzyme assays were performed in leukocyte/platelet-free erythrocyte lysates as described above. The composition of each assay mixture is listed below. After 10 min incubation, the reactions were started by adding substrate or hemolysate and mixing properly. Then, 200 µL of each reaction mixture was transferred into a 96-well plate and changes in absorbance were recorded at 340 nm at 1 min intervals for 20–30 min. All assays were run in triplicates and specific enzyme activity was calculated using the Lambert-Beer law.

$$\text{activity} = (\Delta A \text{ x } V/(\varepsilon \text{ x } l \text{ x } t)) \text{ x } f$$

(A: changes in absorbance, V: volume of reaction mixtures – 200 µL, ε: absorption coefficient of NAD(P)H at 340 nm – 6220 $l.mol^{-1}.cm^{-1}$, l: inside depth of well – 0.58 cm, t: time – min, f: dilution factor)

$$\text{activity } [U{=}\mu mol.min^{-1}] = \Delta A \text{ x } 0{,}055 \text{ x } f$$

$$\text{activity } [U] = \Delta A \text{ x } 0{,}028 \text{ x } f$$

$$\text{specific activity } [U/g \text{ Hb}] = (\Delta A \text{ x } 0{,}028 \text{ x } f)/m_{Hb}$$

(m_{Hb}: hemoglobin levels in leukocyte/platelet-free erythrocyte lysates was determined spectrophotometrically at 414 nm) (Magnotti et al., 2009)

Composition of assay mixtures

HK assay – 0.1 M Tris-HCL/0.5 mM EDTA, pH=8.0; 0.01 M $MgCL_2$; 2 mM glukose; 2 mM ATP; 0.2 mM $NADP^+$; 0.1 U/mL G6PD; 50 µL 1:20 hemolysate; 10 min incubation at 37°C; 30 min measurement; blank without ATP.

GPI assay – 0.1 M Tris-HCl/0.5 mM EDTA, pH=8.0; 0.01 M $MgCl_2$; 0.2 mM $NADP^+$; 0.1 U/mL G6PD; 2 mM F6P; 10 min incubation at 37°C; start by addition of 5 µL 1:20 hemolysate; 20 min measurement; blank without F6P.

6PGA assay – 0.1 M Tris-HCl/0.5 mM EDTA, pH=8.0; 0.01 M $MgCL_2$; 0.2 mM $NADP^+$; 20 µL of 1:20 hemolysate; 10 min incubation at 37°C; start by addition of 0.6 mM 6PGA; 20 min measurement, blank without 6PGA.

G6PD assay – 0.1 M Tris-HCl/0.5 mM EDTA, pH=8.0; 0.01 M $MgCl_2$; 0.2 mM $NADP^+$; 20 µL of 1:20 hemolysate; 10 min incubation at 37°C; start by addition of 0.6 mM 6PGA + 0.6 mM G6P; 20 min measurement, blank without 6PGA+G6P.

PK assay – 0.1 M Tris-HCl/0.5 mM EDTA, pH=8.0; 0.01 M $MgCl_2$; 0.1 M KCl; 1.5 mM ADP; 0,2 mM NADH; 6 U/mL LDH; 20 µL of 1:20 hemolysate; 10 min incubation at 37°C; start by addition of 5 mM PEP; blank without ADP and PEP; 20 min measurement.

A complete panel of RBC enzyme assays (including aldolase, phosphofructokinase, triosephosphate isomerase, phosphoglycerate kinase, bisphosphoglyceromutase, adenylate kinase) was applied in several cases to exclude other but very rare enzymopathies causing HA. The detailed composition of these assay mixtures is described in a research paper by Beutler and colleagues (Beutler et al., 1977).

3.2.1 DETERMINATION OF MICHAELIS-MENTEN CONSTANT (K_M) FOR GLUCOSE AND ATP

All assays were run in the standard HK reaction mixture containing different concentrations of glucose: 0.0125–2.0 mM and/or ATP 0.25–10 mM. The reactions were run in a 1 mL disposable plastic cuvette and changes in absorbance at 340 nm were recorded at 20 s intervals for 30 min. The K_M values were determined by non-linear regression using the Michaelis-Menten model (GraphPad® Prism).

3.2.2 ENZYME STABILITY ASSAYS

The thermal stability assays of HK mutants involved determining the half-life ($T_{1/2}$) and the half-inactivation temperature (T_{50}). Both thermal stability assays were performed in the standard HK reaction mixture, routinely containing 2 mM glucose and 10 mM ATP. For the $T_{1/2}$ assay, RBC lysate

was pre-heated to 40°C for 5–40 min followed by cooling on ice (2 min). After the pre-heating step, 50 μL of the RBC lysate was used in the reaction mixture as described above. $T_{1/2}$ was defined as the time needed to reach a 50% reduction in the initial activity (Rijksen and Staal, 1978). For the T_{50} assay, RBC lysate was pre-incubated at different temperatures (37–44°C) for 5 min, followed by a cooling period (2 min) on ice. The T_{50} was defined as the temperature at which HK maintains 50% of its activity. The pH stability assay was carried out in a 0.1 M Tris-HCl/0.5 mM EDTA buffer of different pH values (6.0–10.0) (Beutler et al., 1977; Rijksen et al., 1983).

3.3 CELL CULTURE OF HUMAN NUCLEATED ERYTHROID CELLS

Human peripheral blood mononuclear cells (PBMCs) were isolated from individuals carrying a heterozygous splice site c.873-2A>G mutation in *HK1* or a hemizygous c.756+3A>G mutation in *PGK1* and healthy donors.

3.3.1 ISOLATION OF MONONUCLEAR CELLS FROM HUMAN PERIPHERAL BLOOD BY DENSITY GRADIENT CENTRIFUGATION

50–100 mL of peripheral blood was diluted with PBS buffer (1:2). The resulting cell suspension was layered over an equal volume of Ficoll-Paque (GE Healthcare) in a 50 mL conical tube. The tubes were centrifuged at 470g for 30 min at 20°C in a swinging-bucket rotor without brake. The upper layer was aspirated and the interphase containing the mononuclear cells was transferred into a new 50 mL conical tube and filled with PBS. The tube was mixed by inverting and centrifuged at 600g for 5 min at 20°C. The supernatant was completely removed and the cell pellet was resuspended in 50 mL of PBS and centrifuged at 220g for 5 min at 20°C. The supernatant was completely discarded and the washing step was repeated. After the second wash, the pellet was resuspended in 12.5 mL of cold red cell lysis buffer and incubated for 10 min at room temperature (RT). The tube was then centrifuged at 600g for 5 min at 4°C and the supernatant was removed. The isolated PBMCs were resuspended in 10 mL of StemSpan SFEM (Stemcell Technology) and counted using the Casy Cell Counter and Analyzer (Roche Life Science).

3.3.2 SELECTION OF PRO-ERYTHROBLASTS

The cells were resuspended to a density 5–10x10^6 cells/mL in StemSpan supplemented with human stem cell factor (hSCF, 100 ηg/mL; Amgen), erythropoietin (EPO, 2 U/mL; Eprex), interleukin-3 (IL-3, 1 ng/mL; Sigma-Aldrich), dexamethasone (Dex, 1 μmol/L; Sigma-Aldrich) and SyntheChol™ NS0 Supplement (Chol, 200-times diluted; Sigma-Aldrich). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 h, the cells were counted and then the culture

medium was replaced with StemSpan supplemented with hSCF (100 ηg/mL), EPO (2 U/mL), Dex (1 μmol/L) and Chol (200-times diluted). At day 5–6, the cell suspension was layered over 20 mL of Percoll (GE Healthcare) in a 50 mL conical tube and centrifuged at 470g for 30 min at 20°C in swinging-bucket rotor without brake. The upper layer was discarded and the interphase layer was transferred into a new 50 mL conical tube. The cell suspension was washed twice with PBS and centrifuged at 600g for 5 min at 20°C.

3.3.3 EXPANSION OF PRO-ERYTHROBLAST

The cell pellet was resuspended in StemSpan, supplemented with hSCF (100 ng/mL), EPO (2 U/mL), Dex (1 μmol/L) and Chol (200-times diluted), to get a final cell concentration of 1.5×10^6 cells/mL. The cell suspension was plated in a culture dish and incubated at 37°C with 5% CO_2. The cells were maintained at 1.5×10^6 cells/mL by daily cell counts and partial medium changes. At day 7–8, the erythroblasts were harvested without a disturbance of the monocyte and macrophage layer. The cell suspension (mainly pro-erythroblasts) was centrifuged at 600g for 5 min at 20°C and one half of the medium volume was replaced by StemSpan supplemented with hSCF (100 ng/mL), EPO (2 U/mL), Dex (1 μmol/L) and Chol (200-times diluted). The cells were maintained at 1.5×10^6 cells/mL by daily cell counts and partial medium changes. After 10–12 days of erythroid proliferation, cells (mainly pro-erythroblasts) were harvested (600g, 5 min at 20°C) to isolate total RNA and/or carry out a Western blot analysis.

The PBMC isolation and maintenance of the cell culture of the patients' samples were done by my colleague Brigitte van Oirschot, Department of Clinical Chemistry and Hematology, University Medical Center Utrecht. Both procedures were done by me for a donor sample which was used in the section 4.4.

3.4 GENOMIC DNA ISOLATION FROM WHITE BLOOD CELLS

Genomic DNA was isolated from peripheral blood using Gentra-Puregene Kit (Qiagen). Lymphocytes from all the blood were separated by lysing RBCs using ice-cold lysis buffer.

5–10 mL of peripheral blood was filled up to 50 mL with ice-cold lysis buffer and mixed by vortexing and inverting. The tube was incubated on ice for 30–40 minutes with occasional mixing. The tube was then centrifuged at 590g for 4 min (4°C). The supernatant was mostly discarded and the pellet was resuspended in 15 mL of ice-cold lysis buffer and kept on ice for 10 min. After incubation, the centrifugation step was repeated. The supernatant was discarded, the pellet was resuspended in 15 mL of ice-cold PBS buffer and centrifuged at 590g for 4 min (4°C). After the final wash, the supernatant was discarded leaving approximately 10–200 μL of the residual liquid. The supernatant was

resuspended in the residual liquid and placed into a new sterile 1.5 mL tube. Then, 300–600 μL of cell lysis solution was added and mixed by vortexing for 10 s. The tube was kept for 1–14 days at RT. Then, 100–600 μL of protein precipitation solution was added and mixed by vortexing for 20 s at high speed. The tube was then centrifuged at 16 000g for 2 min. The supernatant was then transferred into a new 1.5 mL tube with 300 μL of isopropanol. The tube was gently mixed by inverting until the DNA was visible. The tube was centrifuged at 16 000g for 2 min and the supernatant was completely discarded. The pellet was resuspended in 500 μL of 70% ethanol and inverted several times. The tube was centrifuged at 16 000g for 2 min. The supernatant was carefully discarded and dried for 2–5 min at 50°C. Then, 100 μL of DNA hydratation solution was added and the tube was mixed by vortexing for 5 s. The tube was incubated at 65°C for 5 min and then gently shaked overnight at RT. The DNA samples were stored at -80°C.

3.5 PCR REACTION

PCR reactions were performed in a volume of 50 μL (for HK and PGK primers) or 25 μL (for PK primers). The 50 μL PCR reaction was composed of 0.3 μM of each forward and reverse primer, 1.25 U of MasterAmp Taq DNA Polymerase (Eppicentre), 1x PCR buffer (Applied Biosystems), 200 μM of each dNTP (Applied Biosystems) and 100–400 ng of genomic DNA. The 20μL PCR reaction was prepared using 1x HotStarTaq Master mix (Qiagen), 0.5 μM of each forward and reverse primer and 100–400 ng of genomic DNA. All PCR reactions were performed using GeneAmp ® PCR System 2700 (Applied Biosystems) or MJ Mini (Bio-Rad). The list of primers and reaction conditions are listed in the section 8 (Table S 1, Table S 2, Table S 3,Table S 4 and Table S 5).

The PCR products (5–10 μL) were separated in 1–1.5% agarose gel and visualized using ethidium bromide or GelRed (Biotum). The rest of reactions was purified using PCR purification kit (Qiaquick PCR purification kit, MinElute PCR Purification Kit, Qiagen; NucleoSpin Gel and PCR Clean-up; Macherey-Nagel) and forwarded for cycle sequencing.

3.6 CYCLE SEQUENCING

Each 20 μL sequencing reaction contained 1x Ready Reaction Premix, 1x BigDye Sequencing Buffer, 2.3 pM of either forward or reverse PCR primer and 3–10 ng of PCR product. The sequencing reaction conditions are described inTable S 6.

The sequencing products were purified using commercial purification kit (BigDye Xterminator purification kit, Applied Biosystems) according to the manufacture protocol or ethanol precipitation as described further down.

Each sequencing reaction was transferred into a new 1.5 mL tube. Then 64 μL of cold 96% ethanol and 16 μL of cold water were added and the tubes were mixed by vortexing. The tubes were incubated

in the dark for 20 min at RT. After the incubation, the tubes were centrifuged at 16 000g for 20 min (RT). The supernatant was carefully discarded and the pellet was washed with 250 µL of cold 75% ethanol. The tubes were mixed by vortexing and centrifuged at 16 000g for 10 min (RT). The supernatant was drained off and the unsealed tubes were placed in a heat block at 90°C for 1 min. Then, the pellet was dissolved in 25 µL of formamide at 95°C in the heat block for 2 min. Immediately after that, the tubes were placed on ice for 5 min. The sequencing analysis was performed on ABI 3130 automated sequencer (PE Applied Biosystems) or ABI PRISM® 3100 analyzer (Applied Biosystems). The electropherograms were analyzed using sequencing analysis software (Finch TV, version 1.4.0), genome browser BLAST and genome database ENSEMBL.

3.7 RESTRICTION ANALYSIS

A restriction analysis was performed to estimate the frequency of the mutation IV7+3A>G in a control population of >100 normal alleles (53 control subjects). A region encompassing intron 7 of *PGK1* was amplified using PCR according to the previously described conditions (the section 3.5). Then, 25 µL of each PCR product was mixed with 1x CutSmart buffer (NEB) and 10 U/mL of restriction endonuclease *Msl*I (NEB). After 60 min digestion at 37°C, the restriction reaction was terminated by heating at 80°C for 20 min. The products were resolved on 1% agarose gel and visualized using GelRed (Biotum). The patients carrying the mutation IV7+3A>G served as a positive control for each set of samples.

3.8 TOTAL RNA ISOLATION FROM HUMAN PRO-ERYTHROBLAST CELLS

The human pro-erythroblasts were lysed by adding 0.75 mL of TRIzol (Thermo Fisher Scientific) reagent per 5–10x10^6 cells and resuspended by pipetting up and down several times. After 5 min incubation at RT, 0.2 mL of chloroform per 1 mL of TRIzol was added. The tube was vigorously shaked by hand for 15 s and incubated for 2–3 min at RT. The tube was centrifuged at 12 000g for 15 min at 4°C. The upper aqueous phase of the sample was placed into a new 1.5 mL tube and 0.5 mL of 100% isopropanol per 1 mL of TRIzol was added into the tube. The tube was incubated at RT for 10 min. Then, the tube was centrifuged at 12 000g for 10 min at 4°C. The supernatant was removed and the pellet was washed with 1 mL of 75% ethanol. The tube was briefly mixed by vortexing and then centrifuged at 7500g for 5 min at 4°C. The wash was removed and the RNA pellet was dried at 55°C in a heat block for 5–10 min. The RNA pellet was resuspended in Rnase-free water (50–800 µL) and incubated in the heat block at 55–60°C for 10–15 min. Then, the RNA purity and concentration was determined by NanoVue™ (GE Healthcare Life Sciences). All RNA samples were stored at -80°C until the use.

3.9 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Reverse transcription was carried out using a two-step GeneAmp® RNA PCR Core Kit (Applied Biosystems) or SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen)/HotStarTaq Master mix (Qiagen) according to the manufacturers' procedures. First-strand cDNA was synthesized in a 20 µL volume and the reaction mix contained 1 mM each of deoxynucleotide (dATP, dCTP, dGTP, dTTP), 5 mM $MgCl_2$, 1x PCR buffer, 1 U/mL RNAase inhibitor, 2.5 U/mL reverse transcriptase (MuLV, Moloney Murine Leukemia Virus), 2.5 µM of random hexamers and 1–2 µg of RNA. The reaction conditions are listed in Table S 7.

The PCR was carried out in 50 µL reaction volume containing 10 µL of cDNA, 2 mM $MgCl_2$, 1 x PCR buffer, 0.3 µM of each forward and reverse primer (Table S 8) and 25 U/mL of AmpliTaq DNA polymerase. The reaction conditions are listed in Table S 9.

The RT-PCR products (40 µL) were resolved on a high agarose gel (NuSieve Agarose 3:1, Lonza) and visualized using GelRed (Biotum). The RT-PCR products of *HK1* and the most abundant product of *PGK1* were extracted from the gel, purified (NucleoSpin Gel and PCR Clean-up; Macherey-Nagel) and DNA sequences were determined according to the previously described protocol (the section 3.6).

3.10 QUANTIFICATION OF HK-R AND PGK1 TRANTSCRIPTS IN PATIENTS' PRO-ERYTHROBLAST CELLS

Quantitative PCR (qPCR) was performed using FastStart SYBR Green Master (Roche) and ROX Passive Reference Dye (BioRad). Each reaction contained 1x FastStart SYBR Green Master mix, 1x ROX dye, 0.5 µg of cDNA and a pair of specific primers (300 nM) (Table S 10) to avoid the contribution of aberrantly spliced RT-PCR variants in the tested samples. A real-time analysis was performed using ViiA™ 7 Real-Time PCR System (Life Technologies) according to the following cycling conditions: 10 min 95°C, 40 cycles: [30 s 94°C, 30 s 63°C, 45 s 72°C].qPCR specificity was verified by melting curve analysis and agarose electrophoresis. Beta-actin (ACTB) was used as an internal standard. Relative quantification was evaluated on the basis of the comparative CT method ($2^{-\Delta\Delta CT}$).

3.11 “TOPO CLONNING” OF ABERRANT PGK1 TRANSCRIPTS

3.11.1 TRANSFORMATION OF ONE SHOT® TOP10

The less abundant RT products encompassing exons 7 and 8 of *PGK1*, produced in the section 3.9, were subcloned into pCR™4-TOPO vector (TOPO® TA Cloning® Kit for Sequencing, Invitrogen) according to the manufacturer’s protocol. Briefly, the fresh RT-PCR product (2 μL) was mixed with salt solution (1 μL), water (2 μL) and TOPO vector (1 μL). After gentle mixing, the mixture was incubated for 5 min at RT and then chilled on ice. Then 2 μL of the cloning mixture were added into a vial of One Shot® chemically competent *E. coli*, mixed gently and incubated for 5 min on ice. The competent cells were transformed by a heat-shock method (30 s in a 42°C water bath) and immediately placed on ice. Further, 250 μL of S.O.C medium was added into the vial which was shaken at 37°C for 1 h. After shaking, 40 μL of the vial content was mixed with 20 μL of S.O.C medium and the mixture was spread on pre-warmed LB (Luria broth) plates containing kanamycin (50 μg/mL) and incubated overnight at 37°C.

3.11.2 ANALYSIS OF TRANSFORMANTS

Each colony was analyzed for the specific region encompassing exons 7 and 8 of *PGK1* using colony PCR. Each colony was picked with a sterile pipette tip and resuspended in PCR reaction mixture containing 1x HotStarTaq Master mix (Qiagen) and 0.5 μM of each forward and reverse primer (5’-CCGAGCTTCACTTTCCAAGC-3’; 5’-CCAGTGCTCACATGGCTGAC-3’). PCR was performed using the previously described PCR profile (Table S 1).

The PCR products were separated on 1.5% agarose gel and visualized using GelRed. Each positive colony was transferred into 3 mL of LB media containing 50 μg/mL of kanamycinand cultured overnight at 37°C.

3.11.3 PLASMID DNA ISOLATION

After 24 h, plasmid DNA isolation was performed using High-Speed Plasmid Mini Kit (Geneaid) according to the manufacturer’s protocol. Briefly, the harvested bacterial cells were resuspended in 200 μL of PD1 buffer and mixed by vortexing. The resolved bacterial cells were lysed using 200 μl of PD2 buffer and gently mixed by inverting the tube. After 2 min incubation, 300 μl of PD3 buffer was added into the tube and again gently mixed by inverting. The tube was centrifuged at 14–16 000g for 3 min. The supernatant was placed on a PD column with a 2 mL collection tube and centrifuged at 14–16 000g for 30 s. The column was washed with 600 μL of wash buffer and then centrifuged at 14–16,000 g for 30 s. Finally, the column was dried at 14–16 000g for 3 min and then placed

into a new 1.5 mL tube. Plasmid DNA was eluted using 30 µL of elution buffer and the sequence was determined as previously described.

3.12 IMMUNOBLOT ANALYSIS

The cultured pro-erythroblasts of a heterozygous individual carrying mutation c.873-2A>G and a healthy donor were treated with RIPA buffer (Teknova) containing protease inhibitors (100x; Thermo Scientific) and incubated for 45 min on ice. A protein concentration was determined using Bradford method according to the standard procedures. Total cell lysates (23 µg of proteins) were loaded onto a 8% precast SDS-PAGE gel (Bolt® Bis-Tris Plus gel, Life Technologies) and subsequently transferred to a PVDF membrane (Immobilion – FL, Millipore) using a wet tank method. The membrane was further blocked in Odyssey blocking buffer for 2 h and incubated with primary antibodies (anti-HK-1, 1:250, Abcam; anti-actin 1:10 000, Merc Millipore) overnight at 4°C. After the overnight incubation, the membrane was washed out two times with 1xTBS + 0.1% Tween and 1xTBS. The membrane was incubated with secondary antibodies Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit (1:7500; Invitrogen) for 1.5 hour at RT. After the subsequent washing steps (3-times, 1xTBS), the blot was imaged with an Odyssey Infrared Imaging System.

3.13 *IN SILICO* ANALYSIS, 3D STRUCTURAL ANALYSIS, MULTIPLE SEQUENCE ALIGNMENT

In silico analysis and multiple sequence alignment of mutations (p.(Asp293Val), *PKLR* gene; p.(Phe216Tyr), *G6PD* gene; p.(Ser160Pro), p.(Arg472Cys), *GPI* gene; p.(His867Tyr), p.(Thr600Met), *HK1*gene) were generated by PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/). Possible deleterious effects of novel splice site mutations c.873-2A>G, c.493-1G>A (*HK1* gene) and mutation c.756+3A>G (*PGK1*gene) were assessed using Human Splicing Finder (http://www.umd.be/HSF3/). To investigate the structural and functional consequences of four novel missense mutations (p.(His867Tyr), p.(Thr600Met), p.(Ser160Pro), p.(Arg472Cys)), 3-dimensional (3D) structural modeling of the mutants (PDB entry 1DGK (Aleshin et al., 2000); 1IAT (Read et al., 2001)) was performed using the PyMOL program (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.).

4 RESULTS AND DISCUSSION

4.1 THE LITERATURE REVIEW

The presented article provides a comprehensive overview of the current knowledge regarding red blood cell enzymopathies. It summarizes clinically relevant enzymes, their role in RBC metabolism, pitfalls and prospects in the diagnosis of these rare disorders. The meeting abstracts, presented as oral presentations, summarized the current knowledge of the molecular pathophysiology of enzymopathies, highlighted the advances in diagnosis of enzymopathies in our laboratory and evaluated the prevalence of these rare disorders in the Czech and Slovak populations.

PUBLICATIONS RELATED TO THIS AIM:

Articles:

Koralkova P, van Solinge WW, van Wijk R. ***Rare hereditary red blood cell enzymopathies associated with hemolytic anemia-pathophysiology, clinical aspects, and laboratory diagnosis.*** Int J Lab Hematol. 2014; 36(3):388-97.

Meeting abstracts:

Mojzíková R, **Pospíšilová P**, Doležel P, Pospíšilová D, Indrák K, Divoký V. ***Biochemická charakterizace enzymopatií způsobujících vrozenou hemolytickou anémii.*** Transfuze Hematol Dnes. 2011; 17(S2): 23. *(ORAL PRESENTATION)*

Kořalková P, Mojzíková R, Divoká M, Pospíšilová D, Čermák J, Suková M, Divoký V. ***Vrozené erytrocytární enzymopatie v ČR***. Konference vědeckých prací DSP, Olomouc (December 16–17, 2012), Abstract book. *(ORAL PRESENTATION)*

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4.2 DIFFERENTIAL DIAGNOSIS OF CZECH AND SLOVAK PATIENTS WITH HEMOLYTIC ANEMIA OF UKNOWN ETIOLOGY

I declare that my role in the presented results was following: enzyme assays and molecular analyses (*PKLR, G6PD* and *HK1* genes) of the majority of studied patients, *in silico* analyses, 3D modelling of the GPI mutants and manuscript preparation.

The presented study was embodied in a grant project which aimed to characterize the genetic background of alpha-thalassemia and erythroenzymopathies. This section includes two articles and two meeting abstracts. In 2013, performing enzyme assays together with mutation screening became routine methods of diagnosis of patients with HA of unknown etiology in our laboratory. The standard enzyme panel includes four enzyme activity assays determining activities of G6PD, PK, GPI and HK. Furthermore, an extensive panel of RBC enzyme assays comprising determination of PGK, ALD, PFK, bisphosphoglycerate mutase (BPGM), AK, TPI, cytochrome-b5-reductase (Cb5R) is available to exclude very rare enzyme defects. These diagnostic methods are provided only in the specialized laboratories including our department – the only laboratory in the Czech Republic, or possibly in Central Europe which offers the diagnosis of a comprehensive panel of enzymopathies.

Until now, we have analyzed 80 individuals with HA of unknown etiology. Twenty-four Czech and Slovak patients were diagnosed with different types of RBC enzymopathies. Except for the most common erythroenzymopathies, G6PD and PK deficiencies (9 and 12 cases), 2 families with GPI and 1 family with a rare HK deficiency were identified. The defects of GPI and HK were identified in the Czech and Slovak populations for the first time.

Pyruvate kinase deficiency

Pyruvate kinase deficiency is the most common enzyme defect of the Embden-Mayerhof pathway causing HNSHA. PK deficiency has been previously reported in the Czech and Slovak populations (Lenzner et al., 1997). We further diagnosed another 12 patients with PK deficiency (Mojzikova et al., 2014). All patients suffered from hemolysis and hyperbilirubinemia of different severity. None of the affected individuals had a positive family history for HA. The majority of patients were transfusion dependent. Concomitantly, three of them underwent a chelation therapy. The selected laboratory data are summarized in Table 3.

Table 3: Selected laboratory parameters of the studied patients

	Origin	Sex	Age [year]	RBCs [x10^{12}]	Hb [g/L]	Ret [0.5-3.0 %]	Bili [0-17 µmol/L]	Regular transfusions
1	CZ	M	6 m	2.76	77	7.2	33	Yes
2	CZ	M	7	3,12	97	8.1	39	Yes*
3	CZ	F	7	2.82	95	10.6	35	Yes*
4	CZ	M	10 m	2.56	75	7.5	59	Yes
5	SK	M	9	2.65	79	32	38	Yes
6	CZ	F	42	2.95	103	5.9	50	Yes+
7	CZ	F	22	1.81	65	80.5	139	Yes
8	CZ	F	31	3.05	95	6.6	34	Yes
9	CZ	M	3 m	2.49	70	2.1	172	Yes
10	CZ	F	25	2.3	73	25.3	124	Yes
11	CZ	M	50	3.4	121	5.2	49	No
12	CZ	M	21	2.10	72	60.6	ND	Yes

CZ – the Czech Republic; SK – the Slovak Republic; Age = age at the diagnosis; Ret = reticulocytes; Bili = bilirubin

All but two patients showed a decrease in PK activity below 50% of the control range. In patients 7 and 12, a high reticulocyte count masked a low PK activity, thus the PK deficiency was suspected based upon the PK/HK ratio (Table 4). Mutation screening identified eleven mutations in the *PKLR* gene (Table 4). The mutations p.(Arg510Gln), p.(Arg498His), p.(Arg532Trp), p.(Ser332Gly), p.(Arg486Trp) have been previously reported in the Czech and Slovak populations (Lenzner et al., 1997). The most common mutations in our patient group were mutations p.(Arg510Gln) and p.(Arg532Trp). The substitution p.(Arg510Gln) is also one of the most frequent *PKLR* mutation in Northern and Central Europe (Zanella et al., 2005). The mutations p.(Arg116-L117delinsGlnHisCys), p.(Gly275Arg), p.(Ala154Thr) and c.1618+1delG have been described in the literature (Pissard et al., 2006; van Wijk et al., 2009; van Zwieten et al., 2015; Zanella et al., 1997), however, none of these has been detected in the Czech or Slovak populations. Furthermore, the mutations p.(Asp293Val) and p.(Arg518Leufs*12) have not been previously described in the literature.

Table 4: Biochemical and molecular data of patients with PKLR mutation

Patient	PK activity	PK/HK ratio	PKLR mutation	Exon
1	1.63	1.63	**c.1553delG/1553delG p.(Arg518Leufs*12/Arg518Leufs*12)**	11/11
2	1.73	1.75	c.1493G>A/1529G>A p.(Arg498His/Arg510Gln)	11/11
3	1.43	0.51	c.1529G>A/1594C>T p.(Arg510Gln/Arg532Trp)	11/11
4	1.14	0.46	c.1529G>A/1594C>T p.(Arg510Gln/Arg532Trp)	11/11
5	2.64	0.78	c.347-350delinsAACATTG/347-350delinsAACATTG p.(Arg116-L117delinsGlnHisCys/Arg116-L117delinsGlnHisCys)	4/4
6	1.16	0.84	c.1456C>T/1594 C>T p.(Arg486Trp/Arg532Trp)	11/11
7	6.58	1.6	c.1594C>T/1594C>T p.(Arg532Trp/Arg532Trp)	11/11
8	1.56	0.81	**c.878A>T**/1529G>A p.(**Asp293Val**/Arg510Gln)	7/11
9	1.72	2.07	c.823G>A/1594C>T p.(Gly275Arg/Arg532Trp)	7/11
10	2.32	1.12	c.1529G>A/1529G>A p.(Arg510Gln/Arg510Gln)	11/11
11	1.54	1.14	c.994G>A/1456C>T p.(Ser332Gly/Arg486Trp)	8/11
12	6.09	1.40	c. 460G>A/1618+1delG p.(Ala154Thr/-)	5/intr 11

PK [4.6-6.28 U/g Hb]; PK/HK ratio [3.6-6.3 U/g Hb]; intr = intron; novel mutations in bold

The novel mutation p.(Asp293Val) was present together with the mutation p.(Arg510Gln) in patient 8. Multiple sequence alignments revealed that Asp293 is located in a highly conserved region. Detailed *in silico* analyses by PolyPhen and SIFT predicted that Asp to Val substitution may be deleterious. The pathogenic nature of this mutation is supported by the fact that both amino acids display different physical-chemical properties, therefore Asp to Val replacement may affect protein folding and/or catalytic activity of the enzyme.

The second novel mutation p.(Arg518Leufs*12) was associated with atypical severe transfusion dependent HA and neonatal hyperferritinemia. The patient was found to be homozygous for this

mutation. His mother was a heterozygous carrier and the father was unknown. The mutation resulted in a shift of the reading frame, leading to the formation of a premature stop codon. The PK protein (if produced) is predicted to lack the C-terminal end (45 amino acids), which includes the activator binding site Arg532, facilitating enzyme catalysis. Although iron overload is the major factor contributing to pathogenesis of PK deficiency (Zanella et al., 2005), in this case dramatic hyperferritinemia suggested an additional congenital defect. However, mutation screening of genes associated with primary hemochromatosis (*HFE, HFE2, HAMP, SCL40A1 and TFR2*) did not show any causative mutation. The patient was only a carrier of several single nucleotide polymorphisms (SNPs) in these genes which, however, were also present in other PK-deficient patients. This fact ruled out their contribution to the severe phenotype of this patient and could not explain his iron overload. To exclude other causes of severe hyperferritinemia, such as sideroblastic anemia and hereditary hyperferritinemia cataract syndrome, we evaluated the number of ring sideroblasts in the patient's bone marrow and performed molecular screening for the mutation in the IRE (iron responsive element) region of the L-ferritin gene (*FTL*). However, we did not detect the presence of either ring sideroblasts or a causal mutation in *FTL* gene. At the age of 2.5 years, surprisingly, the patient exhibited normal ferritin levels. This may be a result of a combined chelation therapy and a prolonged interval between transfusions. Thus, the severe phenotype involving neonatal hyperferritinemia was likely caused by the p.(Arg518Leufs*12) mutation itself.

Other enzyme deficiencies

As mentioned above, other enzyme defects were detected among the 80 individuals with idiopathic HA. We further diagnosed 9 cases of G6PD deficiency (manuscript in preparation), 2 families with GPI deficiency (manuscript accepted for publication) and 1 patient with very rare HK deficiency (a detailed description is provided in the section 4.3).

G6PD deficiency is the most frequent enzyme disorder in the world. The majority of *G6PD* mutations are asymptomatic, only small percentage of G6PD deficient patients suffers from HNSHA. Based on the severity of clinical manifestations, G6PD deficiency is classified into five classes, from the mildest (Class V) to the most severe (Class I). Class I mutations more frequently affect the exons 6, 10 and 13 encoding the regions that bind the enzyme substrate, dimer interface and $NADP^+$ structural site, respectively (Minucci et al., 2012).

A few mutations in *G6PD* gene have been previously identified in Czech population (Xu et al., 1995). All these G6PD variants (G6PD Praha, G6PD Olomouc, G6PD Varnsdorf – the first described splice site mutation in the *G6PD* gene) belong to the class I mutations which are associated with HNSHA (Minucci et al., 2012).

Our patient group consisted of 7 men and 2 women. All analyzed patients displayed a reduced G6PD activity suggesting G6PD deficiency as the underlying cause of HNSHA. Clinical features of the

patients varied from well compensated hemolysis to severe transfusion dependent HA. Patient 8 suffered from mild methemoglobinemia (7–11%) with no signs of hemolysis. A female patient 9 was a heterozygous carrier of the sickle cell gene; however, hematological characteristics suggested that other defect has modulated her phenotype. The detailed hematological and biochemical data are listed in Table 5.

Table 5: Hematological data of the selected patients

Patient	Origin	Sex	Age [year]	Hb [g/L]	Ret [%]	Bili [µmol/L]	G6PD [U/g Hb]
1	CZ	M	7	111	7.5	70	0.11
2	CZ	M	31	121	5.7	67	2.26
3[+]	CZ	M	36	136	9.2	ND	0.12
4[+]	CZ	M	ND	ND	ND	ND	ND
5[*]	SK	M	16	100	17.8	93	0.38
6[*]	SK	M	1	99	3.7	58	1.73
7	CZ	M	39	150	1.4	233	0.1
8[&]	SK	F	13	137	1.9	9.2 – 17.6	2.83
9[$]	CZ	F	32	125	1.5	12.1	1.81

CZ –the Czech Republic; SK – the Slovak Republic; Age = age at the diagnosis; Ret = reticulocytes [0.5-3.0 %] ; Bili = bilirubin [0-17 µmol/L];[+] siblings; * siblings; [&] a carrier of a heterozygous mutation in *HK1* gene (c.278A>G; p.(Arg93Gln); [$] a HbS carrier

Molecular screening of the *G6PD* gene revealed seven different mutations among the analyzed patients (Table 6). The G6PD variant Olomouc (c.1141T>C, p.(Phe318Leu)) was detected repeatedly in Czech population. Other identified G6PD variants (G6PD Seattle, G6PD Torun, G6PD Mediterranean, G6PD Gond) have been reported in both Czech and Slovak populations for the first time. While patients with the variants G6PD Torun and G6PD Olomouc suffered from severe HNSHA, other detected G6PD variants were characterized by mild or well compensated HA. Clinical manifestation of all studied patients corresponded to the clinical features described by previous studies (Jablonska-Skwiecinska et al., 1999; Minucci et al., 2012; Xu et al., 1995). Furthermore, we identified a novel G6PD variant, c.647T>A, p.(Phe216Tyr), which was also associated with a severe phenotype. The residual activity of the mutant enzyme was < 1% of the control range. Predictions by PolyPhen and SIFT showed that the Phe216Tyr substitution has a potentially damaging effect on the G6PD enzyme. Multiple sequence alignment and a structural model of G6PD revealed that the residue Phe216 is located in a highly conserved region of the substrate binding site. Thus, the substitution Phe216Tyr may abolish enzyme catalysis by affecting the substrate binding. All these findings indicate the pathogenic nature of this G6PD variant; severe phenotype and low residual activity classify p.(Phe216Tyr) into class I.

Table 6: Molecular screening of the G6PD gene among the studied patients

Patient	G6PD mutation	Exon	G6PD variant
1	**c.647T>A p.(Phe216Tyr)**	6	New variant
2	c.844G>C p.Asp282His	8	G6PD Seattle
3^{+}	c.1141T>C p.(Phe318Leu)	10	G6PD Olomouc
4^{+}	c.1141T>C p.(Phe318Leu)	10	G6PD Olomouc
5*	c.1006A>G p.(Thr336Ala)	9	G6PD Torun
6*	c.1006A>G p.(Thr336Ala)	9	G6PD Torun
7	c.563C>T p.(Ser188Phe)	6	G6PD Mediterranean
8	c.477G>C/477G>C p.(Met159Ile/Met159Ile)	5	G6PD Gond
9	c.202G>A/376A>G p.(Vale68Met/Asn126Asp)	4/5	-

G6PD [5.36-7.04 U/g Hb]; novel mutations in bold; $^{+}$ siblings; * siblings

GPI deficiency is the second common enzyme defect of the Embden-Meyerhof pathway. Clinical features in GPI deficiency range from mild to severe hemolytic anemia. GPI deficiency may also be associated with non-hematologic symptoms, in particular with neurologic impairment or mental retardation.

We diagnosed three GPI-deficient patients from two unrelated families. All patients suffered from severe transfusion dependent HA. No neurological defects were present in any of the studied patients. Patients' parents have no signs of chronic hemolysis. All analyzed patients exhibited reduced GPI activity, which reached 15% of the control range. Molecular screening of the *GPI* gene showed that patient 1 was a compound heterozygote for mutations c.478T>C, p.(Ser160Pro) and c.1414C>T, p.(Arg472Cys). Both patients 2 and 3 were homozygotes for the mutation c.1414C>T, p.(Arg472Cys) (Table 7). Both identified mutations are novel, however, substitution of the same residue Arg472 (Arg472His) has been previously identified in a Hispanic patient. A homozygous mutation p.(Arg472His) was associated with a severe phenotype and residual activity of this mutant GPI was around 25% of the control range. A detailed characterization of the recombinant GPI mutant demonstrated increased K_M for F6P, decreased k_{cat} and reduced thermal stability (Beutler et al., 1997; Lin et al., 2009).

Table 7: Summary of the studied GPI-deficient patients

	Patient 1	Patient 2*	Patient 3*
Origin	CZ	SK	SK
Sex	M	M	F
Age [years]	11	19	10
Hb [g/L]	90	101	86
Ret [%]	16.3	33.4	45.6
Bili [µmol/L]	84	127	83
GPI [U/g Hb]	8.51	5.94	6.75
GPI mutations	c.478T>C/1414C>T p.(Ser160Pro/Arg472Cys)	c.1414C>T/1414C>T p.(Arg472Cys/Arg472Cys)	c.1414C>T/1414C>T p.(Arg472Cys/Arg472Cys)

CZ – the Czech Republic; SK – the Slovak Republic; Age = age at the diagnosis; Ret = reticulocytes [0.5-3.0 %] ; Bili = bilirubin [0-17 µmol/L];* siblings, GPI [53 – 62 U/g Hb]

Results from *in silico* analyses (Polyphen and SIFT) revealed that both detected mutations p.Arg472Cys and p.Ser160Pro are not tolerated. To study the probable mechanism by which both mutations affect the protein function, we performed *in silico* modelling of the GPI mutants. The analyses demonstrated that the residue Arg472 is a part of the dimer-domain at the edge of the large external loop domain, a major contact region contributing to the dimer stability. The Arg472Cys substitution likely destabilizes interactions within the dimer interface, and thus may affect either the dimer formation and/or dimer stability. The residue Ser160 is located in the close proximity of the active site, facilitating the binding of the substrate phosphate group. This suggests that the substituted proline likely impairs the binding of the substrate, and thus inhibits catalytic activity of the mutant enzyme (Read et al., 2001).

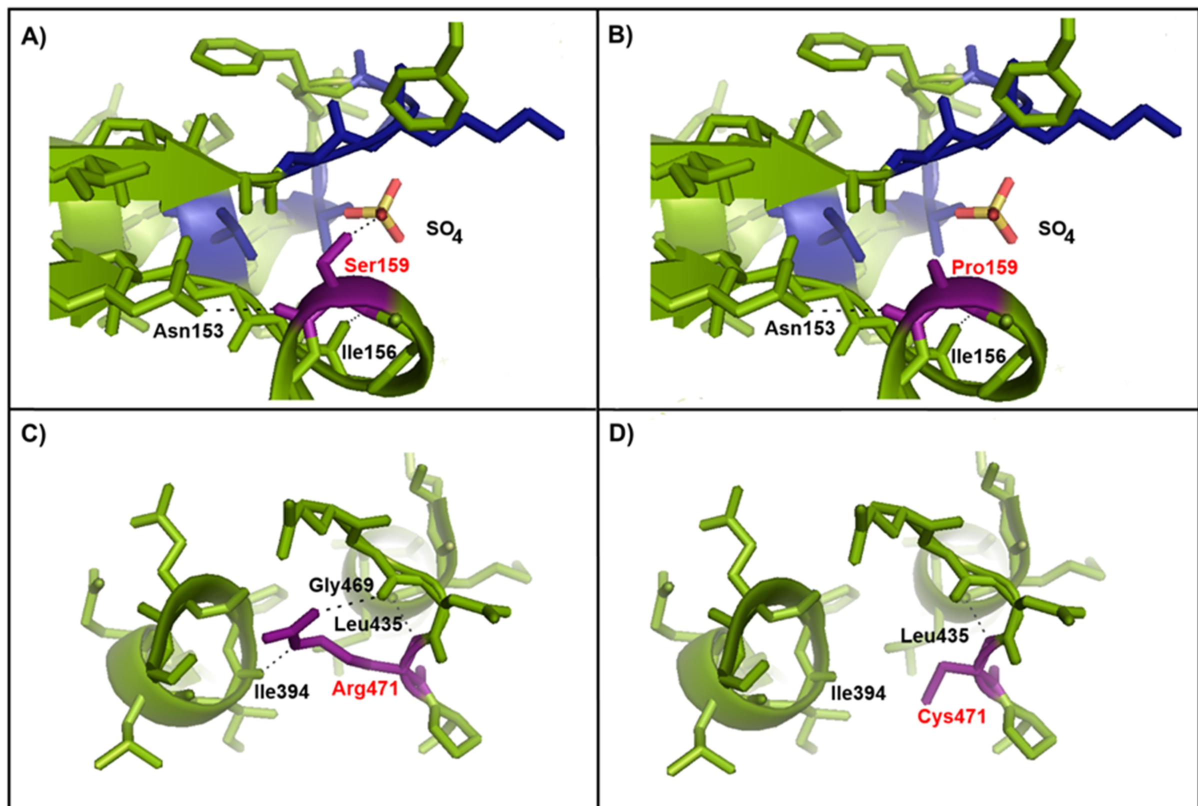


Figure 6: In silico modelling of the Ser160Pro and Arg472Cys GPI mutants. *A) Ser160 (corresponding to Ser 160, shown in purple) is a part of the phosphate-binding site (blue) responsible for the potential hydrogen bond interaction with phosphate group of the GPI substrate (the sulphate was used to mimic the substrate phosphate group). B) Ser160Pro substitution likely results in a disruption of the hydrogen bond (a black dotted line) between the residue at position 159 and the substrate phosphate group and, thus, destabilizes the substrate-enzyme complex. C) Arg472 (corresponding to Arg471, shown in purple) is located at the edge of the large external loop (LEL, 438–468 residues) directly interacting (hydrogen bonding) with neighboring Gly469 residue and with Ile394 of the second monomer, and thus D) disruption of these interactions by Arg472Cys mutationleads to conformational changes within the LEL, serving as a dimer stabilizing hook, and thus to generation of a more labile GPI-dimer. The amino acid numbering begins with alanine as residue 1 instead of initial methionine. The structure illustrations were created with PyMOL program based on Protein data bank ID 1IAT. Black dot lines represent hydrogen bonds.*

Kinetic studies of the mutation p.(Arg472Cys) revealed decreased affinity to F6P and strongly reduced thermal stability. Furthermore, the enzyme activity was almost abolished after short incubation at 45°C. These findings are consistent with the previous functional study of p.Arg472His GPI mutant (Lin et al., 2009) and all together correspond to the model of the proposed pathogenic mechanism. The Ser160Pro mutation was characterized in the individual who was a compound heterozygous with p.(Arg472Cys) mutation. Thus, the contribution of the second mutation has to be expected. Biochemical characteristics of the compound heterozygous mutant showed decreased K_M values and its thermal stability was only slightly affected which may indicate that Arg472Cys homodimers represent only a minor proportion of the mixed dimers pool.

HK deficiency was diagnosed in a patient with severe transfusion dependent HA. The patient was found to be homozygous for mutation c.278A>G, p.(Arg93Gln). Interestingly, another two cases with *HK1* mutation were identified. One patient was a heterozygous carrier of mutation c.493-1G>A. However, phenotype more severe than expected for an *HK1* mutation carrier was probably caused by Gilbert syndrome. The second patient possessed mutation c.278A>G, p.(Arg93Gln). The female patient was also homozygous for mutation c.477G>C p.(Met159Ile) in the *G6PD* gene (Table 6). Detailed description of HK deficiency is in the section 4.3.

In general, enzyme defects are associated with classical hallmarks of hemolysis with no abnormalities in RBC morphology. Routinely used hematology methods are unable to point out a particular enzyme defect. Due to the lack of adequate diagnostic methods, enzymopathies have been previously considered as medical conditions belonging to the category of diagnosis by exclusion. In 1977, the International Committee for Standardization in Hematology established "recommended methods" for the assaying of RBC enzymes (Beutler et al., 1977). These direct activity assays followed by an identification of a causative mutation at DNA level are the only worldwide accepted procedures for erythroenzymopathies diagnosis in current clinical practice. Our laboratory is the only laboratory in the Czech Republic, or possibly in Central Europe which offers a diagnosis of the whole panel of enzymopathies.

In addition to diagnosis of erythroenzymopathies, the direct enzyme assays have been also found to be an effective method for diagnosis of Diamond Blackfan anemia (DBA) and/or to test, for example, the effects of therapeutics on cellular processes. Alterations of erythrocyte ADA activity are observed in the majority of "classical" DBA patients (Fargo et al., 2013). Recently, we have established a direct enzyme assay for erythrocyte ADA in our laboratory. Upregulated ADA activity has become a useful diagnostic maker in a "classical" form of DBA. With regard to testing the effects of potential therapeutics, we studied (in collaboration with a research group from Tübingen), the effect of pharmacologic NF-κB inhibitors, Bay 11-7082, parthenolide and dimethyl fumarate DMF on red blood cell survival and on mechanism of GSH depletion. The inhibitory effects of these pharmacologic compounds were evaluated by monitoring enzymatic activities of G6PD, 6PGD and GR, the key players of cellular redox homeostasis in red blood cells (Ghashghaeinia et al., 2016). Furthermore, the enzyme assays were used for the assessment of anti-oxidative defense in erythrocytes with deficiency of the divalent metal transporter 1 (Zidova et al., 2014).

Taken together, characterization of RBC enzymopathies by biochemical and molecular analyses contributed to a better understanding of pathophysiology of these rare disorders. The direct enzyme assays have a large variety of scientific applications which may help to improve diagnostic testing as well as therapeutic interventions.

PUBLICATIONS RELATED TO THIS AIM:

Articles:

Ludíková B, Mojzíková R, **Pospíšilová P**, Houda J, Sulovská L, Divoká M, Hak J, Procházková D, Divoký V, Pospíšilová D. ***Deficit pyruvátkinázy v dětském věku.*** Čes-slov Pediat. 2012; 67(3): 6–11.

Mojzikova R*, **Koralkova P***, Holub D, Zidova Z, Pospisilova D, Cermak J, Striezencova Laluhova Z, Indrak K, Sukova M, Partschova M, Kucerova J, Horvathova M, Divoky V. ***Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios.*** Br J Haematol. 2014;165(4):556–63.(*Authors contributed equally to this work)

Mojzikova R*, Koralkova P, Holub D, Saxova Z, Pospisilova D, Prochazkova D, Dzubak P, Horvathova M, Divoky V ***Two novel mutations (p.(Ser160Pro) and p.(Arg472Cys)) causing glucose-6-phosphate isomerase deficiency are associated with erythroid dysplasia and inappropriately suppressed hepcidin.*** *Blood Cells Mol Dis.,* manuscript accepted for publication.

Meeting abstracts:

Pospisilova P, Mojzikova R, Partschova M, Zidova Z, Horvathova M, Pospisilova D, Divoky V. ***Hemolytic anemia with hyperferritinemia in Czech pyruvate kinase deficient patient – a novel homozygous frameshift deletion in PKLR gene (p. R518fs) combined with TFR2 (p. R752H) and SLC40A1 (IVS1-24 C>G) gene polymorphisms.*** 19th Meeting of the European Red Cell Society, Ijmuiden, The Netherlands, 10–13 October, 2013: Abstract book, p. 47. *(ORAL PRESENTATION)*

Kořalková P, Mojzíková R, Pospíšilová D, Divoká M, Čermák J, Indrák K, Suková M, Timr P, Striežencová Laluhová Z, van Wijk R, Divoký V. ***Vzácné vrozené anémie v české a slovenské populaci: pokroky v diagnostice erytrocytárních anémií.*** XVII. SLOVENSKO-ČESKÝ HEMATOLOGICKÝ A TRANSFUZIOLOGICKÝ ZJAZD – Bratislava, Slovak Republic (24–27 September, 2015), Abstract book, p.84. *(ORAL PRESENTATION)*

4.3 STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF VERY RARE HEXOKINASE MUTANTS CAUSING HNSHA

I declare that my role in the presented results was following: summarization of the hematological and biochemical data from all the studied patients, enzyme assays and molecular analyses of the studied patients except the patients from Denmark and the Netherlands, kinetic studies of the selected patients, RNA isolation, RT-PCR, qPCR, western blot analysis, *in silico* analyses, 3D modelling of selected HK mutants and manuscript preparation.

The major part of the experimental work was carried out during my fellowship at the Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, the Netherlands. This section contains one original research paper and three meeting abstracts. The publications presented here resulted from collaboration with research groups from the Netherlands, Ireland, Spain and the Slovak Republic. The article describes molecular analysis of six HK-deficient patients representing the largest group studied thus far. It summarizes the current knowledge about HK deficiency and unifies numbering for previously detected mutations according to the erythroid specific HK-R transcript (HK-R NM_033496.2).

Hexokinase is the key enzyme of the Embden-Meyerhof pathway that catalyzes the ATP-dependent phosphorylation of glucose, on which red cell energy metabolism is fully dependent. HK deficiency is an autosomal recessive disorder associated with chronic HSNHA. Affected individuals exhibit mild to severe lifelong HA. However, a complete loss of HK1 is apparently incompatible with life (Kanno et al., 2002).

We analyzed seven patients from 6 unrelated families with a suspected enzyme deficiency. Standard methods including peripheral blood smear examination, cryohemolysis/Pink test, osmotic fragility test, (EMA) binding test, hemoglobin stability test (isopropanol test), and hemoglobin SDS-PAGE were used to exclude hemoglobinopathies and membrane defects. The majority of the patients suffered from chronic hemolysis with variable clinical severity (Table 8). All but one patient showed a decreased HK activity below 50% of the control range and/or increased PK/HK ratio (Table 8). So far, 24 cases of HK deficiency associated with HNSHA have been reported (Mentzer and William, 2014) and only 6 mutations as an underlying cause of HK deficiency have been detected (Bianchi et al., 1998; de Vooght et al., 2009; Kanno et al., 2002; van Wijk et al., 2003). Molecular analysis of our patient group revealed 6 different mutations in *HK1* gene, four of them were novel: c.2599C>T p.(His867Tyr), c.1799C>T p.(Thr600Met), c.873-2A>G and c. 493-1G>A (Table 9, novel mutations in bold). To clarify the pathogenic nature of the novel mutations we performed biochemical and/or RNA studies, a 3-D structural analysis and *in silico* analyses by PolyPhen and SIFT.

Table 8: Hematological and biochemical data of the studied patients

	Patient 1[S]	Patient 2[S]	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Origin	Ireland	Ireland	Czech Republic	Denmark	Netherlands	Czech Republic	Slovak Republic
Sex	M	M	M	M	M	F	F
Age [year]	2	4	8 m	2	22	12	12
RBCs [$x10^{12}$]	2.57; (*--)	2.76; (*--)	2.40; (*--)	2.90; (*--)	4.40; (*--)	4.25; (-*-)	4.58; (-*-)
Hb [g/L]	79; (*--)	95; (*--)	74; (*--)	86; (*--)	160; (-*-)	135; (-*-)	137; (-*-)
Ret [%]	8.8; (--*)	6.2; (--*)	6.3; (--*)	4.7; (--*)	6.0; (--*)	3.1; (--*)	1.9; (--*)
Bili [µmol/L]	54; (--*)	62; (--*)	120; (--*)	124; (--*)	24; (--*)	100; (--*)	17.6; (-*-)
Transfusion therapy	Yes[$]	No	Yes	Yes	No	No	No
Other diagnosis	ND	ND	ND	ND	GS	GS	G6PD Gond
Enzyme activities							
HK	0.64[¥]	0.58[¥]	0.71[#]	1.14[+]	0.65[¥]	0.37[#]	0.33[#]
PK	12.3[¥]	12.6[¥]	3.3[#]	12.5[+]	12.2[¥]	7.2[#]	6.2[#]
PK/HK	19.2[¥]	21.7[¥]	9.5[#]	10.90[+]	18.7[¥]	19.3[#]	18.7[#]

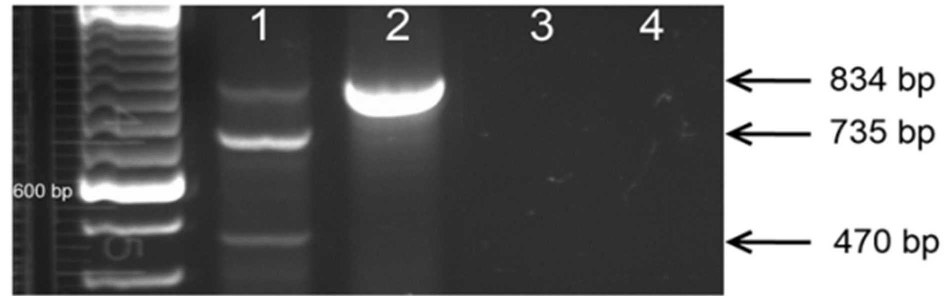
S – siblings; Age – age at diagnosis of HK deficiency, m – months; RBCs – red blood cells; Hb – hemoglobin, M – male; F – female; ND – not determined, GS – Gilbert syndrome.
G6PD – glucose-6phosphate deficiency, variant – GOND; [$]Transfusion therapy has been stopped at the age 3; comparison of hematological data with a reference range: **(*--)** – low; **(-*-)** – normal; **(--*)** – high.
[¥]HK [0.80-1.50 U/g Hb], PK[6.1-12.3 U/g Hb], PK/HK [4.8-11.9]; [#]HK [0.80-1.65], PK[5.1-5.8], PK/HK [3.6 -6.3]; [+]HK [0.62-1.26], PK [8.4-15.2], PK/HK [7.2-15.6].

Table 9: Molecular analysis of HK-deficient patients

	Allele1	Allele 2
	Localization	Localization
Patient 1	c.-193A>G Erythroid promotor	**c.873-2A>G** Intron 7
Patient 2	c.-193A>G Erythroid promotor	**c.873-2A>G** Intron 7
Patient 3	c.278A>G, p.(Arg93Gln) Exon 3	c.278A>G, p.(Arg93Gln) Exon 3
Patient 4	**c.2599C>T, p.(His867Tyr)** Exon 17	**c.2599C>T, p.(His867Tyr)** Exon 17
Patient 5	**c.1799C>T, p.(Thr600Met)** Exon 12	Normal -
Patient 6	**c.493-1G>A** Intron 4	Normal -
Patient 7	c.278A>G, p.(Arg93Gln) Exon 3	Normal -

The mutations are numbered according to the erythroid specific transcript HK-R (NCBI Reference Sequence: NM_033496.2). Novel mutations are in bold.

The novel splice site mutation c.873-2A>G was found in both patients 1 and 2 who concomitantly possessed a mutation c.-193A>G. The maternally inherited mutation c.-193A>G, located in a promotor region, has been previously characterized (de Vooght et al., 2009). A reduced activity of the erythroid specific promoter has been postulated as the underlying mechanism for HK deficiency associated with this mutation (de Vooght et al., 2009). The novel splice site mutation c.873-2A>G was inherited from the patients' father who was found to be a heterozygous carrier of this mutation. *In silico* analysis by Human Splicing Finder predicted that this A>G substitution abolishes the intron 7 acceptor site. To determine how this A>G substitution affects mRNA processing, we performed detailed RNA studies. Sanger sequencing analysis of RT-PCR products revealed that the c.873-2A>G mutation resulted in abnormally spliced transcripts lacking either exon 8 alone or both exons 8 and 9 (Figure 7).



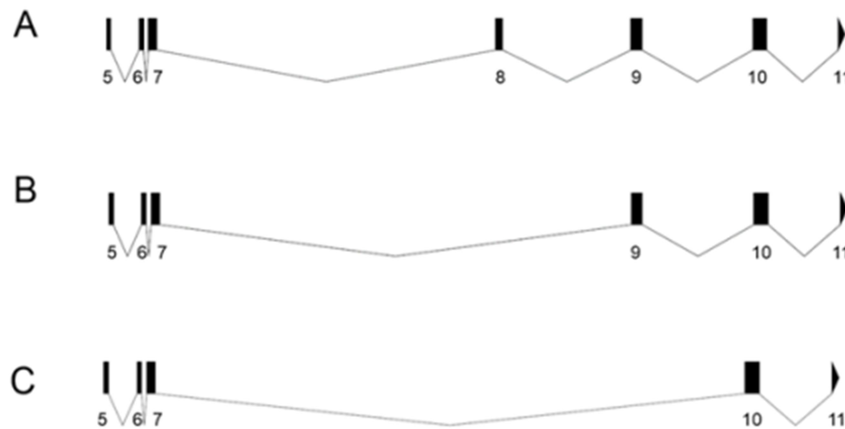


Figure 7: RNA studies of a splice site mutation c.873-2A>G. *Agarose electrophoresis. Visualization of RT-PCR products (a region spanning exons 7 to 9) revealed two additional fragments in the heterozygous subject carrying the mutation c.873-2A>G (line 1) compared to the healthy donor (line 2). Lines 3 and 4 served as negative controls for RT and PCR. Bands of 834 bp (lines 1 and 2) corresponded to a normally spliced variant A. Fragments of 735 bp and 470 bp (line 1) corresponded to the abnormal transcripts B and C lacking either exon 8 or both exons 8 and 9.*

The aberrant proteins (if produced) would lack either the substrate binding site or the substrate binding site and the G6P binding site. Furthermore, pre-mRNA expression of the normally spliced variant of the heterozygous c.873-2A>G carrier was more than 50% lower than in controls (Figure 8). Subsequently, western blot analysis on *in-vitro* cultured pro-erythroblast revealed a marked down-regulation of HK at protein level (Figure 8). Based on these findings, we could conclude that the c.873-2A>G mutation affects pre-mRNA processing, which results in a down-regulation of HK on the protein level.

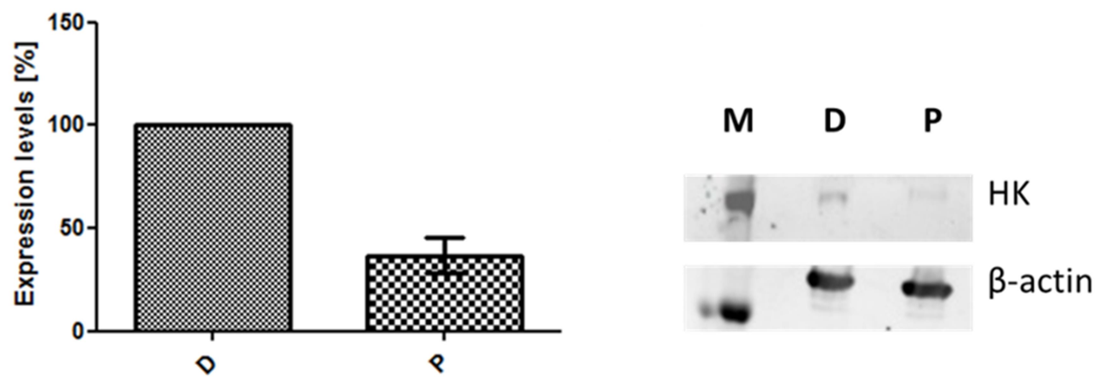


Figure 8: qPCR analysis. *A down-regulated expression of the normally spliced HK transcript of the heterozygous carrier (P) was observed compared to healthy donors (D). Data were normalized to ACTB gene.* ***Western blot analysis.*** *Immunoblotting of ex-vivo cultured pro-erythroblasts demonstrated a marked decrease of HK protein, which corresponded with the decreased expression of thenormally spliced HK transcript. M – marker, D – donor, P – the heterozygous subject carrying the mutation c.873-2A>G.*

A homozygous mutation c.278A>G, p.(Arg93Gln) was detected in patient 3 who suffered from severe transfusion dependent HA, psychomotor retardation and epilepsy (Table 9). Although psychomotor retardation has been previously described in HK deficiency (Gilsanz et al., 1978; Goebel et al., 1972; Magnani et al., 1985), in this case the malformations were likely associated with parental consanguinity and/or CNS bleeding that has occurred during the labor and delivery. Recently, de Voogth et al. have reported this mutation c.278A>G, p.(Arg93Gln) in the heterozygous form in patient with mild HA. They hypothesized that the mutation resulted in a formation of truncated HK variants which are most likely inactive and unstable (de Vooght et al., 2009). Despite the homozygous form of the mutation in our patient, the normally spliced HK variant carrying the c.278A>G mutation is produced in patients' RBCs. However, further examination of the residual HK protein showed a high instability (Figure 9), an increased ATP affinity and a reduced affinity for glucose.

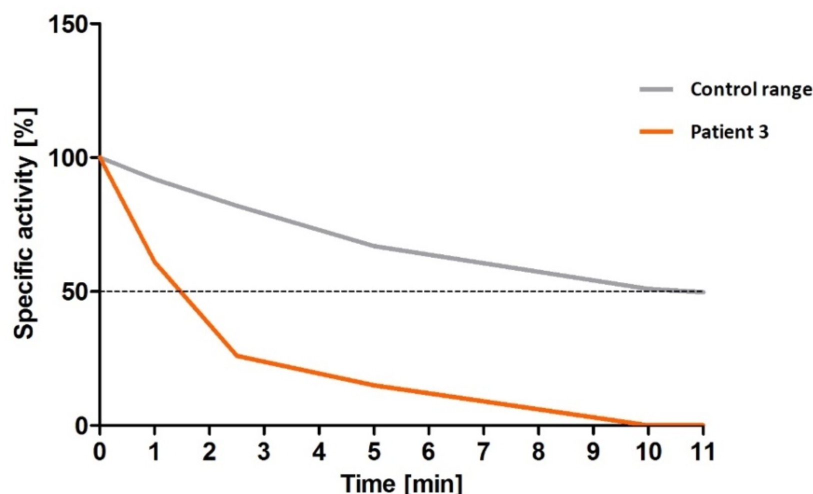


Figure 9: Thermal stability assay. *A high instability of the residual HK protein carrying the c.278A>G mutation (patient 3, colored in orange) compared to the control range*

Interestingly, the same heterozygous mutation c.278A>G, p.(Arg93Gln) was detected in another Czech patient (patient 7). In addition to this, the female patient had a homozygous mutation c.477G>C, p.(Met159Ile) in *G6PD* (Table 6). Despite these molecular defects, no hemolysis was observed and only slight methemoglobinemia (7–11%) was the clinical presentation of this patient.

The novel homozygous mutation c.2599C>T, p.(His867Tyr) was associated with severe HA requiring exchange transfusions and chelation therapy (Table 8, Table 9). *In silico* analysis by PolyPhen-2 and SIFT predicted that a substitution of His to Tyr, respectively, is not tolerated. Subsequent 3D structural analysis of this mutation revealed that His867 is located near the ADP-ligated active site, providing high energy transfer of phosphates to glucose (Figure 10). Any disruption of this process suggested an alteration of enzyme kinetic properties. Subsequent kinetic studies confirmed that the patient's K_M for ATP was 3.3-times increased compared to controls and the K_M for glucose was slightly increased.

The same catalytic process was probably impaired due to a heterozygous mutation c.1799C>T p.(Thr600Met) detected in patient 5 (Table 9). Contrary to the previous substitution, no changes in hydrogen bonds among the affected residues were observed. In this case, the catalytic mechanism was probably disrupted due to the presence of weakly reactive methionine which indirectly inhibits glucose-6-phosphate biding (Figure 10).

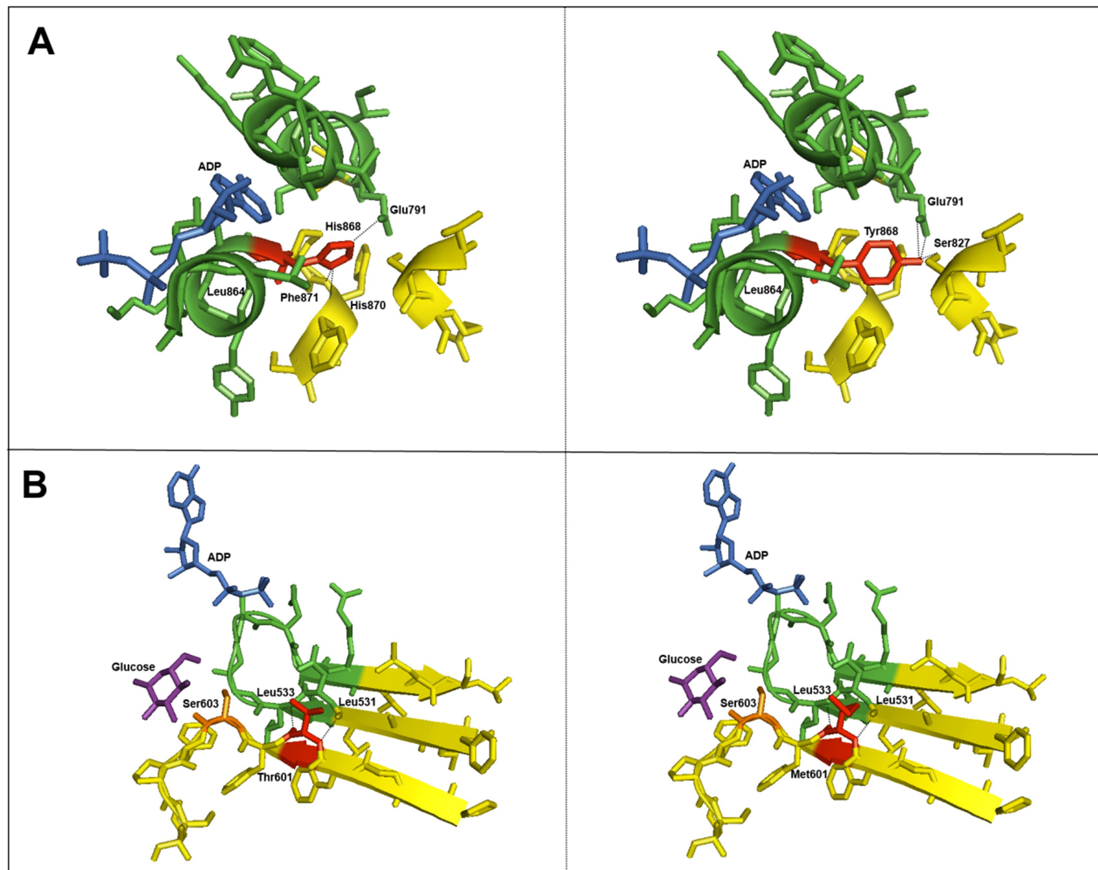


Figure 10: 3D crystal structure of HK-R mutants His867Tyr and Thr600Met (PDB 1DGK – protein databank). *A) His867 (corresponding to His868 in HK-1, shown in red) is located between ADP-ligated active sites (green). No direct interaction between ADP (blue) and His has been described. However, His substitution with Tyr probably leads to a disruption of multiple hydrogen bonds (black dotted lines) among residues Phe871, His870 and to a formation of new hydrogen bonds with Glu791 and Ser827. The changes in hydrogen bonding likely affect electrostatic interactions through the ADP-ligated active site, leading to interaction impairments within the ADP/glucose-complex. B) Met601 (corresponding to Met600 in HK-1, shown in red) is in close proximity of loop 532–539 (green) which is involved in productive binding of ADP (blue) and Ser603 (orange) directly interacting with a molecule of glucose (purple). Although no changes in hydrogen bonding were observed, the Met substitution with Thr in this position may impair electrostatic interactions within an enzyme catalysis site affecting the mechanism of phosphate transfer.*

The novel mutation c.493-1G>A in the heterozygous form was identified in patient 6. The female patient was first diagnosed with Gilbert syndrome, but persistent mild hemolysis and reticulocytosis pointed to the presence of an additional modulator. *In silico* analysis of the mutation c.493-1G>A predicted an activation of a cryptic splice site within intron 4, resulting in formation of aberrant HK variant(s). Based on current findings we can conclude that the aberrant splicing is likely the major factor contributing to HK deficiency in this patient. However, it is not clear whether or not heterozygosity for this mutation is sufficient to modulate patient's phenotype.

This work characterizes genetic background of six patients with a very rare HK deficiency, to our knowledge the largest cohort of studied HK deficient patients. Detailed investigations on the selected mutations clarify their pathogenic nature and thus this work contributes to better understanding of the genotype-phenotype correlation in HK deficiency.

PUBLICATIONS RELATED TO THIS AIM:

Articles:

Koralkova P, Mojzikova R, van Oirschot B, Macartney C, Timr P, Vives Corrons JL, Striezencova Laluhova Z, Lejhancova K, Divoky V, van Wijk R. ***Molecular characterization of six new cases of red blood cell hexokinase deficiency yields four novel mutations in HK1.*** Blood Cells Mol Dis. 2016; 59:71-6.

Meeting abstracts:

Pospisilova P, Mojzikova R, Pospisilova D, Divoky V, Vives-Corrons JL, van Wijk R. ***Two novel homozygous mutations (Agr94Gln and His868Tyr) in HK1 gene associated with hereditary non-spherocytic hemolytic anemia described in Spain and Czech patients.*** 19th Meeting of the European Red Cell Society, Ijmuiden, The Netherlands, October 10–13, 2013. Abstract book, p. 60. *(POSTER PRESENTATION)*

Timr P, **Kořalková P**, Mojzíková R, van Wijk R, Divoký V. ***První případ populace hexokinasy v ČR způsobující nesférocytární hemolytickou anémii.*** XXIV. Konference dětských hematologů a onkologů České a Slovenské republiky, Olomouc, Česká republika October 10–12, 2014. Abstact book, p. 37. *(ORAL PRESENTATION)*

Koralkova P, Mojzikova R, Timr P, Vives Corrons JL, Macartney C, Divoky V, van Wijk R. ***Five new cases of hexokinase deficiency: biochemical and molecular characterization of a novel splice site mutation and 2 novel missense mutations in HK1.*** 20thCongress of the European Hematology Association, Vienna, Austria, June 12–14, 2015. Abstract book – Haematologica, p. 128. *(POSTER PRESENTATION)*

4.4 MOLECULAR CHARACTERIZATION OF A NOVEL NON-HEMOLYTIC ANEMIA VARIANT OF PGK

I declare that my role in the presented research was following: molecular analysis of the studied patients, cell culture of a donor sample, restriction analysis, RNA isolation, RT-PCR, qPCR analysis, TOPO cloning, *in silico* analyses and participation in the manuscript preparation.

The major part of the experimental work was carried out during my fellowship at the Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, the Netherlands. The project was conducted in collaboration with research centers from Belgium and the Netherlands. This section contains one research paper and one meeting abstract. They describe molecular characterization of PGK deficiency in two patients with myoglobinuria, mental retardation, but no signs of hemolysis.

PGK deficiency is a rare X-linked disorder associated with multiple clinical manifestations including hemolytic anemia, mental retardation, exercise intolerance, myoglobinuria, rhabdomyolysis and/or parkinsonism. The majority of patients suffer from hemolytic anemia combined with neurological defects. Myopathy and muscle weakness as the predominant clinical features have been described in several cases. Conversely, a full spectrum of clinical manifestations is rarely present (Beutler, 2007; Sotiriou et al., 2010).

The studied patients were two brothers from healthy, non-consanguineous parents. They suffered from mild retardation, recurrent episodes of muscle weakness and pain after moderate exercise since the age of 4. The younger brother experienced an episode of myoglobinuria and seizures. In the further clinical course, laboratory parameters did not show any signs of hemolysis but a markedly decreased activity of PGK (<15% of the reference range) was detected in both patients. A slight reduction in PGK activity was observed in patients' mother; father was within the control range. To confirm PGK deficiency at the molecular level, we performed mutation screening of the *PGK1* gene. The analysis revealed the presence of hemizygous mutation c.756+3A>G in intron 7 in both patients. The heterozygous form of this mutation was identified in their mother; no mutation was detected in the patients' father. To exclude this mutation as neutral polymorphism, we performed screening of >100 alleles from control subjects. The mutation was not present in the control population. Thus, we analyzed the possible effect of the A>G substitution on the mRNA splicing processing using Human Splicing Finder. This *in silico* analysis predicted that the affected region abolishes the intron 7 donor splice site. To confirm the particular effect of the mutation, we isolated total RNA from *ex-vivo* cultured proerythroblasts of the respective patients and conducted detailed RNA studies. Visualization of RT-PCR fragments of both patients showed two additional fragments compared to a control subject (Figure 11). Unfortunately, the low concentration of both

fragments precluded direct Sanger sequencing. Therefore, to analyze the specific sequence of these abnormal fragments, we subcloned them into pCR^{TM}4-TOPO vector.

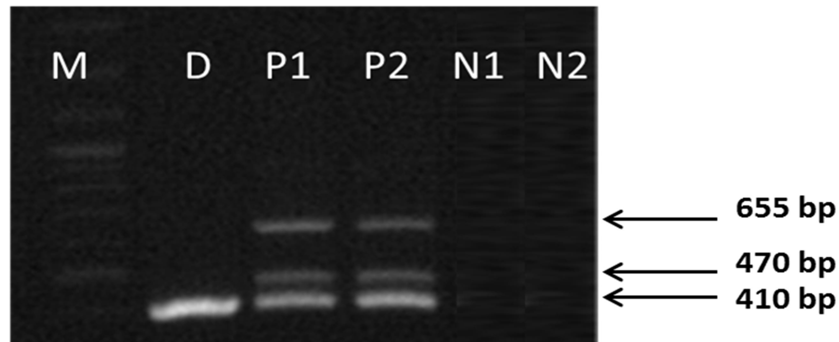


Figure 11: Visualization of RT-PCR fragments of hemizygous c.756+3A>G mutation carriers and a control subject. *A region spanning exons 7 and 8 of PGK1 were amplified. Presence of two additional fragments was observed in both patients (P1 and P2) compared to healthy donor (D). Lines N1 and N2 served as negative control for reverse transcription and PCR.*

The sequencing analysis of positive colonies revealed the presence of two additional aberrant transcripts. Thus, a total of four different PGK variants was detected in the hemizygous carriers of c.756+3A>G mutation compared to the control subject (Figure 11). The most abundant PGK variants A and B consist of 52 bp of intron 7 and/or a complete sequence of intron 7. Both variants result in the introduction of a premature stop codon. The predicted protein (if translated) lacks the C-terminal end, containing 165 amino acids, which facilitates nucleotide binding. The aberrant PGK transcript A, encompassing 52 bp of intron 7, has been previously reported in patients with PGK Fukuroi (c.756+5G>A) and with PGK Antwerp (c.755A>C, p.(Glu252Ala)). Both patients also exhibited recurrent episodes of rhabdomyolysis, myoglobinuria without HA. However, additional clinical manifestations (abnormal brain activity and/or slight changes in muscle fiber morphology) have been reported in both patients (Ookawara et al., 1996; Shirakawa et al., 2006).

The transcripts C and D were characterized by a loss of either 18 bp of exon 7 and/or a complete region of exon 7. The variant lacking 18bp of exon 7 results in deletion of six amino acids (residues 247–252). The loss of complete exon 7 causes a premature codon formation and the predicted protein (if translated) lacks the C-terminal end. None of these PGK variants has been described in the literature (Figure 12).

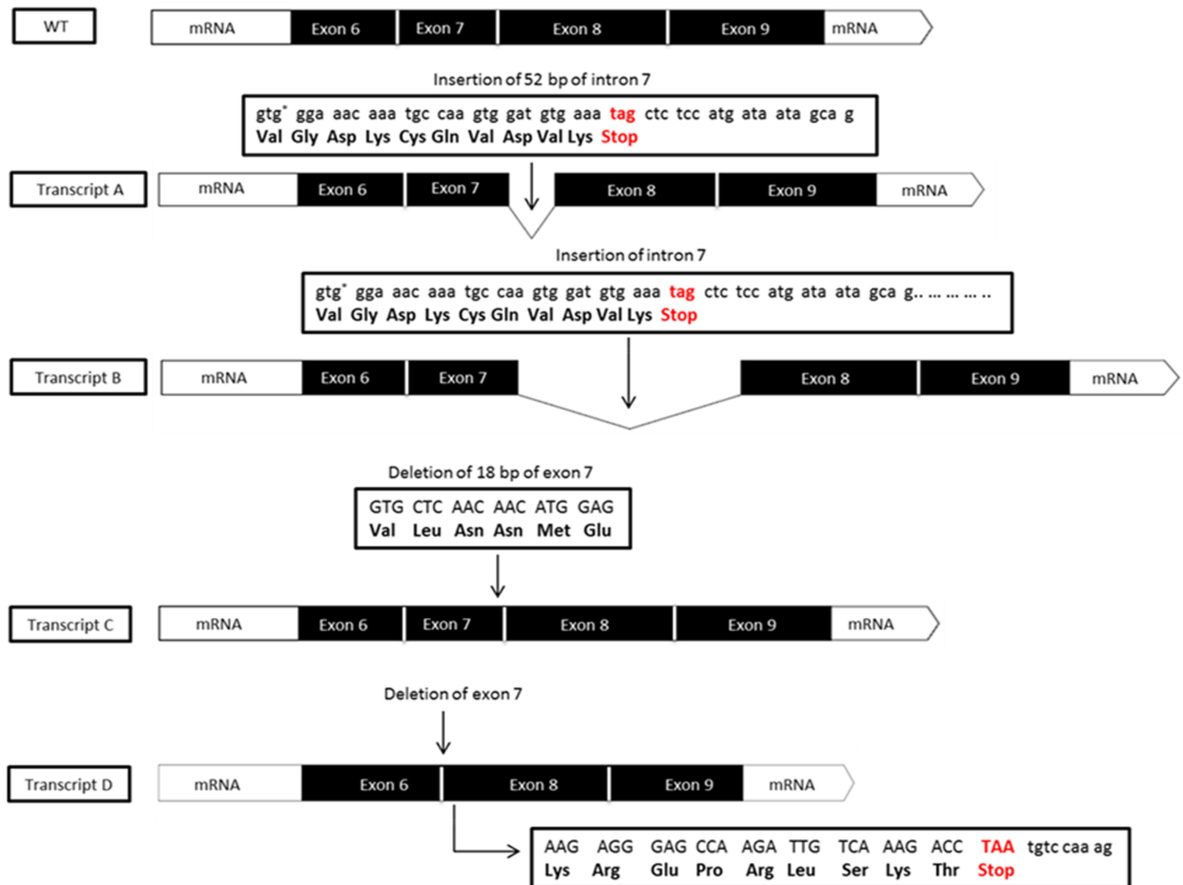


Figure 12: A schematic overview of all detected PGK variants. *The variant WT corresponds to normally spliced variant (band 410 bp). Aberrantly spliced transcripts A and B retain 52 bp of intron 7 or the complete sequence of intron 7. Two additional transcripts C and D, less abundant fragments, lack 18 bp of exon 7 or a region spanning complete exon 7.*

Despite of all these aberrant PGK variants, normal pre-mRNA processing does occur. To quantify the expression levels of the normally spliced variant, we designed a specific pair of primers to avoid the contribution of the additional aberrant PGK variants, and performed qPCR. The analysis showed that the level of the normally spliced PGK variant was 8.5 times down-regulated compared to the healthy individual (Figure 13).

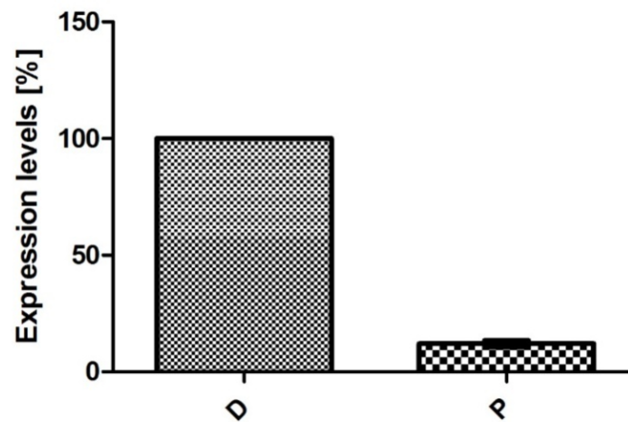


Figure 13: Expression levels of normally spliced PGK pre-mRNA. *ACTB was used as an internal standard. Relative quantification was evaluated based on the comparative CT method (2-ΔΔCT). D – donors, P – patients.*

Taken together, this study showed that c.756+3A>G mutation results in aberrant splicing and ineffective protein production, most likely the underlying cause of PGK deficiency in both patients.

PUBLICATIONS RELATED TO THIS SPECIFIC AIM:

Articles:

Coppens S, **Koralkova P**, Aeby A, Mojzikova R, Deconinck N, Kadhim H, van Wijk R. ***Recurrent episodes of myoglobinuria, mental retardation and seizures but no hemolysis in two brothers with phosphoglycerate kinase deficiency.*** Neuromuscul Disord. 2016; 26(3):207-10.

Abstract meetings:

Coppens S, Deconinck N, van Wijk R, **Koralkova P**, Van Bogaert P, Aeby A. ***Recurrent episodes of myoglobinuria, mental retardation and epilepsy but no haemolysis in two brothers with phosphoglycerate kinase deficiency.*** 19th International Congress of The World Muscle Society – Berlin, Germany (October 7–11, 2014), Neuromuscular Disorders Volume 24, Issues 9–10, p. 873. *(POSTER PRESENTATION)*

4.5 *IN SILICO* MODELLING OF A NOVEL MUTATION OF *PFKM* GENE ASSOCIATED WITH PFK DEFICIENCY

I declare that my role in the presented research was following: *in silico* analyses, 3D structural modelling of the PFK mutant and participation in the manuscript preparation.

This section includes one article. The publication resulted from collaboration of Dr. van Wijk laboratory with research centers from Spain and the Netherlands. The presented article describes the first case of a rare PFK deficiency diagnosed in a 65-year-old female of Spanish origin.

PFK deficiency is a very rare disorder associated with multiple clinical manifestations including muscle cramps, exercise intolerance, myoglobinuria and/or hemolysis. Erythrocyte PFK is composed of PFKL (liver) and PFKM (muscle) subunits which form their hybrids (M4, L4, M3L, M2L2, and ML3). In case of a mutated PFKM subunit, only PFKL subunits remain fully functional in erythrocyte PFK leading to only a partial deficiency of PFK. Thus, patients with PFK deficiency usually exhibit mild or fully compensated hemolytic anemia (Nakajima et al., 2002).

The female patient suffered from recurrent episodes of spasm with muscle weakness and pain after moderate exercise since her childhood. She had a laboratory evidence of moderate anemia. Direct enzyme assays revealed a reduced PFK activity (<30% of the control range) and subsequent molecular screening of *PFKM* gene identified a point homozygous mutation c.929A>G in exon 11. The mutation resulted in the substitution of Asp to Gly at position 309. The mutation has not been described in the literature, thus, *in silico* analyses (SIFT, Polyphen, 3D structural modelling) were performed to test whether or not the substitution p.(Asp309Gly) is the cause of decreased PFK activity and HA due to PFK deficiency.

Both *in silico* tools predicted that the substitution p.(Asp309Gly) is likely deleterious. Subsequent *in silico* 3D structure analysis (a model of rabbit muscle type PFK, PDB entry 3O8L) showed that the residue Asp309 is located in close proximity of the nucleotide (ADP) binding site in the center of PFKM subunit (Figure 14). Interaction between ADP molecule and the ADP binding site initiates a positive allosteric modulation of PFKM enzyme (Banaszak et al., 2011). Although no direct interactions between Asp309 and ADP molecule are observed, the substitution of aspartic acid to glycine likely disrupted multiple hydrogen bonds (Gly177, Gly179, Ser180 and Ser306) closely located to the ADP binding site. This loss of hydrogen bonds may cause a change of

polarity and electrostatic interactions which may affect side-chains of amino acids facilitating ADP binding within the activated allosteric site of the PFKM enzyme; in particular with the neighboring Phe308, which makes stacking interactions with the adenine ring of ADP molecule. This proposed pathogenic mechanism corresponds to the previous study of PFKM Asp543Ala mutation. The Tarui disease-causing mutation Asp543Ala blocks ADP binding and mediates its disastrous effect by increasing allosteric inhibitory efficiency of ATP (Bruser et al., 2012).

Based on these findings, we suggest that p.(Asp309Gly) mutation reduces PFK activity due to decreasing binding of the allosteric activator ADP and increasing the allosteric inhibitory efficiency of ATP.

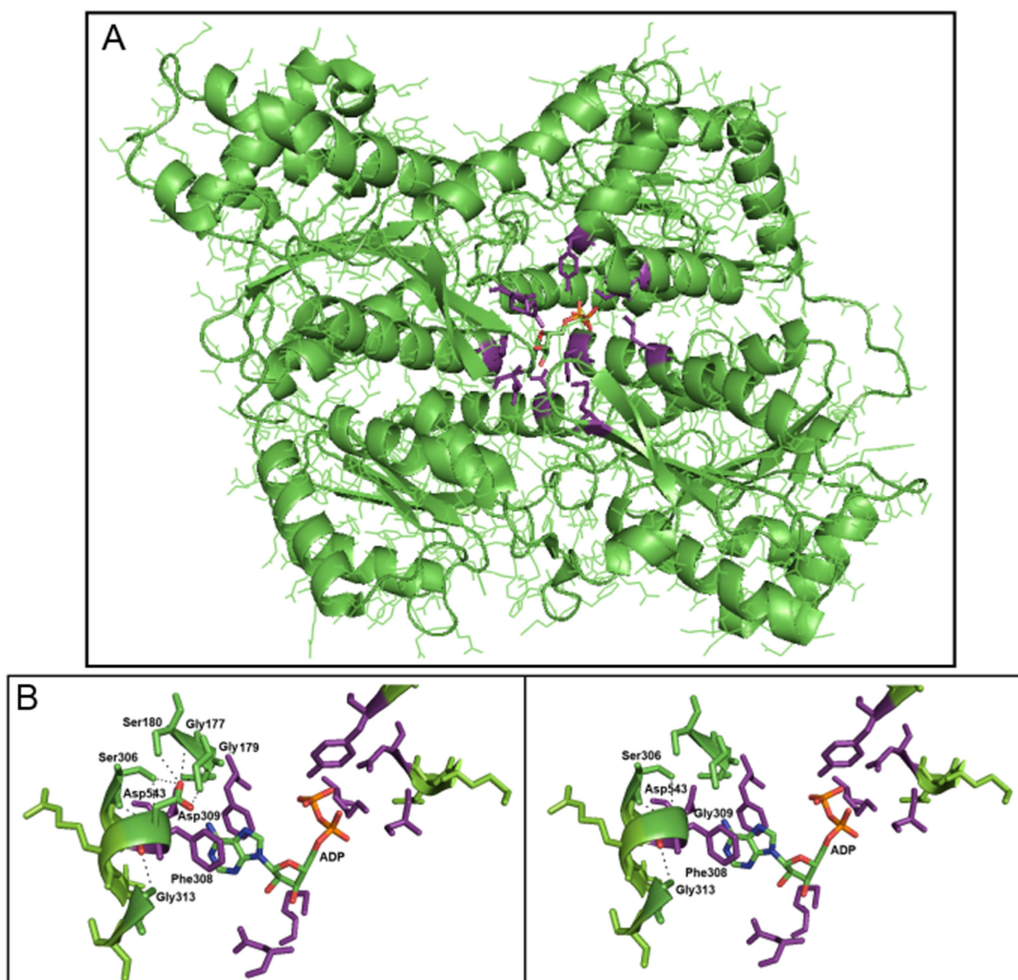


Figure 14: A) 3D crystal structure of PFK subunit from rabbit skeletal muscle. *The ADP binding site (purple) and ADP molecule as positive modulator (atom coloring: carbon – green, nitrogen – blue, oxygen – red, phosphor – orange) in the center of the rabbit subunit of PFKM.* ***B) 3D model of mutation Asp309Gly in PFK subunit.*** *The residue Asp309Gly is located in close proximity of the allosteric nucleotide (ADP) binding site. The substitution Asp to Gly disrupts multiple hydrogen bonds – Gly177, Gly179, Ser180 and Ser306 (black dotted lines). Purple-colored amino acids directly interact with molecule ADP. (PDB 3O8L – protein databank)*

PUBLICATIONS RELATED TO THIS SPECIFIC AIM:

Articles:

Vives-Corrons JL, **Koralkova P**, Grau JM, Mañú Pereira Mdel M, van Wijk R. ***First description of phosphofructokinase deficiency in Spain: identification of a novel homozygous missense mutation in the PFKM gene.*** Front Physiol. 2013; 4:393.

5 SUMMARY

Erythroenzymopathies are a group of inherited disorders arising from deficiency or malfunction of RBC enzymes participated mainly in the Embden-Meyerhof pathway and the hexose monophosphate shunt (Beutler, 1979)

In 2013, direct enzyme assays followed by genetic testing became a routine part of methods applied to patients with HA of unknown etiology in our laboratory. These diagnostic methods are performed only in the specialized laboratories including our department – the only one laboratory in the Czech Republic, or possibly in Central Europe which offers diagnostic testing for the comprehensive panel of enzymopathies. Until now, we have analyzed eighty Czech and Slovak patients with HA of unknown etiology; twenty-four patients were diagnosed with different types of enzyme defect. Except for the most common enzymopathies, G6PD and PK deficiencies (9 and 12 cases), 2 families with GPI and 1 family with a rare HK deficiency were identified. Among the 22 identified mutations, 5 of them have not been described in literature yet. The novel mutations were identified in genes encoding PK (p.(Arg518Leufs*12), p.(Asp293Val)), G6PD p.(Phe216Tyr) and GPI ((p.(Ser160Pro), p.(Arg472Cys)). Furthermore, several G6PD variants (G6PD Seattle, G6PD Torun, G6PD Mediterranean, G6PD Gond) and *PKRL* mutations (p.(Arg116-L117delinsGlnHisCys), p.(Gly275Arg), p.(Ala154Thr), c.1618+1delG)) have been reported in the Czech and Slovak populations for the first time.

In collaboration with research centers from the Netherlands, Spain, Ireland and the Slovak Republic, we have established the molecular basis of HK deficiency in a relatively large number of patients. Among the 6 detected mutations, four of them were novel: p.(His867Tyr), p.(Thr600Met), c.873-2A>G and c.493-1G>A. Further investigations of the selected mutant HKs confirmed their pathogenic nature and indicated possible mechanisms in the pathogenesis of HK deficiency. Through other European collaborations (Belgium, the Netherlands, Spain), we contributed to a characterization of a novel PGK variant c.756+3A>G and to a description of the first case of PFK deficiency in Spain (mutation p.(Asp309Gly)).

Although most enzyme disorders are very rare disorders, these results suggest that the true incidence of these disorders may be higher, and thus direct enzyme activity assays should be considered gold standard procedure in current clinical practice. This work summarizes current knowledge of the molecular pathophysiology of enzymopathies, highlights pitfalls and prospects in the diagnosis of these rare disorders and, as we believe, helps to raise awareness of the prevalence of these rare disorders in the Czech and Slovak populations.

6 LIST OF ALL PUBLICATIONS

Articles:

Ludíková B, Mojzíková R, **Pospíšilová P**, Houda J, Sulovská L, Divoká M, Hak J, Procházková D, Divoký V, Pospíšilová D. ***Deficit pyruvátkinázy v dětském věku.*** Čes-slov Pediat. 2012; 67(3): 6-11.

Vives-Corrons JL, **Koralkova P**, Grau JM, Mañú Pereira Mdel M, van Wijk R. ***First description of phosphofructokinase deficiency in Spain: identification of a novel homozygous missense mutation in the PFKM gene.*** Front. Physiol. 2013; 4:393.

Mojzikova R*, **Koralkova P***, Holub D, Zidova Z, Pospisilova D, Cermak J, Striezencova Laluhova Z, Indrak K, Sukova M, Partschova M, Kucerova J, Horvathova M, Divoky V. ***Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios.*** Br J Haematol. 2014; 165(4):556-63.

Koralkova P, van Solinge WW, van Wijk R. ***Rare hereditary red blood cell enzymopathies associated with hemolytic anemia – pathophysiology, clinical aspects, and laboratory diagnosis.*** Int J Lab Hematol. 2014; 36(3):388-97.

Zidova Z, Kapralova K, **Koralkova P**, Mojzikova R, Dolezal D, Divoky V, Horvathova M. ***DMT1-Mutant Erythrocytes have Shortened Life Span, Accelerated Glycolysis and Increased Oxidative Stress.*** Cell Physiol Biochem. 2014; 34:2221-2231.

Coppens S, **Koralkova P**, Aeby A, Mojzikova R, Deconinck N, Kadhim H, van Wijk R. ***Recurrent episodes of myoglobinuria, mental retardation and seizures but no hemolysis in two brothers with phosphoglycerate kinase deficiency.*** Neuromuscul Disord. 2016; 26(3):207-10.

Koralkova P, Mojzikova R, van Oirschot B, Macartney C, Timr P, Vives Corrons JL, Striezencova Laluhova Z, Lejhancova K, Divoky V, van Wijk R. ***Molecular characterization of six new cases of red blood cell hexokinase deficiency yields four novel mutations in HK1.*** Blood Cells Mol Dis. 2016; 59:71-6.

Ghashghaeinia M, Giustarini D, **Koralkova P**, Köberle M, Alzoubi K, Bissinger R, Hosseinzadeh Z, Dreischer P, Bernhardt I, Lang F, Toulany M, Wieder T, Mojzikova R, Rossi R, Mrowietz U. ***Pharmacological targeting of glucose-6-phosphate dehydrogenase in human erythrocytes by Bay 11-7082, parthenolide and dimethyl fumarate.*** Sci Rep. 2016; 6:28754.

Mojzikova R*, **Koralkova P**, Holub D, Saxova Z, Pospisilova D, Prochazkova D, Dzubak P, Horvathova M, Divoky V ***Two novel mutations (p.(Ser160Pro) and p.(Arg472Cys)) causing glucose-6-phosphate isomerase deficiency are associated with erythroid dysplasia and inappropriately suppressed hepcidin.*** Blood Cells Mol Dis., manuscript accepted for publication.

Meeting abstracts:

Mojzíková R, **Pospíšilová P**, Doležel P, Pospíšilová D, Indrák K, Divoký V. ***Biochemická charakterizace enzymopatií způsobujících vrozenou hemolytickou anémii.*** Transfuze Hematol Dnes. 2011; 17(S2): 23.

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8 SUPPLEMENTS

The PCR reactions were performed according to two standard protocols:

Table S 1: PCR conditions for mutation screening

PK primers			**HK & PGK primers**		
95 °C	15 min		95 °C	10 min	
95 °C	30 s		95 °C	30 s	
T_a	30 s	30 cycles	T_a	30 s	35 cycles
72 °C	30 s		72 °C	45 s	
72 °C	2 min		72 °C	10 min	

Table S 2: List of PK primers

Primer name	**Exon**	**5´-3´ sequence**	**T_a [°C]**	**Product length [bp]**
PK pr F	promoter	TCAAGGTACCATCTTGGGTTCAAAC	60	500
PK pr R	promoter	GCTGCGGGACCATGGAAT	60	
PK 1 F	1	TGGGTGTGCCCCTTTTCTCT	65	430
PK 1 R	1	CCGTGGCTTACATGCTGTGG	65	
PK 2 F	3	ACGAAGGCATGGGGAGGAAG	65	409
PK 2 R	3	TCTACTCCCAGGGCCCAAAA	65	
PK 3_4 F	4,5	AATGGGTTGGATTTGGTTGC	65	499
PK 3_4 R	4,5	TGAGTTCTGAGCCCCGGAGT	65	
PK 5 F	6	GAGCAGTGGGGCTGGGACT	65	405
PK 5 R	6	ATCGGTCTGAGGGCTGATGG	65	
PK 6 F	7	TCCAAGCCCCAGTGTCCTCT	65	476
PK 6 R	7	CGTCGCTCACCTCCAGGATT	65	
PK 7 F	8	CCACGAAGGCGTGAAGAGGT	65	499
PK 7 R	8	CGGTCACCTGTAATCCCCACT	65	
PK 8_9 F	9,10	GGGTGTCAGAGAAGTAGCTTGG	65	600
PK 8_9 R	9,10	TGGACACTCTTCACCCCTGGT	65	
PK 10 F	11	TGACACCTGGAACTGGAACAAAGA	65	385
PK 10 R	11	AGGGAAAACCTGGGACCACA	65	
PK 11 F	12	CTGCAACCTCTACCTCCTGA	65	464
PK 11 R	12	GGACTTAAAGGTGGGGCTTTGG	65	

Table S 3: List of PGK1 primers

Primer name	Exon	5´-3´ sequence	T_a [°C]	Product length [bp]
PGK1_5UTR_F	5UTR	GCCAATAGCGGCTGCTCA		
PGK1_5UTR_R	5UTR	GGCCCAGCCAAAAGCTAAAA	63	378
PGK1-EX1F	1	CGGTGTTCCGCATTCTGCAA		
PGK1-EX1R	1	CTTAGAGCACGAGAGAACA	63	290
PGK1-EX2F	2	CCCATGACTAACCACTATTTTG		
PGK1-EX2R	2	ACATTTCATCCTGTGTACCTAC	63	300
PGK1-EX3F	3	CAATAAGCTAGTTCCCACTAATT		
PGK1-EX3R	3	AAACCAGTAACAACGTGCTTGA	60	320
PGK1-EX4F	4	TGCTGGTAGACTTTGTGGCG		
PGK1-EX4R	4	TGTGTCGGCATTGTATGCTTC	63	423
PGK1-EX5F	5	AGAGGTAAAGCCATCGTTCAGGA		
PGK1-EX5R	5	AGAGCCATAGTGGCAGTTCCAAT	63	505
PGK1-EX6F	6	ATTTTAGTGATAAGGAGCTGGC		
PGK1-EX6R	6	CACCATGCCCAGCCTGTAGTT	63	371
PGK1-EX7F	7	GTGCCTTGAAATAGAAACTCAG		
PGK1-EX7R	7	GGGAATTTTCAGGGCTCATGT	63	332
PGK1-EX8F	8	ACATGAGCCCTGAAAATTCCC		
PGK1-EX8R	8	ATGCTTGTTGATTGGCTGAATC	63	424
PGK1-EX9F	9	CAGCCCTGAGTTCTGGTCTT		
PGK1-EX9R	9	AAGTTCTCCATTCCACCTTCC	63	352
PGK1-EX10F	10	GGAGCACACTGCCTTACAGT		
PGK1-EX10R	10	CACTCCATTCTACCCACCTC	63	303
PGK1-EX11F	11	TGGCATGTTATTGGGAAGATAAA		
PGK1-EX11R	11	ACTAGCTGAATCTTGACATGGA	63	256

Table S 4: List of HK-R primers

Primer name	Exon	5´-3´ sequence	T_a [°C]	Product length [bp]
HK-R pr F	5UTR	TTGGGGTCTTCCTGAGGATCC	65	470
HK-R pr R	5UTR	GCACCTCGACAGGGCAAACT		
HK-R 1F	1	CAAGATGGCCCACCTGTGG	65	241

HK-R 1R	1	TCGCCGCTCATATTCCCTTC		
HK-R 2F	2	GTGCGTGTTCCTGTGCCTGT	65	399
HK-R 2R	2	TGGATGGGAGAGCAGATTGATG		
HK-R 3F	3	GGGAGGCAGACAGGGCCTAC	65	340
HK-R 3R	3	TTGGGATGCCAGTGCTTTGA		
HK-R 4F	4	TGTGTGTGGGAGGGAATGGTT	65	297
HK-R 4R	4	CAACAAAGGGGAAAAGTTGTGGAG		
HK-R 5F	5	GCCCCTATGGGCTTCTGCTT	65	331
HK-R 5R	5	ACTCTCCCCTGCCTGGGTCT		
HK-R 6_7F	6	CACTTCTGCCAAGCGCTGTT	65	555
HK-R 6_7R	6	TGCCCTTTTCATCTCCCATCC		
HK-R 8F	8	GTCTGTCCCAGCCCTCGTGT	65	353
HK-R 8R	8	CACGGACCCAGAAAGCCATG		
HK-R 9F	9	GCTGTCCCTTTCCAGCACCT	65	444
HK-R 9R	9	CCCCGACCACCATTGTCTTG		
HK-R 10F	10	AAGAAAGGGTGGCCGGAGGT	65	471
HK-R 10R	10	GAAGGCATCCACTGGCGAAT		
HK-R 11F	11	TCCTAAAGGGAGGAGTTGCAGCT	65	368
HK-R 11R	11	GGCGGATTTTGGTCATCACG		
HK-R 12F	12	TTTTGCCATGCCTGGCTTGT	65	389
HK-R 12R	12	GGCTGGAGAAGGCCTCAAGG		
HK-R 13F	13	TGAACTCCGTCTCCGATGTTG	65	400
HK-R 13R	13	TATTGCCCGGGCTGGTCTAG		
HK-R 14F	14	GGCTCTCTGACTGCAGTGCAAC	65	278
HK-R 14R	14	CCAAGCTGCTGGGCCAAG		
HK-R 15F	15	CCCTGCCTGTGGAACCTCAG	65	399
HK-R 15R	15	CTTGAAACCTCTGTTCAGACCTCTCC		
HK-R 16F	16	ACGCGGAGTGACCGTGAGAC	65	384
HK-R 16R	16	TGCCCAGGGTCTGCTATTCC		
HK-R 17F	17	GGGCGTCCTTTGGACGTG	65	399
HK-R 17R	17	TCCCCAGCGTAAACCACACC		
HK-R 18F	18	CCCATCACAAAGTCGAAGAATCC	65	368
HK-R 18R	18	GCGTCTCCCAGCGCAACT		

Table S 5: List of G6PD primers

Primer name	Exon	5´-3´ sequence	T_a [°C]	Product length [bp]
G6PD 2 F	2	AATGCCCAGCCGTTCACAAG	65	411
G6PD 2 R	2	TGATCCTGGCGCACTAGCAG		
G6PD 3_4 F	3,4	CCCAGATCACCAAGGGTGGA	65	444
G6PD 3_4 R	3,4	GGGCAGGAGAGGAGGAGAGC		
G6PD 5 F	5	GTGGGACGGGGACACTGACT	65	441
G6PD 5 R	5	CCCCGGACACGCTCATAGA		
G6PD 6_7 F	6,7	GCGAGGAGGTTCTGGCCTCT	65	874
G6PD 6_7 R	6,7	GGACCCCAAACAAGGCTTCC		
G6PD 8 F	8	CCAAGCACCCCACGGTTTTA	65	403
G6PD 8 R	8	CTTCTCCGGGGTTGAGGACA		
G6PD 9 F	9	GCAGGGTGAGCAGAGCCAAG	65	411
G6PD 9 R	9	GGTGTGGACCAGTGCGTGAG		
G6PD 10_11 F	10,11	CACTCACGCACTGGTCCACA	65	617
G6PD 10_11 R	10,11	TGAAAATACGCCAGGCCTCA		
G6PD 12_13 F	12,13	GGGAGCCAGATGCACTTCGT	65	575
G6PD 12_13 R	12,13	GCTGGGCTCGGGTAGTAGCA		

Table S 6: Cycle sequencing conditions

	t [°C]	**Time**	
Initial denaturation	96	1 min	
Denaturation	95	10 s	
Hybridization	50	5 s	25 cycles
Elongation	60	4 min	

Table S 7: Conditions of the first step of RT-PCR

	t [°C]	**Time [min]**	
Extension of hexameric primers	RT	10	
Reverse transcription	42	15	1 cycle
Denaturation	99	5	
Cooling	5	5	

Table S 8: List of primers for RT-PCR

Primer name	Exon	5´-3´ sequence	T_a [°C]	Product length [bp]
HK1_7-9F	7 – 9	TGCCAACATCGTAGCTGTGG	63	834
HK1_7-9R	7 – 9	TGGAAATGAGCCAGGGTCTC	63	
PGK1_6-9F	6 – 9	CCGAGCTTCACTTTCCAAGC	63	735
PGK1_6-9R	6 – 9	CCAGTGCTCACATGGCTGAC	63	

Table S 9: Conditions of the second step of RT-PCR

	t [°C]	Time	
Initial denaturation	95	95 s	
Denaturation	95	30 s	35 cycles
Annealing	63	30 s	
Extension	72	1 min	
Final extension	72	7 min	

Table S 10: List of primers used for qPCR

Primer name	Exon	5´-3´ sequence	T_a [°C]	Product length [bp]
qPCR_HK1_EXON7_8F	7 – 8	TGCCAACATCGTAGCTGTGG	63	339
qPCR_HK1_EXON7_8R	7 – 8	TGGAAATGAGCCAGGGTCTC		
qPCR_PGK1_EX/EX7F	7 – 9	CCGAGCTTCACTTTCCAAGC	63	250
qPCR_PGK1_EX/EX7R	7 – 9	CCAGTGCTCACATGGCTGAC		
qPCR-ACTB_F	2 – 4	CATGGAGAAAATCTGGCACCA	63	440
qPCR-ACTB_R	2 – 4	CCATCTCTTGCTCGAAGTCCA		

9 ACRONYMS

6PGA	6-phosphogluconolactone dehydrogenase
ACTB	Actin β
AD	Autosomal dominant
ADA	Adenosine deaminase
AK	Adenylate kinase
ALD	Aldolase
AR	Autosomal recessive
ARMS	Amplification refractory mutation system
BFU-E	Burst-forming unit erythroid
Bili	Bilirubin
CFU-E	Colony-forming unit erythroid
Chol	SyntheChol™ NS0 Supplement
CZ	The Czech Republic
Dex	Dexamethasone
DHAP	Dihydroxyacetone phosphate
DhSt	Dehydrated stomatocytosis
EMA	Eosin eosin-5'-maleimide binding test
EPO	Erythropoietin
F	Female
G6PD	Glucose-6-phosphate dehydrogenase
GAP	Glyceraldehyde-3-phosphate
GATA-1	GATA-binding factor 1
GLT	Acid glycerol lysis time test
GPI	Glucose phosphate isomerase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Reduced form of glutathione
GSSG	Oxidized form of glutathione
HA	Hemolytic anemia
Hb	Hemoglobin levels
HE	Hereditary elliptocytosis
HK	Hexokinase
HNSHA	Nonspherocytic hemolytic anemia
HS	Hereditary spherocytosis

hSCF	Human stem cell factor
HSt	Hereditary stomatocytosis
Htc	Hematocrit
IL-3	Interleukin 3
Intr	Intron
KLF1	Krüppel like factor 1
K_M	Michaelis-Menten constant
M	Male
metHb	Methemoglobin
MLPA	Multiplex ligation-dependent probe amplification
MuLV	Moloney Murine Leukemia Virus
NaCl	Sodium chloride
NF-E2	Nuclear factor-erythroid-derived 2
OF	Osmotic fragility
OhSt	Overhydrated stomatocytosis
P5N	Pyrimidine-5'-nucleotidase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline buffer
PCR	Polymerase chain reaction
PFK	Phosphofructokinase
PGK	Phosphoglycerate kinase
PK	Pyruvate kinase
qPCR	Quantitative PCR
RBC	Red blood cell
Ret	Reticulocytes
RhAG	Rh associated glycoprotein
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
S	Siblings
SDS-PAGE	SDS–polyacrylamide gel electrophoresis
SK	The Slovak Republic
$T_{1/2}$	The half-life of enzymes
T_{50}	The half inactivation temperature
TPI	Triosephosphate isomerase
γ-GCS	γ-glutamylcysteine synthetase

10 APPENDICES

APPENDICES 4.1

THE LITERATURE REVIEW

International Journal of Laboratory Hematology
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Rare hereditary red blood cell enzymopathies associated with hemolytic anemia – pathophysiology, clinical aspects, and laboratory diagnosis

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SUMMARY

Hereditary red blood cell enzymopathies are genetic disorders affecting genes encoding red blood cell enzymes. They cause a specific type of anemia designated hereditary nonspherocytic hemolytic anemia (HNSHA). Enzymopathies affect cellular metabolism, which, in the red cell, mainly consists of anaerobic glycolysis, the hexose monophosphate shunt, glutathione metabolism, and nucleotide metabolism. Enzymopathies are commonly associated with normocytic normochromic hemolytic anemia. In contrast to other hereditary red cell disorders such as membrane disorders or hemoglobinopathies, the morphology of the red blood cell shows no specific abnormalities. Diagnosis is based on detection of reduced specific enzyme activity and molecular characterization of the defect on the DNA level. The most common enzyme disorders are deficiencies of glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK). However, there are a number of other enzyme disorders, often much less known, causing HNSHA. These disorders are rare and often underdiagnosed, and the purpose of this review. In this brief review, we provide an overview of clinically relevant enzymes, their function in red cell metabolism, and key aspects of laboratory diagnosis.

METABOLISM OF THE RED BLOOD CELL

During maturation red blood cells lose their nucleus, mitochondria, and other organelles. Thus, they are unable to conduct oxidative phosphorylation and protein/lipid synthesis. Despite this limited metabolic machinery, red blood cells are able to fully perform their functional role which mainly involves the transport and delivery of oxygen to tissue. Metabolic pathways involved in cellular function and survival comprise anaerobic glycolysis, the hexose monophosphate shunt, glutathione metabolism, and the

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nucleotide salvage pathway [1–4]. These pathways serve to maintain (i) the integrity and flexibility of the red blood cell membrane, (ii) hemoglobin in its reduced state, (iii) the redox balance of the glutathione pool, and (iv) maintenance of the adenine pool. Red cell metabolism is shown in schematic overview in Figure 1.

Anaerobic glycolysis, also known as the Embden–Meyerhof pathway, is the sole source of energy for the red blood cell. Glucose is metabolized in a multistep process resulting in the production of pyruvate/lactate, ATP, and NADH. ATP drives proper function of K^+/Na^+ pumps, which are involved in maintenance of flexibility and integrity of the red blood cell membrane. NADH is a cofactor of NADH-cytochrome b5 reductase, playing a key role in the reduction of oxidized hemoglobin. Unique to the red cell is the Rapoport–Luebering shunt. This bypass step of glycolysis serves mainly the production of 2,3-bisphosphoglycerate, which together with ATP regulates the affinity of oxygen to hemoglobin [5].

Under standard physiological conditions, 90% of glucose is metabolized through anaerobic glycolysis to produce ATP. The remaining amount of glucose is metabolized through the pentose phosphate pathway whose main function is the production of redox potential in the form of NADPH. NADPH acts as a cofactor of glutathione reductase, which maintains glutathione in its reduced state (GSH), thereby providing protection against oxidative stress [6].

Red cell nucleotide metabolism contributes to maintaining the energy balance in erythrocytes. Nucleotide content in mature erythrocytes is predominantly regulated by the intracellular purine metabolic cycle [7]. Pyrimidines are degraded by pyrimidine-5′-nucleotidase (P5′N) during reticulocyte maturation and are present only as trace amounts in the mature red blood cell. In contrast, adenine derivatives (AMP, ADP, and ATP) represent about 97% of the total nucleotide pool [8]. Because mature red blood cells lack phosphoribosyl-1-pyrophosphate, an essential enzyme for *de novo* synthesis of purines, purine derivatives are reconstituted from intermediates of purine catabolism. This mechanism is known as the salvage pathway [7].

RED BLOOD CELL ENZYMOPATHIES

Hereditary red blood cell enzymopathies are disorders arising from mutations (point mutations, insertions, deletions, splice defects, etc.) in genes coding for red cell metabolic enzymes. Deficiencies or malfunctions of these enzymes generally impair cellular energy balance and/or increase the levels of oxidative stress. Lack of energy and reduced reductive power of red blood cells ultimately affects cellular integrity, leading to premature removal in the spleen and, consequently, decreased red blood cell survival [1, 3]. Most enzyme disorders are inherited in an autosomal recessive form with hemolysis occurring only in homozygous or compound heterozygous individuals. Some enzyme deficiencies are X-linked. A summary of red blood cell enzymopathies and some of its main characteristics is presented in Table 1.

The degree of hemolysis depends on the affected metabolic pathway, the role of the mutated enzyme in this pathway, the functional abnormalities introduced by the underlying mutation, and the possibility for the cell to compensate for the loss of enzymatic activity [2]. The severity of hemolysis is variable, ranging from mild or fully compensated hemolysis without apparent anemia to fatal anemia at birth, severe transfusion-dependent hemolysis, and even death in early childhood. Typical clinical symptoms further include splenomegaly, jaundice, gallstones, and iron overload (also in nontransfused patients). Notably, some enzyme deficiencies are associated with systemic (nonhematological) manifestations such as neurological dysfunction, mental retardation, myopathy, and susceptibility to infection [3].

No curative therapy is available for enzyme defects. Affected individuals with mild anemia or compensated hemolysis do not require any treatment. In more severe cases, treatment mainly consists of supportive therapy (blood transfusions) and chelation therapy to avoid the adverse effects of iron overload. In some cases, splenectomy may be considered. Generally, spleen removal can increase hemoglobin levels but the effect is variable and difficult to predict [9]. Patients with deficiency of enzymes involved in the protection against oxidative stress should avoid the intake of oxidative drugs or food.

ENZYME DISORDERS OF THE EMBDEN–MEYERHOF PATHWAY

The Embden-Meyerhof pathway transforms glucose to pyruvate or lactate and generates ATP. This metabolic

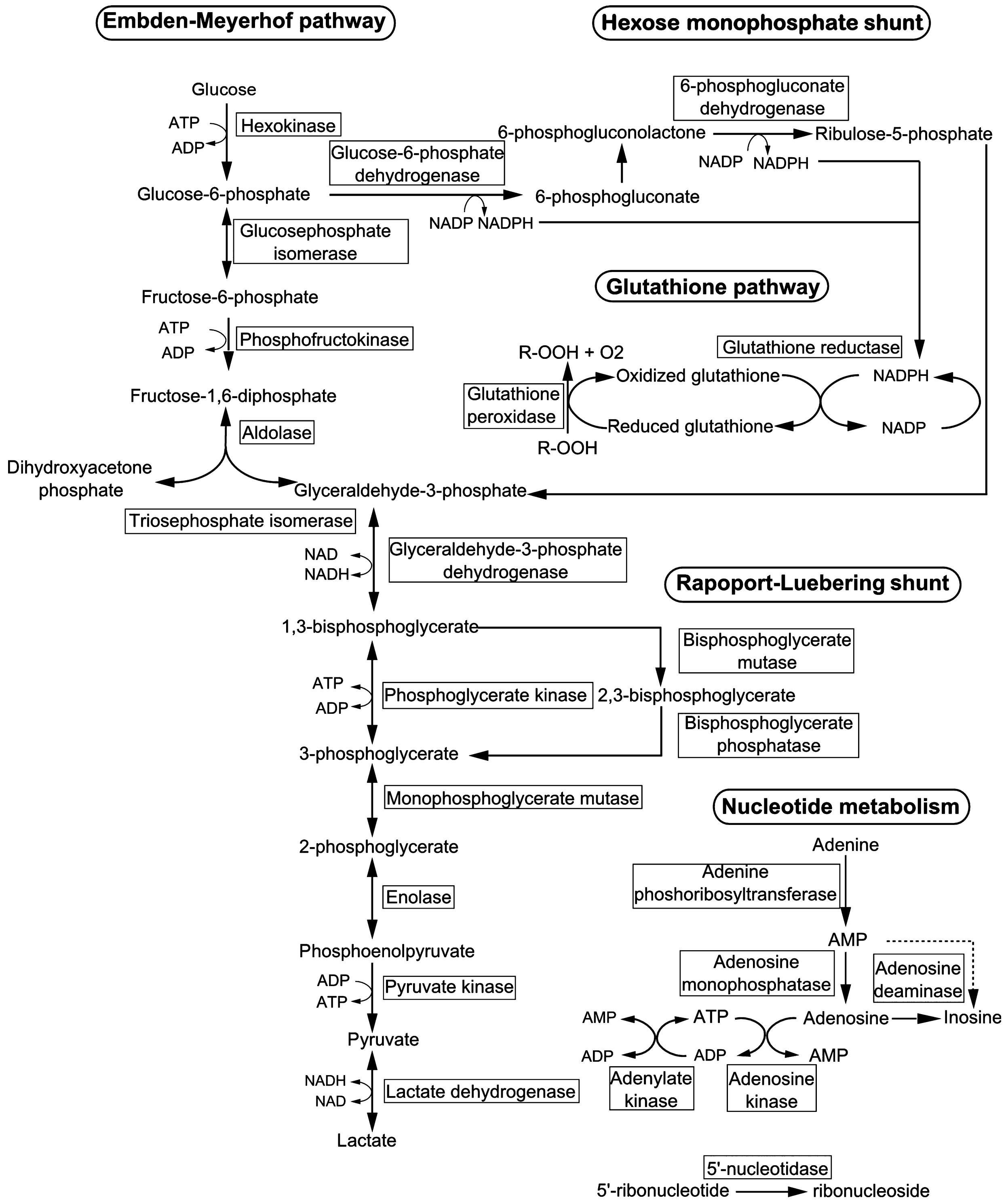

Figure 1. Schematic depiction of the main red blood cell metabolic pathways: the Embden-Meyerhof pathway (anaerobic glycolysis), Rapoport–Luebering Shunt, hexose monophosphate shunt, glutathione pathway, and nucleotide metabolism.

Table 1. Red blood cell enzyme disorders

Enzyme	Role in RBCs	Clinical manifestations	Neurological symptoms	Myopathy	Genetic transmission	No. of reported cases, mutations
Adenosine deaminase, hyperactivity	Nucleotide metabolism	HNSHA; chronic	–	–	AD	3 families, no mutations
Adenylate kinase	Nucleotide metabolism	HNSHA; chronic	–	–	AR	12 families, 7 mutations
Aldolase	Embden-Meyerhof pathway	HNSHA; chronic	±	±	AR	6 cases, 4 mutations
Phosphofructokinase	Embden-Meyerhof pathway	HNSHA; chronic (mild)	–	+	AR	50–100 cases, 17 mutations
Phosphoglycerate kinase	Embden-Meyerhof pathway	HNSHA; chronic	+	+	X-linked	40 cases, 19 mutations
Glucose-6-phosphate dehydrogenase	Hexose monophosphate shunt	HNSHA; induced by oxidant drugs/infection, favism; chronic	–	–	X-linked	>400 × 10^6; >60 families (class I)
Glucosephosphate isomerase	Embden-Meyerhof pathway	HNSHA; chronic	±	–	AR	>50 families, 31 mutations
Glutathione reductase	Glutathione metabolism	HNSHA; induced by oxidant drugs/infection, favism.	–	–	AR	2 families, 3 mutations
Glutathione synthetase	Glutathione metabolism	HNSHA; chronic	+	–	AR	>50 families, 32 mutations
Hexokinase	Embden-Meyerhof pathway	HNSHA; chronic	–	–	AR	20 cases, 5 mutations
Pyrimidine-5′-nucleotidase	Nucleotide metabolism	HNSHA; chronic	–	–	AR	>60 families, 26 mutations
Pyruvate kinase	Embden-Meyerhof pathway	HNSHA; chronic	–	–	AR	>500 families, >200 mutations
Triosephosphate isomerase	Embden-Meyerhof pathway	HNSHA; chronic	+	–	AR	50–100 cases, 18 mutations

RBC, red blood cell; HNSHA, Hereditary Nonspherocytic Hemolytic Anemia; AR, autosomal recessive; AD, autosomal dominant.

pathway is regulated by three rate-limiting steps, involving the catalytic action of hexokinase (HK), phosphofructokinase (PFK), and PK. Cellular NADH and ATP levels also control glycolytic flux. Most enzyme disorders of this pathway are associated with chronic HNSHA. Except for phosphoglycerate kinase (PGK) deficiency, which is X-linked, all enzyme disorders of anaerobic glycolysis are transmitted in an autosomal recessive manner [10].

The most prevalent enzyme disorder of anaerobic glycolysis is PK deficiency. The estimated frequency of PK deficiency is 1 : 20 000 in the general white population [11]. PK is an allosteric enzyme, which catalyzes the irreversible transfer of high-energy phosphate group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. The enzyme is allosterically regulated by FBP (positive regulation) and inhibited by its product ATP. There are four mammalian isozymes of PK: M_1, M_2, L, and R, encoded by 2 different genes. *PKLR*, localized on chromosome 1 (1q21) [12], directs expression of PK-R and PK-L in, respectively, red blood cells and liver. Deficiency of PK-R has two major metabolic consequences: depletion of ATP and accumulation of 2,3-bisphosphoglycerate [13]. Lack of ATP eventually leads to premature removal of red blood cell from the circulation, whereas increased levels of 2,3-bisphosphoglycerate decreases the affinity of hemoglobin for oxygen. The latter partly compensates for tissue hypoxia due to the anemia [14]. More than 220 different mutations in *PKLR* gene associated with PK deficiency are known (http://www.hgmd.org/). Most of the mutations are missense mutations (70%) leading to single amino acid substitutions. The clinical features of PK deficiency are highly variable, ranging from a fully compensated hemolysis to severe transfusion-dependent hemolytic anemia [9]. Few cases of hydrops fetalis and death in neonatal period due to severe PK deficiency have been reported [15]. The severity of anemia is usually stable in adulthood but may worsen during infections or other forms of physiological stress [16]. Iron overload, occurring in the most PK-deficient patients, is considered to be multifactorial [17]. There is no link among the residual PK activity, degree of hemolysis, and clinical severity of PK deficiency.

The second most frequent glycolytic enzyme disorder is glucose-6-phosphate isomerase deficiency (GPI). More than 50 cases with GPI deficiency have been described. In most affected individuals, residual GPI activity is less than 25%. Low enzyme activity results from impaired kinetic properties, reduced thermo stability of the mutant enzyme, or defective protein folding [18]. Clinical features in GPI activity range from mild-to-severe hemolytic anemia. Hydrops fetalis appears to occur more often in GPI deficiency than in other enzymopathies [19]. GPI deficiency may also be associated with nonhematological symptoms, in particular neurological impairment or mental retardation [10, 20].

Phosphofructokinase (PFK), aldolase, and triosephosphate (TPI) deficiencies are more rare enzyme disorders. Erythrocyte PFK is composed of two types of subunits: PFKL (liver) and PFKM (muscle) that may form 5 different isozymes (M_4, M_3L_1, M_2L_2, M_1L_3, and L_4). In case of a mutated PFKM subunit, only a functional PFKL subunit is expressed in red blood cells, causing only a partial deficiency of PFK. Thus, patients with PFK deficiency usually exhibit mild or fully compensated hemolytic anemia. As muscle cells contain only PFKM, the deficiency is more pronounced in muscle cells, leading to myopathy [21]. TPI deficiency is the most severe enzyme defect of anaerobic glycolysis, frequently leading to death in early childhood. TPI catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). In red blood cells, the deficiency leads to hemolytic anemia, which may be due to accumulation of DHAP [22]. However, the deficiency is associated with more severe consequences such as mental retardation and other neurological dysfunctions. These may occur because of the formation of toxic protein aggregates, induced by misfolded TPI [22, 23].

Moderate-to-severe hemolytic anemia is observed in patients with aldolase deficiency. As in TPI deficiency, defects of aldolase are associated with neurological disorders [24]. Few affected individuals have been reported, showing recurrent episodes of rhabdomyolysis and muscle weakness [25, 26]. Severe defects of aldolase probably lead to death in embryonic state [27].

Hexokinase is the enzyme with the lowest *in vitro* activity of all glycolytic enzymes. Deficiency of this key regulatory enzyme is generally associated with severe hemolytic anemia and may lead to death in

the neonatal period [28, 29]. To date, only 20 cases of hexokinase deficiency have been described, making this deficiency a rare red cell enzyme disorder.

Phosphoglycerate kinase (PGK) is the only enzyme of glycolysis in which gene is X-linked. PGK deficiency is associated with chronic hemolysis, neurological impairment (including mental retardation and ataxia), and myopathy (exercise intolerance or muscle weakness) [30]. There are only few affected individuals who suffer the full spectrum of clinical features. Thus, PGK deficiency shows a very wide clinical phenotype. Some cases of PGK deficiency with myopathy but without hemolysis have been described [30–34].

Deficiencies of other glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, monophosphoglycerate mutase, and enolase have been described but they appear not to be associated with hemolysis [35].

Enzyme disorders of the hexose monophosphate shunt and glutathione metabolism

The red blood cell is continuously exposed to various forms of oxidative stress. To protect cellular hemoglobin and other macromolecules against oxidative damage, reductive power is required. The reduced form of glutathione (GSH) constitutes the cell's main source of reductive power. NADPH, generated by the hexose monophosphate shunt, is required to maintain glutathione in its reduced form. Key enzymes of this defense mechanism are G6PD, glutamate cysteine ligase, glutathion synthetase, and glutathione reductase [6]. Generally, disorders of these enzymes cause hemolysis only under conditions of increased levels of oxidative stress (acute hemolytic anemia).

G6PD catalyzes the first step of the hexose monophosphate shunt thereby producing NADPH (Figure 1). G6PD deficiency is an X-linked disorder and the most frequent enzyme disorder worldwide with more than 400 million people affected worldwide [36]. G6PD-deficient variants are divided into five classes according to the level of residual activity and the severity of phenotype. Most variants belong to classes II, III, or IV and are associated only with acute hemolysis. In contrast, the rare class I variants, characterized by very low activity of G6PD (<1%), are associated with chronic HNSHA. Most G6PD-deficient individuals are asymptomatic but may develop acute hemolytic crises upon conditions that increase the levels of oxidative stress such as the intake of specific drugs, fava beans, or the occurrence of infections. For further reading on G6PD-deficiency, we refer the reader to excellent reviews [37, 38] and references herein.

Glutathione reductase (GR) is important in the regeneration of GSH from oxidized glutathione (GSSG) (Figure 1). Hereditary GR deficiency is a very rare disorder characterized by increased susceptibility to oxidative stress. The clinical phenotype resembles a deficiency of G6PD with affected individuals being generally asymptomatic and the induction of acute hemolytic crisis by the intake of drugs or fava beans [6, 39]. Importantly, acquired deficiency of GR may also occur, as a result of insufficient flavin intake. GR requires flavonoids for enzymatic activity. Such acquired deficiencies are without clinical consequences [35].

The tripeptide glutathione is synthesized in red blood cells from cysteine, glutamine, and glycine in a 2-step process catalyzed by, respectively, glutamate cysteine ligase (GCL) and glutathione synthetase (GS). Defects of both enzymes decrease cellular GSH content and, hence, increase its susceptibility to oxidative stress. Both disorders are rare: >50 families with GS deficiency have been described, whereas only 12 families with GCL deficiency have been reported. Apart from acute hemolytic episodes, the deficiency may display additional nonhematological symptoms such as mental retardation (GCL deficiency) and 5-oxyprolinuria (GS deficiency) [40].

Deficiencies of glutathione peroxidase and other enzymes involved in red blood cell anti-oxidant defense such as catalase have been reported. They do not cause hemolysis [35].

Enzyme disorders of nucleotide metabolism

Purine metabolism maintains red blood cell ATP and GTP levels. Ninety-seven percent of total nucleotides are adenine derivatives (AMP, ADP, and ATP). Guanine derivatives constitute 1–3%. Pyrimidines are removed during the maturation process and are therefore only present in trace amounts [8, 41]. There are a number of enzymes involved in nucleotide metabolism. The most important ones are pyrimidine-5′-nucleotidase (pyrimidine metabolism) and adenylate kinase and adenosine deaminase (purine metabolism)

(Figure 1). Defects of all three enzymes are associated with HNSHA.

Pyrimidine-5′-nucleotidase (P5N) deficiency is probably the third most prevalent enzyme disorder associated with HNSHA [42]. During red blood cell maturation, P5N removes pyrimidine nucleotides from the red blood cell by catalyzing the hydrolysis of UMP and CMP, rendering diffusible nucleosides and free phosphates [41, 43]. P5N deficiency results in the accumulation of pyrimidine nucleotides. They form insoluble aggregates, which are visible as basophilic stippling on a peripheral blood smear (Figure 2). Although not specific, prominent basophilic stippling is characteristic of P5N deficiency. P5N deficiency is an autosomal recessive disorder associated with mild-to-severe chronic hemolytic anemia, splenomegaly, and jaundice [42].

Adenylate kinase and adenosine deaminase are both important in maintaining the adenine pool. Deficiencies of both adenylate kinase and hyperactivity of adenosine deaminase probably deplete the cell from adenine nucleotides and, consequently, ATP, eventually resulting in chronic hemolytic anemia. Adenylate kinase deficiency is an autosomal recessive disorder, which has been described only in 12 families. Affected individuals exhibit moderate or severe hemolytic anemia. Some patients with psychomotor impairment have been reported [44]. Hyperactivity of adenosine deaminase is a very rare autosomal dominant disorder for which no causative mutations yet have been described [45].

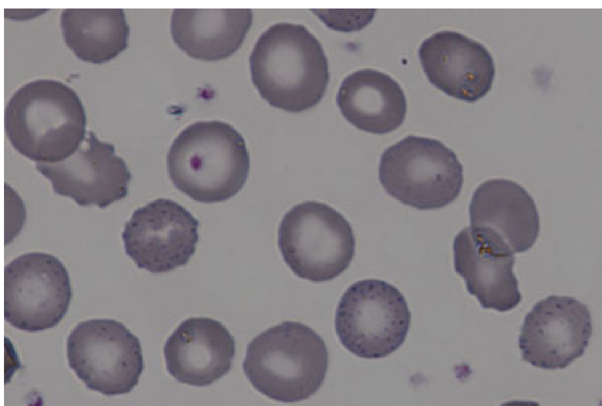

Figure 2. Peripheral blood smear of a patient with pyrimidine-5′-nucleotidase deficiency showing prominent basophilic stippling.

LABORATORY DIAGNOSIS OF RED BLOOD CELL ENZYME DISORDERS

Red blood cell enzymopathies are associated with normocytic normochromic anemia and common signs of hemolysis, that is, increased plasma levels of bilirubin and lactate dehydrogenase, low levels of haptoglobin and high reticulocyte count [46]. The diagnosis of HNSHA due to a red blood cell enzymopathy generally is a diagnosis that is based on exclusion: a negative direct Coombs test, a normal osmotic fragility/cryohemolysis test, no specific morphological abnormalities, and no evidence for an abnormal hemoglobin. Differential diagnosis of a red blood cell enzymopathy requires demonstration of reduced enzymatic activity and confirmation of the diagnosis on the DNA level. Importantly, there is no relationship between the degree of loss of enzymatic activity and clinical severity.

Nowadays, there are a number of quantitative or semiquantitative screening assays for determination of the most prevalent enzymopathies (deficiencies of G6PD and PK). G6PD deficiency screening tests such as fluorescent spot tests or cytochemical assays are based on the direct/indirect detection and/or quantitation of NAPDH produced by G6PD [37]. Direct tests measure the production of NAPDH which has its absorption maximum at 340 nm and fluorescence emission at 460 nm. Indirect tests translate NADPH production into a colorimetric readout using chromophores such as brilliant cresyl blue, resazurin, or formazan derivatives [47]. PK deficiency screening tests are usually indirect colorimetric assays based on the detection of chromophore products. Although these assays are usually rapid and inexpensive, they are able to detect only the most common enzyme defects. Thus, the classical methods described by Beutler still represent the method of choice for the diagnosis of red blood cell enzymopathies [48]. The general basis comprises the determination of a specific enzyme activity using a spectrophotometric assay. Enzymatic activity is directly related to changes in absorbance over time. Specific reactions need to be prepared for each enzyme, and changes in substrate (either NAD^+, $NADP^+$, NADH, and NADPH) are monitored at

340 nm for a set amount of time. The specific enzymatic activity of each particular enzyme is expressed as IU/gHb [48]. In red blood cell enzymopathies, enzyme activity of affected individuals is usually reduced to 25% of normal. As stated, there is no correlation between the residual activity of enzyme and severity of the clinical picture. Despite the many benefits of these assays (high sensitivity and high specificity), there are several pitfalls, which may influence results and their interpretation. Enzymatic activities are influenced by factors such as (i) the conditions during storage and shipping of blood samples (i.e., *in vitro* stability of enzymes), (ii) the proper removal of leukocytes and platelets from the red blood cell sample (leukocytes and platelets may express isozymes, encoded by other genes, which interfere with red cell enzyme activity measurements), (iii) blood transfusions (blood sample will contain donor erythrocytes), (iv) the age of the patient (the expression of many enzymes is age-related), and (v) the presence of a high number of reticulocytes in the patient's blood sample. The activity of many enzymes (in particular HK, PK, G6PD and P5N) is red blood cell age-related. Thus, the youngest cells have the highest enzymes activity (Figure 3). In the case of a high reticulocyte count, any deficient enzyme may show a normal or even increased *in vitro* activity [1, 48]. The evaluation of the activity of an age-dependent enzyme (*e.g.,* PK) should therefore always be related and compared with another such enzyme (*e.g.,* HK). If for instance PK is normal or low normal but HK is increased, this is indicative of a (masked) PK deficiency.

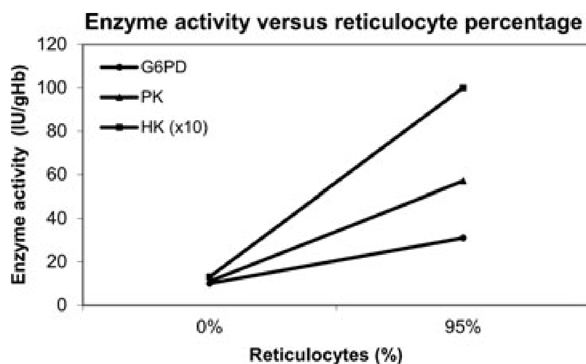

Figure 3. Influence of reticulocytosis on enzymatic activities of hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase.

As stated, red blood cell morphology in enzymopathies is essentially normal. However, some nonspecific abnormalities may be present. For example, P5N deficiency is accompanied by prominent basophilic stippling. Basophilic stippling, however, can also be seen in patients with sideroblastic anemia, thalassemia, or lead poisoning. Heinz bodies (precipitated hemoglobin), blister cells, bite cells may occur in G6PD deficiency or enzyme deficiencies of glutathione metabolism [49]. Heinz bodies can also be found in red blood cells from patients with unstable hemoglobin or thalassemia. Anisocytosis, poikilocytosis, and contracted red blood cells (echinocytes) can be present in PK deficiency [9].

A number of methods (radioactive, capillary electrophoresis, or high-perform liquid chromatography) have been described for determination of red cell P5N activity [42]. However, the reproducibility of most of these methods does not meet standards for use in clinical practice. A simple screening test P5N deficiency, or any other enzyme involved in nucleotide metabolism, comprises the spectrophotometric measurement of pyrimidine and purine nucleotides in deproteinized hemolysate. This method uses the differences in absorption maxima of purines (260 nm) pyrimidines (280 nm) [48]. The ratio between these reflects overall cellular purine and pyrimidine content. For instance, an accumulation of pyrimidine nucleotides, as seen in P5N deficiency, decreases the purine/pyrimidine nucleotide ratio.

In case of a suspected red blood cell enzyme deficiency, the molecular characterization of the defect on the DNA level is essential for confirmation of the diagnosis. Mutations are usually unique, which makes it necessary to sequence the entire coding region, including flanking intronic splice site sequences. Importantly, *cis*-acting, often erythroid-specific, regulatory regions may harbor mutations interfering with gene expression and thereby causing a red cell enzyme disorder [50, 51]. The identification of a causative mutation enables proper genetic counseling and prenatal diagnosis. DNA investigations may be the only way to establish the diagnosis in neonatal patients or patients who are transfusion-dependent. Moreover, molecular characterization is required for a better understanding of the complex genotype-to-phenotype correlation in red blood cell enzymopathies.

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APPENDICES 4.2

DIFFERENTIAL DIAGNOSIS OF CZECH AND SLOVAK PATIENTS WITH HEMOLYTIC ANEMIA OF UKNOWN ETIOLOGY

Deficit pyruvátkinázy v dětském věku

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Souhrn

Účel studie: Deficit pyruvátkinázy (PK) je druhý nejčastější enzymatický defekt vedoucí k hemolytické anemii. Cílem práce je demonstrovat klinické a laboratorní nálezy u deficitu PK u prvních dětských pacientů v České republice definovaných na molekulární úrovni.

Pacienti a metody: Čtyři děti (10 měsíců – 7 let) byly vyšetřeny pro závažnou nesférocytární hemolytickou anemii s hladinou hemoglobinu 64–97 g/l. Všechny děti vyžadovaly po porodu fototerapii a transfuzi, dvě dokonce výměnnou transfuzi. V klinickém nálezu dominovala bledost, subikterus sklér a kůže. Standardními metodami byla vyloučena porucha membrány erytrocytu, hemoglobinopatie a kongenitální dyserytropoetická anemie. Byla stanovena aktivita PK a provedena sekvenace genu kódujícího PK. Pro zvýšení hladin feritinu byla vyšetřena hladina hepcidinu.

Výsledky: Všechny děti mají makrocytární anemii. Hodnoty bilirubinu se pohybují v rozmezí 38–89 μmol/l. Aktivita PK je u všech dětí snížená (23–32 %). Dva pacienti jsou smíšení heterozygoti pro mutace c.1529C>A (p.Arg510Gln) a c.1594C>T (p.Arg532Trp) a jeden pro mutace c.1493G>A (p.Arg498His) a c.1529C>A (p.Arg510Gln). V jednom případě se jedná o homozygotní deleci v exonu 11, která nebyla dosud popsána. Tento pacient s nejtěžším průběhem nemoci má vysokou hladinu feritinu, avšak sníženou hladinu hepcidinu, což se může podílet na přetížení železem. Byla vyloučena koincidence s hemochromatózou typu I.

Závěr: Deficit PK může být u novorozence příčinou závažné anemie a hyperbilirubinemie vyžadující někdy výměnnou transfuzi a opakované podání transfuzí v kojeneckém věku, jejichž frekvence se většinou postupně snižuje. Deficit PK může být provázen i přetížením železem s nutností chelatační léčby. V české populaci je onemocnění zatím poddiagnostikováno.

Klíčová slova: glykolýza, pyruvátkináza, nesférocytární hemolytická anemie, přetížení železem, hepcidin

Summary

Pyruvate kinase deficiency in children

Purpose of the study: Pyruvate kinase deficiency (PK) is the second most common enzymatic defect leading to hemolytic anemia. The aim is to demonstrate the clinical and laboratory findings in PK deficiency in the first pediatric patients in the Czech Republic who were defined at the molecular level.

Patients and methods: Four children (10 months – 7 years) were examined for severe nonspherocytic hemolytic anemia with hemoglobin levels 64–97 g/l. All the children required transfusions and phototherapy after delivery, two of them even an exchange transfusion. The clinical finding was dominantly characterized by pallor, subicterus of sclerae and skin. Erythrocyte membrane disorder, hemoglobinopathies and congenital dyserythropoietic anemia were excluded using standard methods. PK activity was determined and sequencing of the gene encoding PK was done. Because of increased ferritin levels, hepcidin level was measured.

Results: All children have macrocytic anemia with bilirubin values from 38 to 89 micromol/l. PK activity is decreased in all children (23–32%). Two patients are mixed heterozygotes for mutations c.1529C>A (p.Arg510Gln) and c.1594C>T (p.Arg532Trp) and one for the c.1493G>A (p.Arg498His) and c.1529C>A

(p.Arg510Gln) mutations. In one case, a homozygous deletion in exon 11, which has not yet been described, was found. This patient with the most severe course of the disease has a high ferritin level, but a reduced level of hepcidin, which may contribute to iron overload. Coincidence with type I hemochromatosis was excluded.

Conclusion: PK deficiency in the newborn may cause severe anemia and hyperbilirubinemia sometimes requiring an exchange transfusion and repeated administration of transfusions in infancy; their frequency is usually gradually reduced. PK deficiency may also be associated with iron overload and the need for chelation therapy. In the Czech population this disease is still underdiagnosed.

Key words: glycolysis, pyruvate kinase, nonspherocytic hemolytic anemia, iron overload, hepcidin

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ÚVOD

Deficit pyruvátkinázy (PK) je nejčastější enzymatickou abnormalitou glykolytické dráhy a spolu s deficitem glukózo-6-fosfátdehydrogenázy (G6PD) nejčastější příčinou nesférocytární hemolytické anemie [1, 2]. Poprvé byl popsán počátkem šedesátých let minulého století Valentinem a spolupracovníky [3]. Jedná se o vrozené autozomálně recesivně dědičné metabolické onemocnění s prevalencí 1:20 000 [2].

Pyruvátkináza hraje klíčovou roli v glykolytické dráze. Katalyzuje jeden ze dvou kroků při tvorbě adenozintrifosfátu (ATP).

Vzhledem k tomu, že zralé červené krvinky neobsahují mitochondrie, je glykolýza jedinou dráhou generující ATP, který je nezbytný pro další metabolické pochody, pro zpětnou regulaci glykolýzy, membránový transport a pro zachování flexibility a integrity membrány erytrocytu. Mimo jiné zde dochází ke generaci redukované formy nikotinamidadenindinukleotidu (NADH) nezbytného pro redukci methemoglobinu na hemoglobin a 2,3 difosfoglycerátu (2,3-DPG), regulátoru afinity hemoglobinu ke kyslíku. Deficit PK se tak projeví neefektivní glykolýzou vedoucí ke snížené životaschopnosti erytrocytů a extravaskulární hemolýze.

PK je aktivní ve formě tetrameru. U savců byly popsány čtyři různé izoenzymy PK (M1, M2, L, R), které jsou specifické pro jednotlivé tkáně [4], z nichž pouze R forma se vyskytuje výhradně v červených krvinkách [5]. Jaterní (PK-L) a erytrocytární (PK-R) izoenzymy jsou kódovány genem *PKLR*, který se nachází na chromozomu 1 (1q21) [4]. Kódující oblast je rozdělena do dvanácti exonů [6]. Exon 1 je specifický pro transkripci erytrocytární formy, exon 2 pro jaterní formu, zbývající exony pak jsou společné. Dva další izoenzymy PK-M1 a PK-M2 jsou kódovány genem *PKM* nacházejícím se na 15. chromozomu. PK-M1 se vyskytuje převážně v srdci, mozku a kosterním svalstvu, PK-M2 ve fetálních tkáních, leukocytech a trombocytech. Dosud bylo popsáno přes 180 mutací *PKLR* genu, které jsou asociovány s defektem PK. Většinou se jedná o mutace měnící smysl kodonu a způsobující jednoduchou aminokyselinovou záměnu, která má za následek snížení enzymové aktivity na různých úrovních [7]. Mezi nejčastější mutace patří: 1) c.1529G>A typická pro USA a severní a střední Evropu, 2) c.1456C>T častá v jižní Evropě a 3) c.1468C>T vyskytující se v Asii [2, 3, 7].

Klinické symptomy deficitu pyruvátkinázy se objevují u jedinců s homozygotní mutací nebo u složených heterozygotů. Heterozygoti jsou přenašeči bez projevů onemocnění, výjimečně se u nich mohou projevit příznaky mírné hemolýzy. Průběh onemocnění je velmi variabilní, od kompenzované anemie bez klinických projevů přes lehkou anemii s exacerbacemi hemolýzy při infekcích až po těžkou anemii s nutností opakovaného podávání transfuzí. Vždy je přítomna retikulocytóza. U novorozenců s deficitem PK se poměrně často objevuje významná anemie a hyperbilirubinemie s rizikem rozvoje jádrového ikteru, která si v některých případech může vyžádat výměnnou transfuzi. Byly popsány i případy hydropsu plodu [8] a život ohrožující anemie u novorozence. U nemocných s chronickou anemií se mohou vyvinout žlučové kameny, a to již v první dekádě života. U pacientů s deficitem PK byly popsány i tranzientní aplastické krize způsobené parvovirem B19. K vzácným komplikacím patří chronické vředy na dolních končetinách, akutní pankreatitidy, abscesy slinivky, útlak míchy extramedulární hematopoetickou tkání a tromboembolická onemocnění [9]. Hemolýza nebývá provokována léky, někteří autoři však popisují zvýšení hemolýzy po podání vyšších dávek salicylátů.

Podezření na enzymový deficit obecně podporuje obraz normocytární, méně často makrocytární anemie. Biochemické parametry, jako je zvýšení nekonjugovaného bilirubinu a laktátdehydrogenázy (LD) a snížení hladiny haptoglobinu, odpovídají známkám extravaskulární hemolýzy. Při morfologickém hodnocení erytrocytů v nátěru periferní krve bývá přítomná polychromazie, anizocytóza, v některých případech ojedinělé normoblasty (jaderné erytroidní buňky), vzácně echinocyty a akantocyty.

Vzhledem k absenci jednoznačného klinického

i laboratorního diagnostického znaku, který by odpovídal deficitu PK, je vždy nutné nejprve vyloučit běžně se vyskytující vrozené hemolytické anemie, zejména hereditární sférocytózu, případně nestabilní hemoglobinopatie. K odlišení sférocytózy je možné použít test kryohemolýzy a test s eosin-5--maleimidem, jejichž výsledky jsou u enzymového defektu normální. K vyloučení hemoglobinopatií je vhodné provést elektroforézu hemoglobinu, isopropanolový test či test tepelné stability erytrocytů, které jsou u enzymopatií negativní. Výskyt Heinzových tělísek může odpovídat nestabilní hemoglobinopatii nebo souviset s enzymopatiemi v metabolismu glutathionu (G6PD aj.). Pomocným vyšetřením může být test autohemolýzy, který je u většiny pacientů patologický a v případě deficitu PK se upravuje pouze po přidání ATP, nikoli však glukózy. K přesné diagnóze deficitu PK však stejně tak jako ostatních erytrocytárních enzymopatií slouží průkaz snížené kinetické aktivity enzymu. Diagnózu definitivně potvrzuje molekulárně genetické vyšetření na DNA/RNA úrovni prokazující kauzální mutaci genu *PKLR*.

Terapie onemocnění je symptomatická, kauzální léčba zatím neexistuje. Někteří nemocní jsou závislí na transfuzní léčbě. Splenektomie je vyhrazena pro nejtěžší případy onemocnění s těžkou anemií, u kterých může vést k omezení nutnosti transfuzní léčby a ke stabilizaci hodnot hemoglobinu. U části pacientů s deficitem PK se rozvíjí přetížení organismu železem, které si může vyžádat léčbu chelátory. Byla popsána i úspěšná transplantace kostní dřeně u malého dítěte [10]. Je zkoumána možnost genové léčby.

V české populaci bylo popsáno jen několik případů dospělých pacientů s deficitem PK s prokázanou mutací genu pro PK [11]. V tomto článku prezentujeme klinický a laboratorní profil prvních dětských pacientů s průkazem kauzální mutace pro PK.

PACIENTI

Soubor pacientů tvoří čtyři děti ve věku 10 měsíců – 7 let s diagnózou chronické hemolytické anemie nejasné etiologie. U všech pacientů byla při základním vyšetření krevního obrazu a markerů hemolýzy zjištěna různě závažná anemie s projevy extravaskulární hemolýzy.

Pacienti č. 1 a 2

První dva pacienti jsou sourozenci, děvče a chlapec ve věku 4 roky a 10 měsíců. Obě děti se narodily v termínu, dívka byla hypotrofická s porodní hmotností 2100 g a porodní délkou 44 cm, chlapec se narodil s hmotností 3130 g a délkou 51 cm.

Obě děti měly protrahovaný novorozenecký ikterus s nutností fototerapie, u chlapce bylo dokonce nutné provést výměnnou transfuzi. Makrocytární anemie s výraznou retikulocytózou je u obou dětí provázena mírnou hyperbilirubinemií a v prvním roce života si u obou vyžádala opakované podání transfuze erytrocytární masy. V klinickém nálezu u dětí dominuje bledost a subikterus sklér a kůže, mírná hepatosplenomegalie a systolický šelest v prekordiu bez průkazu vrozené srdeční vady při echokardiografickém vyšetření.

Po prvním roce věku byly u dívky podávány transfuze erytrocytární masy již méně často, většinou při poklesu hemoglobinu v souvislosti s infekcí. U dívky a obou rodičů nebyly překvapivě při screeningovém vyšetření aktivity G6PD a PK na jiném pracovišti prokázány patologické hodnoty. Rodiče dětí jsou zdraví, netrpí žádným hematologickým ani metabolickým onemocněním.

Pacient č. 3

Třetím nemocným je 11měsíční chlapec, který byl po propuštění z novorozeneckého oddělení odeslán na hematologickou ambulanci pro suspektní vrozenou hemolytickou anemii. Je z prvního těhotenství šestnáctileté matky. Má závažnou perinatální anamnézu. Byl narozen v termínu s porodní hmotností 3020 g, po porodu byl těžce hypoxický a byla nutná intubace a napojení na umělou plicní ventilaci po dobu 6 dnů. Ihned po porodu byla zjištěna vysoká hladina bilirubinu (90 µmol/l), těžká anemie a trombocytopenie. Byla provedena výměnná transfuze. V dalším průběhu byla nutná po dobu 5 dní fototerapie a opakovaně byla podána transfuze erytrocytární masy.

Při klinickém vyšetření v hematologické ambulanci byl nápadný mírný ikterus s šedým nádechem kůže dítěte, lehce ikterické skléry a výrazná hepatosplenomegalie. Dítě bylo přijato k hospitalizaci k vyšetření pro anemii a pro podezření na vrozenou metabolickou vadu. Otec nežije s rodinou a o jeho zdravotním stavu nelze získat dostačující informace.

Pacient č. 4

Poslední pacient je 7letý chlapec odeslaný k dovyšetření anemie nejasného původu. Chlapec byl anemický již v novorozeneckém věku, po porodu měl časnou nekonjugovanou hyperbilirubinemii, bez sérologického konfliktu, která si vyžádala osmidenní fototerapii. Vzhledem k postupné anemizaci v měsíci věku byla nutná další transfuzní léčba. Koncentrace hemoglobinu (Hb) se pohybovala kolem 60 g/l, hyperbilirubinemie (224 µmol/l) přetrvávala. K vyloučení poruchy membrány erytrocytu byl proveden test kryohemolýzy s negativ-

ním nálezem. Byl vyšetřen test stability Hb, fetální hemoglobin (HbF) alkalickou denaturací a autohemolýza, vše s negativním výsledkem.

Pacient v roce věku podstoupil punkci kostní dřeně, při které byla zjištěna aktivovaná erytropoeza bez dalších specifických patologických nálezů. V dalším sledování měl středně těžkou makrocytární anemii s hladinou Hb kolem 100 g/l, retikulocytózu a lehkou nekonjugovanou hyperbilirubinemii (30 μmol/l). Při screeningu enzymopatií na jiném pracovišti rovněž nebylo potvrzeno snížení aktivity PK nebo G6PD. Matka 7letého chlapce dříve užívala preparáty železa pro sideropenickou anemii.

METODY

U všech dětí bylo provedeno opakované standardní vyšetření krevního obrazu včetně nátěru periferní krve a biochemické vyšetření markerů hemolýzy a metabolismu železa. Test kryohemolýzy, elektroforéza hemoglobinů, průkaz Heinzových tělísek a test tepelné stability byly provedeny podle standardních postupů.

Stanovení aktivity pyruvátkinázy a G6PD

Aktivita G6PD byla stanovena spektrofotometricky pomocí komerčního kitu (The Microplate Neonatal G6PD Screening Assay, Bio-Rad Laboratories, UK).

Aktivita PK byla stanovena následovně: Pro přípravu enzymových lyzátů byla použita periferní krev (heparin) s následným odstraněním leukocytů a destiček filtrací přes směs mikrokrystalické celulózy s α-celulózou. Po odstranění plazmy centrifugací byla připravena buněčná suspenze ve fyziologickém roztoku (1:1). Výsledný enzymový lyzát byl připraven přidáním buněčné suspenze do stabilizačního roztoku obsahujícího EDTA a merkaptoethanol (1:9).

Aktivita pyruvátkinázy (E.C.2.7.1.40) byla stanovena spektrofotometricky v temperované směsi (37 °C) obsahující laktátdehydrogenázu, ADP, fosfoenolpyruvát a NADH sledováním poklesu absorbance při 340 nm (Infinite 200 NanoQuant, Tecan, Švýcarsko). Specifická aktivita (U/g), která se uvádí v literatuře, byla vztažena k množství hemoglobinu v lyzátu stanoveném rovněž spektrofotometricky při 414 nm [12].

Sekvenace genů *PKLR* a *HFE1*

Genomická DNA byla izolována z periferní krve pomocí QIAamp DNA kitu (Qiagen, USA). Všechny exony byly amplifikovány pomocí PCR (PCR primery a podmínky jsou k dispozici na vyžádání). Pro sekvenační reakci odpovídajících PCR fragmentů byl použit BigDye terminator kit v 1.1 (Applied Biosystem, USA) a sekvenační analýzy byly provedeny na sekvenátoru ABI Prism 3100 (Applied Biosystems, USA).

Stanovení hladiny hepcidinu

Hladiny hepcidinu (bioaktivní forma hepcidinu-25) byly stanoveny v plazmě. Část plazmy určená k měření hladiny hepcidinu byla rozdělena do alikvotů, aby se předešlo opakovanému rozmrazování, a zamražena při -80 °C. K měření hladin hepcidinu byl použit komerční ELISA kit (DRG Instruments, Německo).

Parametry metody podle výrobce byly následující: dynamický rozsah 0,9–140 ng/ml, senzitivita této metody byla stanovena na 0,9 ng/ml, reprodukovatelnost – intra assay CV 4,86 %, inter assay test CV 11,42 %.

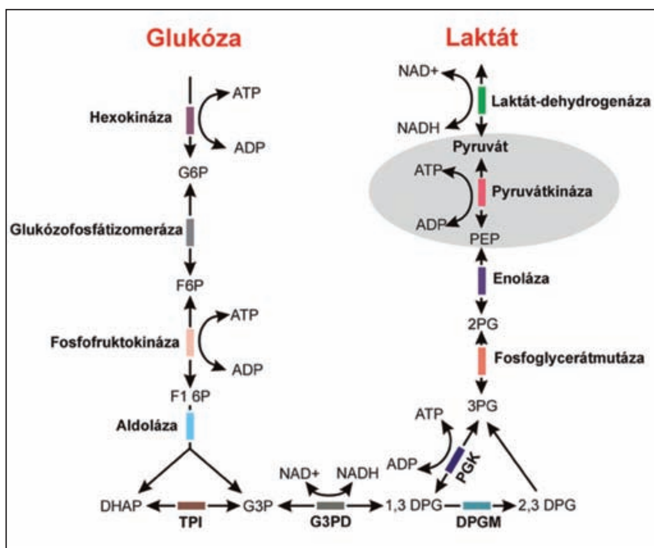
VÝSLEDKY

U 3 dětí (pacienti 1, 2 a 4) se jednalo o makrocytární, v jednom případě o přechodně normocytární anemii (výsledek zkreslen předchozí aplikací erytrocytární masy – pacient č. 3). Hladina Hb se po-

Tab. 1. Vyšetření krevního obrazu a biochemických nálezů u pacientů.

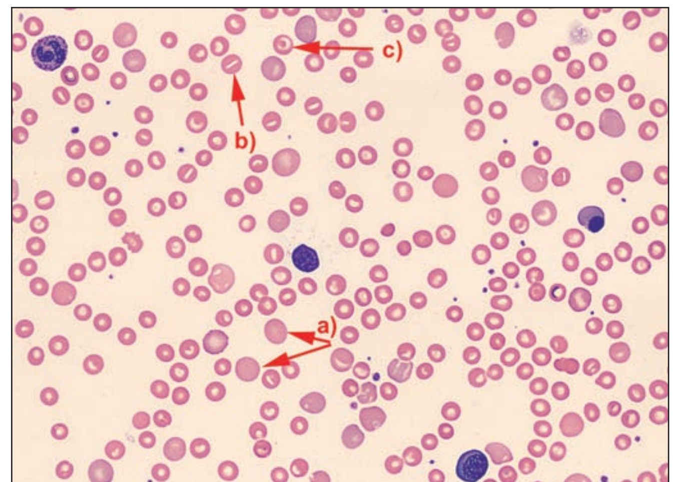
Pacient č.	Věk/ pohlaví (měsíce)	Hemoglobin (g/l)	Erytrocyty (10^9/l)	MCV (fl)	MCH (pg)	Retikulocyty	Bilirubin (μmol/l)	LD (μkat/l)	Feritin (μg/l)
1	50/Ž	71	2,20	102,3	32,3	0,124	54	15,40	19
2	10/M	64	2,46	92,6	31,5	0,190	81	13,16	716
3	11/M	77	2,76	90,5	27,9	0,101	89	3,75	4853
4	86/M	97	3,12	88,8	31,1	0,081	38	5,58	205

MCV: střední objem erytrocytů
MCH: střední koncentrace hemoglobinu
LD: laktátdehydrogenáza
U všech pacientů je přítomna kolísavá makrocytóza a výrazná retikulocytóza, mírná hyperbilirubinemie a výrazné zvýšení LD. U pacientů č. 2 a 3 je výrazně zvýšená hladina feritinu.



Obr. 1. Schéma glykolytické dráhy.
PGK: fosfoglycerátkináza, G3PD: glyceraldehyd-3-fosfátdehydrogenáza, TPI: triózofosfátizomeráza, DPGM: difosfoglycerátmutáza
Glykolýza je metabolická dráha přeměny glukózy na dvě molekuly pyruvátu za čistého výtěžku dvou molekul ATP a dvou molekul NADH. Probíhá v ***cytosolu*** *buněk. Skládá se z deseti kroků, každý z nich katalyzuje jiný enzym. Posledním nevratným krokem je přeměna fosfoenolpyruvátu na pyruvát za vzniku ATP. Tato přeměna je katalyzovaná pyruvátkinázou. Přenosem vodíku z NADH na poslední produkt glykolýzy – pyruvát dochází k redukci pyruvátu na laktát.*

Fig. 1. Diagram of glycolytic pathway.
PGK: phosphoglycerate kinase, G3PD: glyceraldehyde-3-phosphate dehydrogenase, TPI: triosaphosphateisomerase, DPGM: diphosphogylcerate mutase
Glycolysis is the metabolic pathway of conversion of glucose into two molecules of pyruvate, a pure yield of two molecules of ATP and two molecules of NADH. It takes place in the cytosol of cells. It consists of ten steps, each is catalysing another enzyme. The last step is the irreversible conversion of phosphoenolpyruvate to pyruvate to produce ATP. This transformation is catalyzed by pyruvatekinase. The hydrogen transfer from NADH to the last product of glycolysis – pyruvate is causing a reduction of pyruvate to lactate.



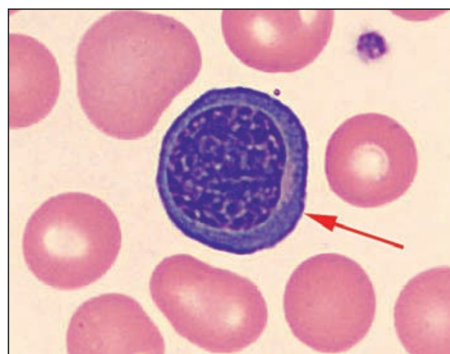
Obr. 2. Nátěr periferní krve pacienta č. 3 – anizocytóza, polychromazie (a), ojedinělé stomatocyty (b) a terčovité erytrocyty (c).
Fig. 2. The No. 3 patient's blood smear – anisocytosis, polychromasia (a), isolated stomatocytes (b) and target erythrocytes (c).

hybovala v rozmezí 62 až 100 g/l a retikulocyty byly v rozmezí 10–19 %. Při biochemickém vyšetření byla u všech pacientů výrazně zvýšená hladina LD a jen lehce vyšší hladina nekonjugovaného bilirubinu (tab. 1). U pacientů č. 2 a 3 byla prokázána výrazně zvýšená hladina feritinu: 716 μg/l a 4853 μg/l.

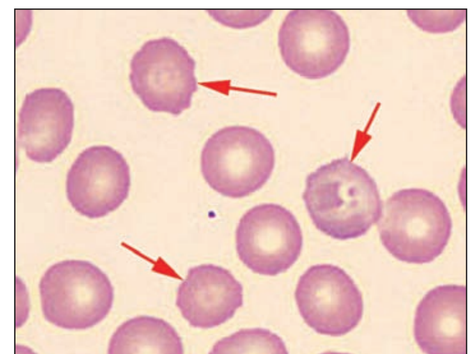
V nátěru periferní krve byly u všech pacientů patrné nespecifické změny morfologie erytrocytů: polychromazie, anizocytóza a u pacientů č. 2 a 3 rovněž terčovité erytrocyty a stomatocyty. U pacienta č. 3 byly navíc přítomny echinocyty a ojedinělé normoblasty.

Porucha membrány erytrocytů a nestabilní hemoglobinopatie byly u všech dětí vyloučeny testem kryohemolýzy, elektroforézou hemoglobinů, průkazem Heinzových tělísek a testem tepelné stability, které byly ve všech případech negativní.

Vyšetření kostní dřeně u pacientů č. 1, 3 a 4 prokázalo výraznou akcentaci prekurzorových buněk erytropoezy (40 až 74,1 %) se známkami mírné dysplazie, avšak bez nálezu zvýšeného procenta vícejaderných erytroblastů a prstenčitých sideroblastů. Byla tedy vyloučena kongenitální dyserytropoetická anemie i sideroblastická anemie.



Obr. 3. Detail nátěru periferní krve pacienta č. 3 s nálezem jaderné erytroid - ní buňky.
Fig. 3. Detail of the patient's peripheral blood smear No. 3 with finding of nucleated erythroid cells.



Obr. 4. Detail nátěru periferní krve pacienta č. 2 s ojedinělými echinocyty.
Fig. 4. Detail of the patient's peripheral blood smear No. 2 with isolated echinocytes.

Tab. 2. Výsledky biochemických a molekulárně genetických analýz.

	Aktivita G6PD (U/g Hb)	Aktivita PK (U/g Hb)	Mutace	Erytropoeze v kostní dřeni (%)	Hepcidin (ng/ml)
Pacient č. 1	8,48	1,43	c.1529C>A/c.1594C>T	74,1	54,3
Pacient č. 2	8,13	1,14	c.1529C>A/ c.1594C>T	-	53,2
Pacient č. 3	5,83	1,63	homozygotní delece	73,2	11,8
Pacient č. 4	5,77	1,73	c.1493G>A/c.1529C>A	40	24,5
Kontroly	5,0–9,93	4,6–6,28	-		

G6PD – glukózo-6-fosfátdehydrogenáza; PK – pyruvátkináza; aktivity G6PD a PK a koncentrace hepcidinu byly stanoveny v tripletech, normální hladiny: 13,3–54,4; rozmezí kontrolních hodnot aktivit G6PD a PK bylo stanoveno u 10 zdravých kontrol

U všech nemocných byla stanovena aktivita pyruvátkinázy, která byla ve všech případech snížená a odpovídala přibližně 30 % hodnoty kontrolních aktivit. Sekvenováním genu pro PK byla u všech 4 pacientů prokázána kauzální mutace. Oba sourozenci (pacienti č. 1 a 2) jsou smíšenými heterozygoty pro známé mutace c.1529C>A (p.Arg510Gln) a c.1594C>T (p.Arg532Trp) nacházející se v exonu 11. U pacienta č. 3 se jedná o dosud nepopsanou homozygotní aminokyselinovou deleci v exonu 11, která vede k posunu čtecího rámce a tvorbě proteinu, kterému chybí část aktivační domény. Tato mutace nebyla doposud popsána a její funkční studie dále probíhají. Pacient č. 4 je také smíšený heterozygot pro dvě již popsané mutace, a to mutaci c. 1493G>A (p.Arg498His) a opět c.1529C>A (p.Arg510Gln).

Vzhledem ke zvýšení hladiny feritinu byla u všech pacientů vyšetřena hladina hepcidinu, která byla u pacienta č. 3 snížená (11,3 ng/ml), u pacienta č. 2 na dolní hranici normy. U pacientů č. 1 a 4 byla hodnota hepcidinu normální.

Vzhledem k neobvykle vysoké hladině feritinu bylo u pacienta č. 3 provedeno i molekulárně genetické vyšetření k vyloučení současně přítomné mutace pro hemochromatózu typu 1 (HFE), aby se vyloučila nebo potvrdila možná koincidence deficitu PK s některou z dalších poruch metabolismu železa – vrozenou hemochromatózou. Nejčastější mutace spojené s HFE1 – c.845C>A (p.Cys 282Tyr), c.187C>G (p.His63Asp), c.193A>T (p.Ser65Cys) – však nebyly sekvenační analýzou prokázány.

DISKUSE

Na popsaném souboru pacientů poukazujeme na různorodost klinických projevů při deficitu PK. Zajímavé je, že mutace c.1529C>A, kterou jsme detekovali u 3 pacientů, je svým nálezem sice typická pro střední a severní Evropu, ale ve slovanské populaci, pro kterou je charakteristický nález mutace c.1594C>T, se objevuje jen zřídka [11].

I přesto, že není známa přímá korelace mezi genotypem a fenotypem u různých mutací, dá se předpokládat, že mutace měnící čtecí rámec, promotorovou oblast nebo větší delece, které vedou k výrazné redukci aktivity PK, mohou vést k závažnému fenotypu, jako je intrauterinní smrt plodu nebo život ohrožující anemie u novorozence. Na základě studie italských autorů [7], která se zabývá vztahem mezi závažností anemie a konkrétními mutacemi, se dá předpokládat, že některé homozygotní mutace, které mohou být i důsledkem konsanguinity, odpovídají lehkému, střednímu nebo těžkému stupni anemie. U pacientů s mutací c.1456C>T a c.1594C>T jde tak často o mírnou anemii (více než 100 g/l Hb). Mutace c.1529C>A by měla podle uvedených autorů vést ke středně těžké anemii (Hb 80–100 g/l, 10 a méně transfuzí) [3, 7]. U smíšených heterozygotů se dá fenotyp odhadnout jen obtížně, což potvrzují i klinické projevy u obou sourozenců (pacienti č. 1 a 2). I přesto, že oba jsou smíšenými heterozygoty pro mutace c.1529C>A a c.1594C>T, které jsou zmiňované spíše s mírnějšími projevy anemie, oba sourozenci mají těžkou formu anemie s nutností opakovaných transfuzí.

Na patogenezi rozvoje přetížení železem u pacientů s deficitem PK se mohou podílet opakované transfuze, hemolýza, ale i neefektivní erytropoeza. K přetížení železem může přispívat i koincidence deficitu PK s vrozenou poruchou metabolismu železa. Až 20 % evropské populace má mírnou alteraci v metabolismu železa spojovanou s heterozygotním přenašečstvím mutací c.187C>G (p.His63Asp) a c.845C>A (p.Cys282Tyr) v *HFE* genu způsobujících vrozenou hemochromatózu. Poslední studie prokázaly abnormality v *HFE* genu u 35 % pacientů s PK deficitem, kteří nejsou závislí na transfuzích [13]. Tyto abnormality jsou spojeny se zvýšením hladiny železa (Fe) v séru a feritinu [14]. Přetížení železem i bez mutace genu pro hemochromatózu bylo popsáno u polytransfundovaných pacientů a pacientů s PK deficitem po splenektomii [14].

Diskutuje se i o možné roli snížení hladiny hepcidinu, klíčové molekuly regulující přísun železa do organismu [15]. Hladina hepcidinu je kontrolována zejména erytropoetickou aktivitou prostřednictvím

signálních molekul GDF15 a TWSG1, které reagují na anemii. Stav erytropoezy je pravděpodobně nadřazeným faktorem všem ostatním mechanismům ovlivňujícím metabolismus Fe a hladinu železa v organismu. Hladina hepcidinu se snižuje u anemií, především u stavů se zvýšenou potřebou Fe, jako je sideropenická anemie, což vede ke zvýšenému vstřebávání železa ze střeva. Zvyšuje se naopak u stavů s vysokou hladinou železa v organismu, např. u vrozených hemochromatóz nebo chorob spojených se sekundárním přetížením železem a nezávisle u akutního zánětu. Bylo prokázáno, že u deficitu PK se kombinují oba faktory regulující protichůdně produkci hepcidinu: na jedné straně anemie s vyššími nároky na přísun železa při větším obratu erytropoezy a na straně druhé zvýšená nabídka železa s vysokou hladinou feritinu. Těžká anemie je v tomto případě nadřazena signálům o přetížení organismu železem a produkce hepcidinu je negativně regulována cestou aktivace GDF 15. Výsledkem je potom snížení jeho hladiny s následným zvýšením resorpce Fe i přes jeho zvýšené zásoby, což může přispívat ke zvyšování zásobního železa a k rozvoji přetížení železem. U pacienta č. 3 jsme také prokázali snížení hladiny hepcidinu, která se mohla spolupodílet na neobvykle vysoké hladině feritinu. U pacienta č. 2 byla hladina hepcidinu na dolní hranici normy i při vysoké hladině feritinu. Při přetrvávající výrazné hyperferitinemii bude potřeba u pacienta č. 3 zvážit sekvenování *HFE2A* genu kódujícího hemojuvelin a *HFE3* genu kódujícího transferinový receptor 2 [16].

I když je deficit pyruvátkinázy nejčastější enzymatickou abnormalitou glykolytické dráhy, která způsobuje dědičnou nesférocytární hemolytickou anemii, bylo v České republice u dětí zatím popsáno pouze několik případů. A to i přesto, že podle celosvětové prevalence 1:20 000 [2, 7] by v České republice mělo být teoreticky diagnostikováno 500 osob trpících touto nemocí. Jednou z příčin může být určení nesprávné diagnózy, a to z několika možných důvodů. U polytransfundovaných pacientů může být stanovená aktivita PK zkreslená, zejména pokud se stanovuje v odběru bezprostředně po transfuzi nebo v krátkém intervalu od transfuze, protože se tak měří vlastně i aktivita PK dárcovských erytrocytů. Je proto důležité, aby se v těchto případech aktivita PK stanovovala až ve vzorku odebraném těsně před další transfuzí, kdy už prakticky nehrozí „kontaminace“ dárcovskými erytrocyty. Dalším faktorem zkreslujícím vyšetření může být přítomnost leukocytů a trombocytů ve vyšetřovaném materiálu, protože jak leukocyty, tak trombocyty obsahují jinou izoformu enzymu (PK-M2), která má až 300krát vyšší aktivitu než erytrocytární forma PK-R [7]. Vzhledem k tomu, že aktivita řady enzymů souvisí se stářím populace červených krvinek, může mít i zvýšený počet retikulocytů za následek zvýšení aktivity některých enzymů, zejména hexokinázy a pyruvátkinázy.

ZÁVĚR

Cílem tohoto sdělení, které popisuje soubor prvních dětských pacientů s průkazem kauzální mutace genu *PKLR*, je upozornit na variabilitu klinických i laboratorních příznaků u pacientů s deficitem PK, která může být vzácně provázena závažným přetížením železem, a na úskalí diagnostiky této enzymopatie.

U pacientů s deficitem PK a s rozvojem závažného přetížení železem je vždy nutno zvážit možnost výskytu přidružené vrozené hemochromatózy. Je třeba pečlivě monitorovat stav zásob železa a v indikovaných případech včas zahájit chelatační léčbu jako prevenci rozvoje cirhózy jater při neléčené hemosideróze jater [14].

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Nové knihy z Edice Lékař a pacient

LÉČBA DIABETU INZULINOVOU PUMPOU U DĚTÍ KROK ZA KROKEM – nejen pro rodiče a edukační sestry

MUDr. David Neumann, Ph.D.

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Rodiče dětí s diabetem, stejně jako i edukační sestry, se setkávají s řadou situací, které musí správně vyřešit, aby předešli akutnímu nebo dlouhodobému zhoršení nemoci.
Právě jim je určena naše knížka – tedy především těm, kteří se k léčbě inzulinovou pumpou u dětí chystají anebo právě začali. Měla by jim být každodenním pomocníkem při zvládání nejrůznějších problémů tak, aby byl dítěti umožněn co nejbohatší výběr činností a cukrovka nezasahovala do jeho psychického, dovednostního a sociálního vývoje.
Krok za krokem jsou probrány jednotlivé fáze a možné situace, počínaje úvodním nastavením přes zvládnutí glykemií po jídlech a v noci až po období „experimentů“, kdy dítě a rodič zjišťují, co všechno je s „pumpičkou“ možné. Důraz klade autor na pochopení bezpečnosti léčby a nástrah, které nese. Podrobně proto čtenáře seznamuje mj. se specialitami moderních pump, jak pomáhají, ve kterých situacích jsou prospěšné, kdy ne a jaké jsou alternativní možnosti léčby.
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bjh research paper

Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the *PKLR* gene (p.Arg518fs), and low hepcidin to ferritin ratios

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Summary

Pyruvate kinase (PK) deficiency is an iron-loading anaemia characterized by chronic haemolysis, ineffective erythropoiesis and a requirement for blood transfusion in most cases. We studied 11 patients from 10 unrelated families and found nine different disease-causing *PKLR* mutations. Two of these mutations - the point mutation c.878A>T (p.Asp293Val) and the frameshift deletion c.1553delG (p.(Arg518Leufs*12)) - have not been previously described in the literature. This frameshift deletion was associated with an unusually severe phenotype involving neonatal hyperferritinaemia that is not typical of PK deficiency. No disease-causing mutations in genes associated with haemochromatosis could be found. Inappropriately low levels of hepcidin with respect to iron loading were detected in all PK-deficient patients with increased ferritin, confirming the predominant effect of accelerated erythropoiesis on hepcidin production. Although the levels of a putative hepcidin suppressor, growth differentiation factor-15, were increased in PK-deficient patients, no negative correlation with hepcidin was found. This result indicates the existence of another as-yet unidentified erythroid regulator of hepcidin synthesis in PK deficiency.

Keywords: red blood cell, pyruvate kinase deficiency, iron overload, hepcidin, ferritin.

Erythrocyte pyruvate kinase (PK) deficiency, the most common cause of nonspherocytic haemolytic anaemia in central and northern Europe, is characterized by a variable degree of haemolysis and increased risk of iron overload. The mechanism of haemolysis is not clearly understood; however, the disruption of glycolysis leading to ATP depletion is one of the major contributing factors (Zanella *et al*, 2005). An appropriate diagnosis can be made only by measuring the erythrocyte PK enzyme activity and by direct mutation analysis of the gene encoding PK (*PKLR*). More than 220 mutations in the *PKLR* gene (1q22) have been identified worldwide since the first mutation was described in 1961 (Valentine *et al*, 1961). The disease is transmitted as a recessive trait, usually affecting only homozygotes or compound heterozygotes. The clinical manifestations vary from mild to severe anaemia requiring repeated transfusions. Rare cases of

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neonatal death and non-immune hydrops foetalis have also been reported (Ferreira *et al*, 2000). Similar to patients with β-thalassaemia, PK-deficient patients may develop secondary iron overload. Although repeated blood transfusions were considered to be the major cause, iron accumulation also affects non-transfused PK-deficient patients (Zanella *et al*, 1993). The pathogenesis of iron overload appears to be multifactorial, involving chronic haemolysis, ineffective erythropoiesis, splenectomy and, eventually, coinheritance of hereditary haemochromatosis (Zanella *et al*, 2001). Importantly, the levels of hepcidin, the negative regulator of iron absorption in the gut and iron recycling from macrophages (Ganz, 2004), are reduced in patients with PK deficiency (Finkenstedt *et al*, 2008). Based on recent analyses, growth differentiation factor-15 (GDF15) is one of the candidate molecules proposed to suppress hepcidin production and enhance iron loading in the setting of ineffective erythropoiesis (Tanno & Miller, 2010; Tanno *et al*, 2010). The negative correlation between hepcidin and GDF15 was first reported in patients with β-thalassaemia (Tanno *et al*, 2007). Although GDF15 levels are increased in PK deficiency, the magnitude of GDF15 elevation is markedly lower than that in β-thalassaemia (Tanno *et al*, 2010). Here, we analysed *PKLR* mutations and iron status parameters in a cohort of 11 patients with PK deficiency. Our genetic data, together with calculations of the hepcidin/ferritin ratio, indicate the involvement of an as-yet unknown erythroid-derived hepcidin suppressor in PK deficiency.

Design and methods

Patients, haematological and biochemical analysis

We investigated 11 patients with congenital nonspherocytic haemolytic anaemia from 10 unrelated Czech and Slovak families. The patient cohort consisted of five adults, three children and three infants. The Ethics Committee of Palacky University Hospital approved the collection and analysis of samples. Informed consent was obtained according to the Declaration of Helsinki. Red blood cell parameters were measured using a Sysmex XE-500 analyser (Sysmex Corp., Kobe, Japan). Serum parameters of iron metabolism, including iron, ferritin and transferrin saturation as well as bilirubin levels, were determined with standard biochemical methods. PK and hexokinase (HK) activities were measured in erythrocyte lysates that were free of leucocytes and platelets according to the methods recommended by the International Committee for Standardization in Haematology (Beutler *et al*, 1977). PK and HK activities were expressed as u/g haemoglobin (Hb). HK activity was included to estimate the contribution of young red blood cells to the total PK activity. The reference values for enzyme activities were established from 10 normal subjects. GDF15 serum concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit for human GDF15 according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The normal range for GDF15 was determined from eight healthy controls (four children and four adults). The serum erythropoietin (EPO) concentrations were measured by radioimmunoassay (Zadrazil *et al*, 2007).

Hepcidin measurement

The method for hepcidin measurement was established based on the protocol published by Li *et al* (2009). Briefly, each sample was prepared from 200 μl of human serum. Calibration standards were prepared from unmodified synthetic human hepcidin (DTHFPICIFCCGCCHRSKCGMCCKT) dissolved in rabbit serum. Hepcidin labelled with a stable isotope [$^{13}C_9$, $^{15}N_1$-Phe_4] (50 ng) was added to each sample as an internal standard. Both hepcidin variants were synthesized by Thermo Fisher Scientific (Ulm, Germany). The samples were extracted on an Oasis HLB μElution plate and separated by reverse-phase liquid chromatography using an UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to a QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA). Subsequent detection of hepcidin was performed by selected reaction monitoring method. The acquired data were processed using Analyst® software (AB SCIEX, version 1.5.1). The assay was validated by Intrinsic LifeSciences LLC (La Jolla, CA, USA), and the normal control range was determined in 18 healthy individuals (10 children and eight adults).

Molecular analysis

Genomic DNA was isolated from peripheral blood drawn in EDTA using a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). The exons of the following were amplified by polymerase chain reaction (PCR): *PKLR* (accession: NM_000298.5) (Lenzner *et al*, 1997); genes known to be responsible for hereditary haemochromatosis (Camaschella & Poggiali, 2011), including *HFE* (accession: NM_000410.3), *HFE2* (haemojuvelin) (accession: NM_213653.3), *HAMP* (hepcidin) (accession: NM_021175.2), *TFR2* (transferrin receptor 2) (accession: NM_003227.3) and *SLC40A1* (ferroportin 1) (accession: NM_014585.5); and a gene encoding the light chain of ferritin *(FTL)* (accession: NM_000146.3). The iron response element (IRE) regions of the 5′-UTR of *SLC40A1* and *FTL* were also amplified. PCR primers and conditions are available upon request. The PCR products were purified with a QIAquick kit (Qiagen) and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the ABI Prism 310 Genetic Analyser (Applied Biosystems) with software provided by the manufacturer.

Statistical methods

Student's *t*-test was used to determine the statistical difference between the patients and controls using Origin 6.1

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software (OriginLab Corporation, Northampton, MA, USA). The Spearman coefficient for correlation analyses was calculated using Statistica 10 software (StatSoft, Inc., Tulsa, OK, USA). *P* values <0·05 were considered statistically significant.

Results

Patients' characteristics and PK activity

The clinical findings of nine unrelated patients and two siblings obtained at the time of diagnosis are summarized in Table I. All patients suffered from mild to severe haemolytic anaemia (with Hb levels ranging from 65 to 121 g/l) and hyperbilirubinaemia (33 to 172 μmol/l). All but one of these patients had increased reticulocyte counts (4·8 to 80·5%). Neonatal jaundice was reported in most of the patients. Seven patients were transfusion-dependent; three were concomitantly treated with chelators and two underwent splenectomy. Two paediatric patients received transfusions during infancy, one adult patient was on intermittent transfusion therapy and only one patient had never received a transfusion in his lifetime. In 10 patients, erythrocyte PK activity was below the normal reference values (4·60–6·28 u/g Hb) (Table II). In one case (Patient 7), the activity was false normal (6·58 u/g Hb) due to substantial reticulocytosis (80·5%) and PK deficiency was therefore established based on low PK/HK activity ratio (1·6; normal range 3·6–6·3) (Table II). Reduced PK activity compared with the controls was also detected in the available healthy parents, confirming a congenital defect in the child. All parents tested were later shown to be heterozygous carriers of specific *PKLR* mutations (Table II).

Mutational analysis of the PKLR gene

Direct sequence analysis of the *PKLR* gene in the patient cohort revealed nine different mutations in either the homozygous state (four patients) or the compound heterozygous state (seven patients) (Table II). Seven of the mutations are known; five of these (c.1594C>T, c.1529G>A, c.1493G>A, c.1456C>T, and c.994G>A) have previously been identified in Czech and Slovak populations (Lenzner *et al*, 1997). The c.823G>A substitution and the insertion-deletion (c.347_350delinsAACATTG) were identified in these populations for the first time. The clinical picture and the severity of the disease in our patients bearing the above-mentioned PK mutations were comparable to the phenotype previously reported for patients with identical mutations (Baronciani *et al*, 1995; Zanella *et al*, 1997, 2005, 2007). The c.878A>T and c.1553delG mutations are novel and have not been previously described in the literature.

The maternally inherited c.878A>T missense mutation was detected in a heterozygous state with the previously reported, paternally inherited c.1529G>A substitution (Fig 1A, Table II), which is the most common *PKLR* mutation in the northern and central European population (Zanella *et al*, 2005). The c.878A>T mutation involves the alteration of positively charged aspartic acid to hydrophobic valine on the

Table I. Clinical and haematological data of the patients.

Patient	Sex	Age	Neonatal jaundice	ExTx	Spleen	Hb, g/l	Ret,% [0·5–3·0]	Bilirubin, μmol/l [0–23]	Ferritin, μg/l [6–200]*	TfS [0·21–0·48]	Hepcidin, μg/l [13·1–104·8]	GDF15, ng/l [166–344]	EPO, iu/l [4·3–29]
1	M	9 years	Yes	Yes	Out	79	32	38	2000§	0·79	n.d.	3052	n.d
2	M	6 months	Yes	Yes	In	77	7·2	33	4852	0·97	90·7	935	46·2
		33 months				98	4·8	49	145§	n.d.	16	1399	78·1
3	F	7 years	Yes	Yes†	In	95	10·6	35	16	0·26	5·7	851	454
4	M	10 months	Yes	Yes	In	75	7·5	59	303	n.d.	15·5	876	123
5	M	7 years	Yes	Yes†	In	97	8·1	39	205	0·50	24·5	386	n.d
6	F	42 years	No	Yes‡	In	103	5·9	50	645	0·37	11·4	2581	46·8
7	F	22 years	Yes	Yes	Out	65	80·5	139	372§	0·88	9·1	514	122
8	F	31 years	Yes	Yes	In	95	6·6	34	948	0·54	29·1	805	126
9	M	3 months	Yes	Yes	In	67	2·1	172	802	n.d.	n.d.	2163	n.d
10	F	25 years	Yes	Yes	In	73	25·3	124	29·7	0·20	5·7	1259	n.d
11	M	50 years	No	No	In	121	5·2	49	906	0·34	68·8	854	51·5

Patients 3 and 4 are siblings; ExTx: exchange transfusion; TfS: transferrin saturation; GDF15: growth differentiation factor-15; EPO (serum erythropoietin); n.d.: not determined.

*normal range for ferritin levels, women: <200, men: <300, children: <70 μg/l (Brugnara, 2009). The control range for GDF15 was determined from eight healthy controls (four children and four adults).

†Dependent on blood transfusion during the first year of life due to severe anaemia.

‡Intermittent ExTx.

§Patients on chelation therapy. Two sets of data are shown for Patient 2; the first set represents values at the time of diagnosis, the second corresponds to values after 6 months of chelation therapy.

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Table II. Biochemical and molecular data of the patients and their family members.

Patient	PK activity, u/g Hb	PK/HK	*PKLR* mutation	Amino acid alteration
1	2·64	0·78	c.[347_350delinsAACATTG]; [347_350delinsAACATTG]	p.[Arg116_Leu117delinsGlnHisCys]; [Arg116_Leu117delinsGlnHisCys]
1 (mother)	2·19	2·92	c.[347_350delinsAACATTG];[=]	p.[Arg116_Leu117delinsGlnHisCys];[=]
1 (father)	2·19	1·97	c.[347_350delinsAACATTG];[=]	p.[Arg116_Leu117delinsGlnHisCys];[=]
2	1·63	1·63	c.[1553delG];[1553delG]	p.[Arg518Leufs*12];[Arg518Leufs*12]
2 (mother)	2·57	2·3	c.[1553delG];[=]	p.[Arg518Leufs*12];[=]
3	1·43	0·51	c.[1529G>A];[1594C>T]	p.[Arg510Gln];[Arg532Trp]
4	1·14	0·46	c.[1529G>A];[1594C>T]	p.[Arg510Gln];[Arg532Trp]
4 (mother)	1·33	1·75	c.[1529G>A];[=]	p.[Arg510Gln];[=]
4 (father)	1·57	1·51	c.[1594C>T];[=]	p.[Arg532Trp];[=]
5	1·73	1·75	c.[1493G>A];[1529G>A]	p.[Arg498His];[Arg510Gln]
6	1·16	0·84	c.[1456C>T];[1594C>T]	p.[Arg486Trp];[Arg532Trp]
7	6·58	1·6	c.[1594C>T];[1594C>T]	p.[Arg532Trp];[(Arg532Trp)]
8	1·56	0·81	c.[878A>T];[1529G>A]	p.[Asp293Val];[Arg510Gln]
8 (father)	3·41	3·48	c.[1529G>A];[=]	p.[Arg510Gln];[=]
8 (mother)	4·04	3·98	c.[878A>T];[=]	p.[Asp293Val];[=]
9	1·72	2·07	c.[823G>A];[1594C>T]	p.[Gly275Arg];[Arg532Trp]
9 (father)	2·45	2·62	c.[823G>A];[=]	p.[Gly275Arg];[=]
9 (mother)	–	–	c.[1594C>T];[=]	p.[Arg532Trp];[=]
10	2·32	1·12	c.[1529G>A];[1529G>A]	p.[Arg510Gln];[(Arg510Gln)]
11	1·54	1·14	c.[994G>A];[1456C>T]	p.[Gly332Ser];[Arg532Trp]

PK: pyruvate kinase (normal activity range: 4·60–6·28 u/g Hb); PK/HK: pyruvate kinase and hexokinase activity ratio (normal range: 3·6–6·3). The normal activity range was determined in 10 healthy controls.

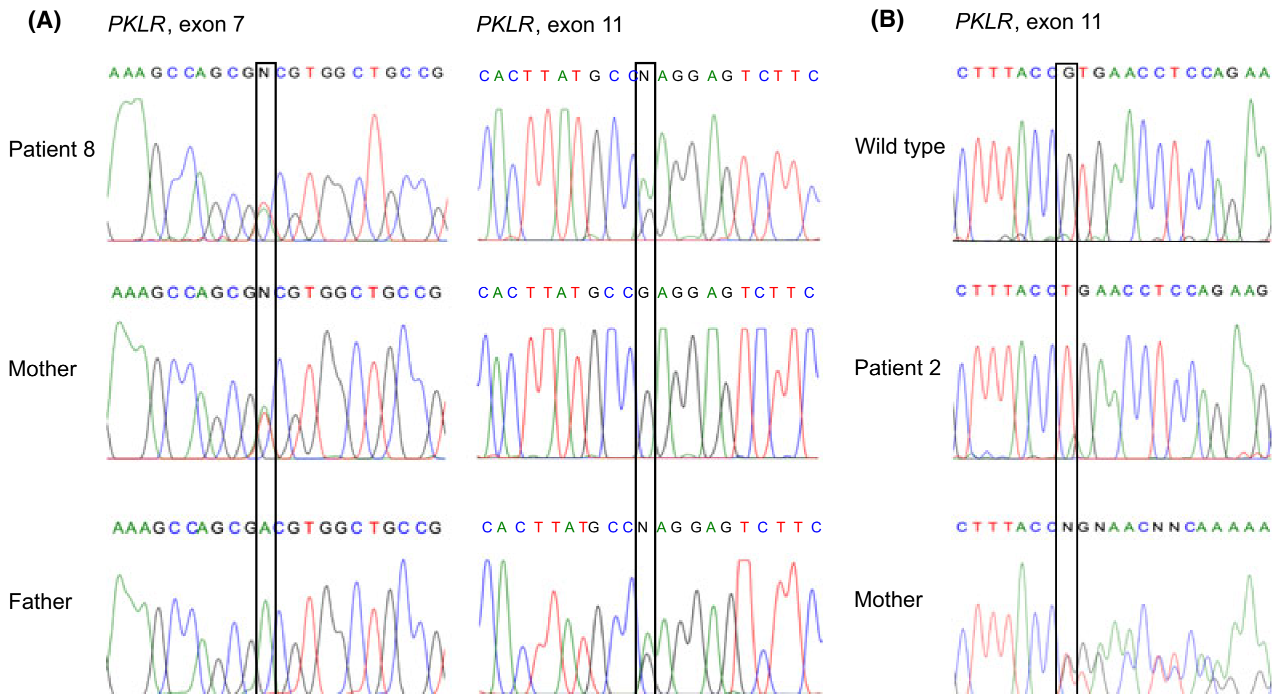

Fig 1. Detection of two novel *PKLR* mutations by sequencing amplified DNA. (A) In the family of Patient 8, the propositus carried the newly described c.878A>T mutation in exon 7 and the c.1529G>A mutation in exon 11; the mother was heterozygous for the c.878A>T mutation, and the father was heterozygous for the c.1529G>A mutation. (B) In the family of Patient 2, the propositus was homozygous for the second newly described mutation in the *PKLR* gene, a single nucleotide deletion (c.1553delG) in exon 11 leading to a frameshift (p.(Arg518Leufs*12)) and a premature stop codon. One allele was inherited from his heterozygous mother; his father was unavailable for the analyses.

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surface of the A domain of PK. This alteration might increase the hydrophobicity of PK, which is thermodynamically less favourable for protein folding. PK activity was markedly reduced to 1·56 u/g Hb in an affected patient (Patient 8) who presented with a mild phenotype (Hb 95 g/l, reticulocytes 6·6%, and bilirubin 34 μmol/l; Table I).

The novel PKLR frameshift deletion associated with atypical severe phenotype involving neonatal hyperferritinaemia

Patient 2 carried the second previously unidentified frameshift deletion, c.1553delG (p.(Arg518Leufs*12), short p.Arg518fs), in a homozygous state; the mother is a heterozygous carrier of this deletion (Fig 1B, Table II). Because the father is unknown and thus unavailable for analyses, a possible hemizygous genomic deletion (loss of heterozygosity) was excluded by quantitative PCR-based copy number analysis (Supplementary methods; data not shown). The mutation causes premature termination of translation at codon 529, with predicted formation of a truncated protein lacking 46 C-terminal amino acids including the critical activator binding site Arg532 (Valentini *et al*, 2002). The activity of PK was reduced in both the patient (1·63 u/g Hb) and his mother (2·57 u/g Hb). The patient's clinical picture was very severe from birth, with a need for repeated blood transfusions. The patient exhibited profound anaemia (Hb, 77 g/l), with signs of hypoxia, neonatal jaundice (bilirubin, 90 μmol/l), thrombocytopenia, prominent hepatosplenomegaly and dramatic hyperferritinaemia (in the range of 3500–5000 μg/l), and transferrin saturation reaching almost 100%. Elevated levels of liver enzymes (not shown) suggested possible liver damage, which led us to perform liver biopsy. Massive iron deposits in both hepatocytes and Kupffer cells corresponding to grade IV haemosiderosis were detected. Taken together, these findings suggested the presence of another co-inherited defect in the child aside from PK deficiency. Sideroblastic anaemia was ruled out by the absence of sideroblasts in the bone marrow. The nucleotide sequence of the IRE region of the *FTL* gene, which encodes L-ferritin, was not mutated; thus, a possible association with hereditary hyperferritinaemia cataract syndrome (HHCS) was excluded (Brooks *et al*, 2002). Sequence analysis of all known genes that cause primary haemochromatosis (*HFE, HFE2, HAMP, SLC40A1* and *TFR2*) (Camaschella & Poggiali, 2011) did not reveal any causative mutations. Only five different previously described SNP variations were identified in *TFR2* (c.2255G>A (p.Arg752His) - rs41295942), *SLC40A1* (c.44-24G>C - rs1439816, c.-330CGG[8] - rs16836041, c.663T>C (p.Val221 =) - rs2304704) and *FTL* (c.163T>C (p.Leu55 =)) (Table SI). Of these polymorphisms, only rs1439816:G>C was previously shown to be associated with the clinical aggressiveness (liver damage) of hereditary haemochromatosis 1 caused by the p.Cys282Tyr *HFE* mutation; however, this SNP had no effect on ferritin levels in the control group, and it was therefore concluded that the negative effect of the rs1439816:C allele was restricted to pathological conditions (Altès *et al*, 2009). Because nine of our remaining PK-deficient patients were also homozygous for the rs1439816:C allele (Table SI), this polymorphism probably does not contribute to the severe phenotype of this patient and cannot explain his iron overload. Recently, at an age of two and a half years, the patient's ferritin levels dramatically decreased (to 145 μg/l), which may be a result of combined chelation therapy lasting 6 months and a prolonged interval between transfusions. Based on these new findings, we conclude that the severe neonatal phenotype of this patient was caused by the *PKLR* mutation itself.

Iron status in PK-deficient patients

One of the main health complications in PK deficiency is secondary iron overload with multifactorial pathogenesis (Zanella *et al*, 1993, 2001). In our cohort, nine patients had elevated serum ferritin (median, 724 μg/l), including the patient with no transfusion history (Patient 11). Of nine available cases, five showed a concomitant increase in transferrin saturation (from 0·50 to 0·97) (Table I).

Firstly, we assessed a possible effect of splenectomy on iron loading. One of our two splenectomized patients (Patient 7) showed a progressive increase in ferritin levels after spleen removal. Nevertheless, sustained haemolysis was observed in this patient after spleen surgery, which probably contributed to a further increase in her ferritin levels. On the other hand, no significant difference in ferritin was recorded for the second splenectomized patient (Patient 1); splenectomy improved this patient's anaemia, nevertheless his presplenectomy ferritin levels were already dramatically elevated (2213 μg/l). This patient is a child; thus, the contribution of spleen removal to iron loading may become more apparent over time.

We next searched for the presence of known *HFE* mutations (p.His63Asp, p.Cys282Tyr), which have been proposed as a condition that may predispose a patient to increased iron absorption (Arruda *et al*, 2000; Zanella *et al*, 2001). Abnormal *HFE* genotypes were identified in four of our patients (Table SI). One of these patients (Patient 3), who was a p.[His63Asp];[Cys282Tyr] *HFE* compound heterozygote, was the only paediatric patient from the group with normal ferritin levels.

Hepcidin levels in PK deficiency

To address the role of hepcidin in the pathogenesis of iron overload in our patient cohort, we established a proteomics-based method for hepcidin measurement. As expected, the levels of hepcidin were reduced in PK-deficient patients (median, 15·8 μg/l) (Table I) compared with healthy age-matched controls (median, 27·6 μg/l) (Table I), reflecting the impact of ineffective erythropoiesis in PK deficiency. The difference, however, was not statistically significant. We

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therefore calculated the hepcidin/ferritin ratio which represents a more accurate estimation of proper hepcidin production with respect to iron loading, and found it to be very low in our PK-deficient patients (median, 0·06) compared with a healthy age-matched group of controls (median, 0·35) (Fig 2). Even the patient with the highest hepcidin level (Patient 2) showed a dramatically reduced ratio, which was detected in repeated measurements (ranging from 0·01 to 0·04) and eventually increased to 0·11 as a response to 6 months of chelation therapy and improved anaemia (Hb, 98 g/l) (Table I and Fig 2). The direct effect of chelation therapy on hepcidin levels and hepcidin/ferritin ratio is difficult to address in our cohort, given the complexity of positive and negative signals regulating hepcidin, the small number of patients receiving chelation therapy, and the lack of hepcidin pre-chelation values for remaining patients.

Candidate erythroid signals regulating hepcidin

To clarify the involvement of erythropoiesis in iron metabolism in PK deficiency, serum levels of GDF15 and EPO, two markers of erythropoietic activity known to be associated with hepcidin production, were evaluated. The levels of GDF15, a putative negative regulator of hepcidin production in the setting of ineffective erythropoiesis (Tanno & Miller, 2010), were determined using a commercial enzyme-linked immunosorbent assay. PK-deficient patients showed elevated GDF15 compared with healthy age-matched controls (median, 905·5 μg/l and 223 μg/l, respectively) (Table I), confirming previously reported results (Zanella *et al*, 2005). Importantly, no correlation between GDF15 and hepcidin or GDF15 and hepcidin/ferritin ratio was observed. In addition, all available patients from our cohort showed increased levels of EPO (median, 100·1 iu/l) (Table I), probably reflecting tissue hypoxia. Also, EPO levels did not correlate with hepcidin or hepcidin/ferritin ratio.

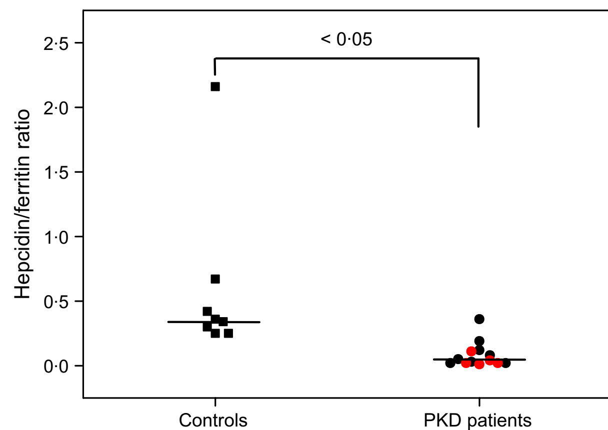

Fig 2. Hepcidin/ferritin ratio in PK-deficient patients. The hepcidin/ferritin ratio was significantly lower in PK-deficient patients compared to healthy controls ($P < 0.05$). The red dots in the graph indicate the hepcidin/ferritin ratio calculated from five repeated measurements (1·5 months, 9·5 months, 10 months, 20·5 months, and 33 months) for Patient 2 with a p.(Arg518Leufs*12) mutation in the *PKLR* gene, which is associated with hyperferritinaemia occurring from the neonatal period through to infancy (2 years of age). Only two hepcidin/ferritin ratios (calculated from those hepcidin and ferritin values shown in Table I) were included into the statistical analysis for this patient. The thick horizontal lines represent the medians for each group. PKD, pyruvate kinase-deficient.

Lastly, as inflammatory processes are known to independently influence hepcidin levels (Ganz, 2004), it is important to note that none of our patients had signs of inflammation at the time of the analyses. In concordance, a substantial increase in hepcidin (from 90·7 μg/l to 386 μg/l) was observed in one of our patients (Patient 2) during bacterial infection.

Discussion

In this report we characterized *PKLR* mutations and systemic iron metabolism in eleven patients with PK deficiency from ten unrelated families. Nine different disease-causing *PKLR* mutations were identified; two of these mutations - the point mutation c.A878T (p.D293V) and the frameshift deletion c.1553delG (p.R518fs) - were novel. The patient affected by the frameshift deletion presented with an unusually severe phenotype involving neonatal hyperferritinaemia, which eventually ameliorated by two and a half years of age, probably in response to combined chelation therapy and a prolonged interval between transfusions.

PK-deficient patients usually develop secondary iron overload over time as a consequence of chronic haemolysis, ineffective erythropoiesis and transfusion therapy; splenectomy and inheritance of *HFE* mutations have been proposed as additional risk factors (Zanella *et al*, 2001). Consistently, all our transfusion-dependent patients presented iron overload; one patient has developed hyperferritinaemia independently of blood transfusions. Similar findings, iron overload independent of blood transfusions in PK deficiency, were previously reported by Zanella *et al* (1993). Although splenectomy reduces the symptoms of haemolysis, it is also known to be a risk factor for iron loading. Due to a low number of splenectomized patients in our cohort, however, the involvement of spleen surgery cannot be adequately addressed. The contribution of co-inherited *HFE* mutations to excessive iron accumulation is also difficult to assess, especially because three of the *HFE* mutant patients were children and HFE-related haemochromatosis is an adult-onset disease (Pietrangelo, 2010). Moreover, one paediatric patient with abnormal *HFE* genotype did not have any signs of iron overload. Based on these observations we can conclude that the *HFE* genotype does not play a major role in determining iron loading in PK-deficient paediatric patients; however, it may be important to follow up these patients due to a potential higher risk of iron overload in adulthood (Zanella *et al*, 2001).

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Analysis of factors regulating iron homeostasis revealed that hepcidin levels were relatively low for the degree of iron loading in all PK-deficient patients with increased ferritin. Similar results were published for β-thalassaemia intermedia (Kearney *et al*, 2007; Origa *et al*, 2007), which clearly confirm the suppression of hepcidin by an erythroid signal that overrides iron loading-induced signalling. Although the levels of the putative hepcidin suppressor, GDF15, were increased in our patient cohort, the levels were considerably lower than those reported for β-thalassaemia (Tanno *et al*, 2010). No correlation between GDF15 and hepcidin or hepcidin/ferritin ratio indicates that GDF15 can only be used as a marker of accelerated ineffective erythropoiesis in PK deficiency (Tanno & Miller, 2010; Tanno *et al*, 2010).

Besides elevated GDF15 in the patients' serum, we also found increased levels of EPO (Ashby *et al*, 2010; Ganz, 2011) that again did not correlate with hepcidin. This evidence further suggests that EPO by itself is an indirect suppressor of hepcidin as mice with disrupted erythropoiesis are not able to attenuate hepcidin synthesis in response to EPO administration (Pak *et al*, 2006; Vokurka *et al*, 2006). In addition to GDF15 and EPO, a suppressive effect of twisted gastrulation protein homolog 1 (TWSG1) (Tanno *et al*, 2009) and hypoxia (Yoon *et al*, 2006; Ganz, 2011) should be considered with respect to the regulation of hepcidin production. Nevertheless, the role of TWSG1 has not been clearly confirmed so far (Tanno *et al*, 2010). Although hypoxia inducible factor (HIF) signalling was reported to directly and also indirectly (through EPO) downregulate hepcidin (Gordeuk *et al*, 2011), the exact signalling remains elusive. Altogether, our findings indicate that the main erythroid-derived regulator of hepcidin synthesis in PK deficiency remains to be identified.

In conclusion, we have shown that the hepcidin/ferritin ratio is consistently low in PK deficiency, which reflects relatively low hepcidin levels, even in PK deficiency associated with hyperferritinaemia.

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Author contributions

RM and PK designed the study, performed enzyme assays and some molecular analyses, collected and analysed data and contributed to manuscript writing. DH established the hepcidin assay. ZZ determined GDF15 levels. DP, JC, ZSL, KI and MS treated the patients, collected patient material and provided clinical information. MP and JK participated in molecular analyses. MH contributed to the design of the study, interpretation of the results and wrote the manuscript. VD participated in the design of the study, interpretation of the results and revision/editing of the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Methods S1. qPCR-based copy number analysis.

Table SI. *HFE*, *TFR2* and *SLC40A1* SNPs.

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Supplementary Method.

Copy number of *PKLR* exon 10 was assessed by SYBR Green I based absolute quantification, using standard curve method. Concentration values of *PKLR* were normalized to *RPPH1* housekeeping gene that is commonly used as a copy number variation (CNV) reference, as it's an autosomal gene with no reported CNV in population. The reaction was run in triplicates. The copy number value of Patient 2 was compared to six non-PK-deficient patient samples. The primer sequences are available upon request.

Table SI. *HFE*, *TFR2* and *SLC40A1* SNPs.

Patient	*HFE* mutation	*TFR2* p.Arg752His	*SLC40A1* CGG(7_8)	*SLC40A1* c.44-24G>C
1	wt	wt	CGG[7];[8]	[C];[C]
1 (mother)	wt	wt	CGG[8];[8]	[C];[C]
1 (father)	wt	wt	CGG[7];[7]	[C];[C]
2	wt	[Arg752His];[=]	CGG[8];[8]	[C];[C]
2 (mother)	wt	wt	CGG[8];[8]	[C];[C]
3	[His63Asp];[Cys282Tyr]	wt	CGG[8];[8]	[C];[C]
4	[His63Asp];[=]	wt	CGG[8];[8]	[C];[C]
3,4 (mother)	[Cys282Tyr];[=]	wt	CGG[8];[8]	[C];[C]
3,4 (father)	[His63Asp];[=]	wt	CGG[7];[8]	[C];[C]
5	[His63Asp];[=]	wt	CGG[8];[8]	[C];[C]
6	[His63Asp];[(His63Asp)]	wt	CGG[8];[8]	[C];[C]
7	wt	wt	CGG[7];[8]	[C];[C]
8	wt	wt	CGG[7];[8]	[C];[C]
8 (mother)	wt	wt	CGG[7];[7]	[C];[G]
8 (father)	wt	wt	CGG[7];[8]	[C];[C]
9	wt	wt	CGG[7];[8]	[C];[G]
9 (mother)	wt	wt	CGG[8];[8]	[C];[C]
9 (father)	wt	wt	CGG[7];[8]	[C];[G]
10	wt	wt	CGG[8];[8]	[C];[C]
11	wt	wt	CGG[8];[8]	[C];[C]

Rem.: *HFE* screened for p.Cys282Tyr, p.His63Asp, p.Ser65Cys, and p.Val256Ile; *TFR2* for p.Arg752His and *SLC40A1* for microsatellite polymorphism c.-330CGG(7_8) and c.44-24G>C.
CGG[7];[8] in *SLC40A1*column is short for c.-330CGG[7];[8]. [C];[C] and [C];[G] in *SLC40A1*column is short for c.[44-24G>C];[44-24G>C] and c.[44-24G>C];[=], respectively.

Title:

Two novel mutations (p.(Ser160Pro) and p.(Arg472Cys)) causing glucose-6-phosphate isomerase deficiency are associated with erythroid dysplasia and inappropriately suppressed hepcidin

Short title: glucose-6-phosphate isomerase deficiency causing hemolytic anemia

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Abstract

Glucose-6-phosphate isomerase (GPI) deficiency, a genetic disorder responsible for chronic nonspherocytic hemolytic anemia, is the second most common red blood cell glycolytic enzymopathy. We report three patients from two unrelated families of Czech and Slovak origin with macrocytic hemolytic anemia due to GPI deficiency. The first patient had 15% of residual GPI activity resulting from two new heterozygous missense mutations c.478T>C and c.1414C>T leading to substitutions p.(Ser160Pro) and p.(Arg472Cys). Two other patients (siblings) inherited the same c.1414C>T p.(Arg472Cys) mutation in a homozygous constitution and lost approximately 89% of their GPI activity. Erythroid hyperplasia with dysplastic features was observed in the bone marrow of all three patients. Low hepcidin/ferritin ratio and elevated soluble transferrin receptor detected in our GPI-deficient patients suggest disturbed balance between erythropoiesis and iron metabolism contributing to iron overload.

Key words: Glucose-6-phosphate isomerase deficiency, Hemolytic anemia, Dysplastic erythropoiesis, Hepcidin to ferritin ratio, Soluble transferrin receptor, Iron loading

Introduction

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a homodimeric enzyme, which participates in the second step of glycolysis in interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). The *GPI* gene is located at the 19q13.1 and has 18 exons spanning 59 kb. It encodes 558-aminoacid monomer with a molecular weight of 63 kDa [1]. The human GPI crystal structure [2] provides a framework for modeling of GPI mutations. GPI deficiency is the third most common cause of hereditary nonspherocytic hemolytic anemia (HNSHA), after glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiencies, and is inherited in autosomal recessive fashion [3]. Thirty five mutations (29 missense, 3 nonsense, 2 splice site and 1 frameshift) [1,4,5,6,7] have been reported since GPI deficiency was first described in 1967 [8]. The phenotype varies from mild to severe anemia associated with splenomegaly in homozygotes and compound heterozygotes; simple heterozygotes are hematologically normal. GPI deficiency may be associated with hydrops fetalis during the neonatal period [9]. Some patients may also exhibit neurological dysfunction [10,11,12], which is explained by neuroleukin function of GPI in its monomeric

form [13,14,15], and granulocyte dysfunction associated with rare GPI variants with very low residual GPI activity [10,16]. Coincidence of GPI and G6PD deficiencies has been reported in some cases [6,17,18]. The clinical and biochemical features of mouse model of GPI deficiency resemble those present in human patients [19]. GPI deficiency is one of the few erythroenzymopaties which benefits from splenectomy just as pyruvate kinase, phosphoglycerokinase and hexokinase deficiencies [20].

Analyses of diverse erythroid and iron metabolism defects have shown that disrupted erythropoiesis negatively affects iron homeostasis and vice versa. In many cases, the molecular pathophysiology involves disordered production of hepcidin; the key molecule inhibiting iron absorption, mobilization, and recycling [21]. It has been shown that anemias characterized by ineffective erythropoiesis, like β-thalassemia [22] and congenital dyserythropoietic anemia [23], are associated with hepcidin suppression. As a result, iron absorption and recycling are augmented, leading to the development of secondary iron overload independently of transfusions. We recently published on disturbed balance between erythropoiesis and iron metabolism in two other types of hereditary hemolytic anemia: PK deficiency [24] and erythrocyte membrane defects [25]. Also in these disease states stimulated, ineffective erythropoiesis causes disordered production of hepcidin. Active erythropoiesis in the bone marrow is essential for hepcidin suppression as patients with Diamond Blackfan anemia (DBA), characterized by red cell aplasia, or mice with ablated bone marrow hematopoiesis are unable to attenuate hepcidin production [26,27,28]. The information on iron homeostasis in GPI deficiency has been limited; several cases with ferritinemia developed independently of transfusions have been reported in the literature [7,29].

Here we present, for the first time in the Czech and Slovak populations, two families with three cases of GPI deficiency caused by two novel GPI mutations, p.(Ser160Pro) and p.(Arg472Cys). We performed kinetic, thermostability and molecular modeling study to determine how these mutations could affect enzyme structure and function. In addition, we evaluated patients' bone marrow and revealed erythroid hyperplasia with dysplastic changes and reduced granulopoiesis. We also demonstrate disrupted balance between erythropoiesis and iron metabolism in these patients; this imbalance should be considered as a contributing factor for the development of iron overload in GPI deficiency.

Patients and methods

We investigated three patients with chronic nonspherocytic macrocytic hemolytic anemia from two unrelated families of Czech and Slovak origin (**Table 1**). Patient 1 is a 10-year-old Czech boy who suffered from severe transfusion-dependent hemolytic anemia (Hb 90 g/L, reticulocytes 4.5%) since birth till the age of 6 years when he was splenectomized and regular blood transfusions were eliminated. Bone marrow biopsy showed hypercellular bone marrow with reduced granulopoiesis, erythroid hyperplasia and dysplastic changes in late erythroid precursors; however, no mutations in *CDAN1* gene (for codanin-1) were found. Patients 2 and 3 are 19- and 11-year old siblings (boy and girl born to non-consanguineous parents) of Slovak origin with chronic hemolysis (Hb 76-101 g/L and reticulocytes 13-14%) and severe neonatal jaundice treated with exsanguination transfusion. Normo-/hypercellular bone marrow with erythroid hyperplasia showing dysplastic features and reduced granulopoiesis were also noted in these patients (**Figure 1**). Both patients were regularly transfused until the splenectomy was performed at the age of his 6 and her 7 years. Due to persistent hyperferritinemia, both patients are subjected to Exjade-chelation therapy. Before splenectomy, all patients presented with splenomegaly. Neurological functions of the patients were normal and no clinical signs of neutrophil dysfunction were observed. The parents, free of any signs of chronic hemolysis or other clinical symptoms, were undertaken for investigations as well.

All participants gave informed consent to this study which was approved by the Ethics Committee of Palacky University Hospital.

Hematological and enzyme studies

Routine hematological and biochemical studies were performed according to standard methods. Erythrocyte enzyme activities were determined based on previously described standardized procedures [30]. For the platelet-leukocyte assay, 5 μl of packed 200-times diluted platelet-leukocyte cells were added to the same 1 mL reaction mixture and the specific leukocyte GPI activities were expressed as the enzyme activity per milligram of total protein measured by the Biuret assay.

K_M values for GPI wild-type and mutant enzymes for G6P and F6P were measured as described previously [5]; G6P concentration varied between 0.1 and 2.5 mM and F6P between 25 and 1000 μM. Lineweaver-Burk plot was used to analyze enzyme data.

Heat stability of GPI variants was determined in hemolysate in 0.1 M Tris/0.5 mM EDTA buffer (pH 8.0) with 0.5% BSA, incubated at 37ºC, 40ºC and 45ºC for 10-60 minutes. The T_{50} (incubation temperature leading to 50% enzyme inactivation in 10 minutes) was elevated from incubation conducted between 30ºC and 90ºC. A control sample exposed to 30ºC for 10 minutes has served as the reference value for 100% activity.

Growth differentiation factor 15 and hepcidin measurements

Serum growth differentiation factor-15 (GDF15) and hepcidin concentrations were measured by commercial ELISA kit and by previously described quantitative SPE-LC-MS/MS assay, respectively [24].

Molecular analysis and enzyme modeling

DNA analyses are described in the Supplemental methods (Online supplemental documents). A ribbon plot of the structure of GPI monomer was obtained from the crystallographic structure of the dimer (protein data bank 1IAT). The 3D models of the mutant forms of GPI were generated using program PyMOL (www.pymol.org).

Results

Patients' characterization

The patients exhibited severe macrocytic hemolytic anemia associated with reticulocytosis, hyperferritinemia (**Table 1**) and erythroid hyperplasia in the bone marrow (**Figure 1**). Although marked dysplastic changes were noted in the erythroid precursors of all patients, no mutation in genes known to be associated with congenital dyserythropoietic anemia including *CDAN1*, *SEC23B*, and *KLF1* has been detected. Serum vitamin B12 and folic acid levels were within normal limits in all studied patients (not shown). Other tests and values, such as direct Coombs' test, Ham's test, sucrose lysis test, heat instability test, cryohemolysis test, Hb-electrophoresis and HbA_2 and HbF values were negative/normal. No Heinz bodies were seen with brilliant cresyl blue staining.

Glycolytic enzyme activities

The peripheral blood glycolytic enzyme activities in the patients and their parents are summarized in **Table 2**. Elevation in erythrocyte enzyme activities, such as hexokinase (HK), G6PD, gluconolactone dehydrogenase (GLD) and PK is likely caused by contribution of

relatively young erythrocyte populations. Both, PK/HK and G6PD/GLD ratios were within the control range. All three patients have low residual erythrocyte GPI activities – reduced by more than 85%. The parents showed decreased GPI activities as well, corresponding to about 30% of normal control activity. The patients' platelet-leukocytes GPI activities reached approximately 30%, whereas the parents' ones showed intermediate enzyme activities (data not shown).

GPI sequence analysis

DNA sequencing revealed that Patient 1 (family 1) is a compound heterozygote for c.478T>C (exon 5) and c.1414C>T (exon 16) mutations in *GPI* gene that lead to p.(Ser160Pro) and p.(Arg472Cys) substitutions, respectively (**Figure S1**, Online supplemental documents). His father is a heterozygote for c.478T>C and his mother is a heterozygote for c.1414C>T mutation. Patients 2 and 3 (family 2) are homozygotes for the same c.1414C>T mutation p.(Arg472Cys) (**Figure S1,** Online supplemental documents). Their parents are carriers of this mutation. Both mutations were confirmed by *Hae*III and *Mlu*CI digestion. Mutation c.478T>C creates a new restriction site for *Hae*III, whereas c.1414C>T mutation for *Mlu*CI endonuclease (**Figure S2**, Online supplemental documents).

GPI kinetic studies and *in silico* modeling

The results from kinetic experiments of GPI variants are shown in **Table 3**. While in the Patient 1 the kinetic properties and thermostability resulted from the putative heterogeneous mixture of the enzyme hetero- or homodimers, the homozygosity of p.(Arg472Cys) substitution in the Patient 2 and 3 allowed precise characterization of this variant. The GPI mutant from the compound heterozygous Patient 1 showed an increased affinity for G6P and/or F6P. Its thermal stability was only slightly affected; $T_{1/2}$ was within normal limits, whereas T_{50} was decreased. The homozygous p.(Arg472Cys) mutant had a decreased affinity for F6P and a slightly increased G6P affinity. Its thermal stability was strongly reduced. Furthermore, the enzyme activity was almost abolished after short incubation at 45°C.
Analysis of the 3D GPI structure using *in silico* modelling (**Figure 2**) showed that Ser160 interacts with the substrate phosphate group and directly participates in the enzyme catalysis. The Ser160Pro substitution likely alters binding to the substrate, and thus affects catalytic activity of the mutant enzyme. Arg472 is a part of a dimer-domain located at the edge of the large external loop (LEL), which stabilizes both GPI monomers into a dimer conformation.

Arg472 forms hydrogen bonds with residues Gly470 and Ile395 (located on other monomer). The Arg472Cys mutation likely leads to a disruption of these interactions which probably destabilizes the GPI dimer conformation (**Figure 2**).

Erythropoietic activity, iron metabolism and hepcidin

The information on iron status and erythropoietic activity in GPI deficiency has been limited [7,29]. Two our patients showed signs of iron overload *i.e.* increased serum iron, transferrin saturation (TSAT) and ferritin (**Table 4**). One patient had normal iron status parameters (**Table 4**) as a result of chelation therapy, which had been initiated because of persistent hyperferritinemia, before and after splenectomy (ferritin 540±70 µg/L, TSAT 77%). Nevertheless, the analysis of hepcidin and hepcidin/ferritin ratio revealed inappropriately low hepcidin levels with respect to the iron stores in all three patients (0.089, 0.039 and 0.001; control range: 0.2-2.2) (**Table 4**). This suggests pathological hepcidin suppression likely by stimulated erythropoiesis as documented by increased levels of soluble transferrin receptor (sTfR – 17, 24.5 and 21.6; control range: 1.9-4.4 mg/L) (**Table 4**), erythropoietin (EPO; 34.1, 63.8 and 147; control range: 4.3-29 IU/L) and in agreement with erythroid hyperplasia in their bone marrow (**Figure 1**). In addition, two patients showed marked elevation of GDF15 (777 and 989; control range: 166-344 ng/L) (**Table 4**), a marker of ineffective erythropoiesis [31]. This is also consistent with dyserythropoiesis and dysplastic changes in erythroid precursors detected in patients' bone marrow (**Figure 1**).

The combination of hepcidin with ferritin and sTfR levels can differentiate patients with thalassemia trait and erythrocyte membrane defects from healthy controls and may be helpful in assessing disease severity [25,32]. We have, therefore, reevaluated our published patients with PK deficiency [24] and included into comparative analysis also transfusion-dependent patients with DBA, who presented with iron overload, but had hepcidin levels appropriate for the degree of iron loading, because of the absence of erythropoiesis in their bone marrow [26]. As can be seen from **Figure 3A,** all disease categories (GPI deficiency, PK deficiency and DBA) differ from healthy controls and show altered (hepcidin/ferritin)/sTfR index (**Figure 3B**). DBA patients are shifted left in the graph and have increased (hepcidin/ferritin)/sTfR index due to low sTfR (**Figure 3**) reflecting erythroid aplasia. All patients with GPI deficiency and the majority of PK-deficient patients are shifted to the right and top in the graph and have reduced (hepcidin/ferritin)/sTfR index (**Figure 3**) reflecting inappropriate hepcidin suppression by stimulated/ineffective erythropoiesis.

Discussion

GPI deficiency is the second most common human erythrocyte enzymopathy of glycolysis leading to chronic hemolytic anemia after PK deficiency. However, GPI deficiency occurs quite rarely and is characterized by relatively high heterogeneity of *GPI* gene mutations. Up to now, around 50 GPI-deficient cases have been reported [7].

Here, we report on two novel GPI mutations p.(Arg472Cys) and p.(Ser160Pro) associated with HNSHA. The p.(Arg472Cys) mutation was found in the homozygous state as well as in the compound heterozygous form with p.(Ser160Pro) mutation. *In silico* analysis by Polyphen and SIFT revealed that both substitutions p.(Arg472Cys) and p.(Ser160Pro) are not tolerated. Interestingly, substitution of the same residue Arg472 has been previously reported; the Arg472His mutant was distinguished by increased K_M for F6P, decreased k_{cat} and low thermal stability [33,34]. A 3D structural model of the Arg472 GPI mutant showed that the Arg472Cys substitution likely destabilizes interactions within the dimer interface, and thus may affect either the dimer formation and/or dimer stability [2]. Indeed, kinetic studies of p.(Arg472Cys) mutant revealed decreased affinity to F6P and strongly reduced thermal stability. These findings are consistent with the previous functional study of the p.(Arg472His) GPI mutant and all together correspond to a model of the proposed pathogenic mechanism.

Based on 3D modelling of the p.(Ser160Pro) mutant, we found that the substituted proline likely affects binding of the substrate, and thus inhibits GPI catalytic activity. Biochemical characteristics of the heterozygous p.(Arg472Cys)/p.(Ser160Pro) mutant showed decreased K_M values. K_M with approximately one half the value of control indicates higher affinity for G6P and F6P and their tighter binding to the enzyme and reduced release of the product leading to delayed enzyme catalysis. Its thermal stability was only slightly affected which may indicate that Arg472Cys homodimers represent only minor proportion of the mixed dimers pool. Thus it seems that the impaired kinetic properties altered by Ser160Pro mutation have the main impact on the red blood cell metabolism in the case of a compound heterozygous state (p.(Arg472Cys)/p.(Ser160Pro)). However, in order to determine the Ser160Pro mutant pathophysiology unambiguously it would be necessary to analyze its homozygous form.

Analyses of erythropoietic markers together with iron status parameters revealed that inappropriately low hepcidin production caused by stimulated/ineffective erythropoiesis is making GPI deficient patients susceptible to iron overload independently of transfusions, similar to patients with β-thalassemia [22], PK deficiency [24] or erythrocyte membrane defects [25]. In connection with two other risk factors for iron loading present in GPI deficiency, splenectomy and chronic hemolysis [35], these patients should be carefully monitored in order to overcome potential damaging effect of excessive iron stores. As proposed by Guimarães et al. [32], the (hepcidin/ferritin)/sTfR index can be used to monitor possible disease development especially with respect to more profound disruption of erythropoiesis-hepcidin-iron store axis.

In summary, we describe new p.(Arg472Cys) and p.(Ser160Pro) GPI mutations in the first cases of GPI deficiency in the Czech and Slovak populations. As the p.(Arg472Cys) mutation occurs in homozygosity, we were able to characterize its consequences on enzyme kinetic, thermostability and structural properties. We also present bone marrow abnormalities in all three GPI-deficient patients, i.e. erythroid hyperplasia with dysplastic features and reduced granulopoiesis, without coexistence of congenital dyserythropoietic anemia. In addition, we detected imbalance between erythropoiesis and iron metabolism in GPI-deficient patients, as evidenced by inappropriately low levels of hepcidin with respect to their iron loading. Elevated markers of erythropoietic activity (sTfR and EPO) and ineffective erythropoiesis (GDF15) in patients with PK deficiency (our earlier work [24]) and GPI deficiency (this study) suggest major role of stimulated/ineffective erythropoiesis on hepcidin suppression in

these red blood cell glycolytic enzymopathies similar to other types of iron-loading anemias [21]. In this regard, the role of erythroferrone (ERFE), an erythroid regulator for hepcidin suppression, needs to be considered [36]. ERFE is produced by erythroid precursors in the bone marrow in response to EPO via JAK2/STAT5 signaling pathway. The inability of *Erfe*$^{-/-}$ mice to downregulate hepcidin effectively after phlebotomy or EPO administration [36] together with elevated ERFE expression in β-thalassemia intermedia mouse model [37] confirms its involvement in both acute hepcidin response to increased erythroid activity and hepcidin suppression by ineffective erythropoiesis. However, determination of ERFE levels in different pathological conditions including GPI deficiency depends on a completion of development and full validation of ELISA assay for ERFE measurements in the human plasma [38].

Contributors

R.M. designed the study, performed enzyme assays and molecular analysis, collected and analyzed data, and wrote the paper; P.K. participated in molecular analysis, performed enzyme modeling, and wrote the paper; D.H. measured hepcidin level; Z.S. determined GDF15 levels; D.P. and D.P. treated patients, collected patient material, and provided clinical information; P.D. participated in data analysis; M.H. participated in study design, data analysis, interpretation of results, and wrote the paper; and V.D. participated in data analysis, in interpretation of results, and contributed to the manuscript writing.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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Table 1: Laboratory values of the GPI-deficient patients and their family members.

Parameters	Family 1			Family 2			
	Patient 1	Father	Mother	Patient 2	Patient 3	Father	Mother
Age*/Sex	11/M	39/M	36/F	19/M	10/F	42/M	39/F
RBC (10^{12}/L)	2.53	4.83	3.89	2.51	2.21	5.01	4.18
Hb (g/dL)	90	152	128	101	86	161	126
Htc	0.3	0.44	0.38	0.34	0.29	0.46	0.37
MCV (fL)	119	91.3	96.4	133	136	91.6	89.2
Ret (%)	16.3	0.6	1.1	33.4	45.6	1.3	1.0
Bilirubin (µmol/L)	84	5	11	127	83	-	-
Haptoglobin (g/L)	<0.04	-	-	<0.04	<0.04	-	-
LDH (µkat/L)	4.58	2.83	2.49	11.42	17.86	-	-

Legends:*Age at diagnosis of GPI deficiency; Normal control ranges: RBC (children: 4.5-5.3; female: 3.8-4.9; male: 4.3-5.7 x10^{12}/L); Hb (children: 13-16; female: 12-16; male: 13-18 g/dL); MCV (78-100 fL), reticulocytes (0.5-3.0 %); bilirubin (0-23 µmol/L); haptoglobin (0.3-2 g/L); LDH (2.34-4.68 µkat/L).

Table 2: Panel of red blood cell enzymes assayed in both patients' families.

Enzyme	Control range	Family 1			Family 2			
		Patient 1	Father	Mother	Patient 2	Patient 3	Father	Mother
G6PD [IU/g Hb]	[5.36-7.04]	10.50±0.478	6.34±0.066	6.44±0.456	8.43±0.815	10.86±1.760	6.69±0.833	7.09±1.045
GLD [IU/g Hb]	[5.87-6.71]	10.54±0.494	6.05±0.42	7.23±0.32	9.81±0.635	10.39±0.660	6.05±0.114	8.86±0.611
PK [IU/g Hb]	[5.12-5.78]	10.55±0.241	7.01±0.314	6.65±0.234	16.28±0.373	15.5±1.962	5.84±0.414	7.31±0.194
GPI [IU/g Hb]	[53-62]	8.51±1.404	29.05±4.13	21.56±1.25	5.94±0.3112	6.75±0.570	21.18±0.733	33.42±1.400
P5´N (pyr/pur)	[1.8<]	2.29	2.21	1.95	2.04	2.37	2.01	2.16
HK [IU/g Hb]	[0.81-1.65]	2.74±0.768	1.59±0.061	1.29±0.071	5.62±0.048	5.85±0.284	0.93±0.078	1.29±0.022
G6PD/GLD	[0.80-1.20]	1.00	1.05	0.89	0.86	1.05	1.11	0.80
PK/HK	[3.60-8.17]	3.85	4.41	5.16	2.90	2.65	7.48	5.67

Legends: G6PD: glucose-6-phosphate dehydrogenase; GLD: gluconolactone dehydrogenase; PK: pyruvate kinase; GPI: glucosephosphate isomerase; P5´N: pyrimidine-5´-phosphate nucleotidase; HK: hexokinase; enzyme activities are shown as average ± SD of three independent measurements.

Table 3: Molecular and biochemical data for the newly described GPI-variants.

Parameter	Family 1	Family 2	Control (wt)
Nucleotide substitutions	c.[478T>C];[1414C>T]	c.[1414C>T;[1414C>T]	-
Amino acid substitutions	p.[Ser160Pro];[Arg472Cys]	p.[Arg472Cys];[Arg472Cys]	-
GPI activity (%)	15	11	100
$T_{1/2}$ at 37 ºC	>120 min	>120 min	>120 min
$T_{1/2}$ at 40 ºC	>120 min	6 min	>120 min
$T_{1/2}$ at 45 ºC	>120 min	1 min	>120 min
T_{50}	47	39	50
K_M for G6P (mM)	0.861±0.220	1.22±0.168	2.04±0.615
K_M for F6P (µM)	31.38±1.638	155.06±25.668	57.82±1.414

Legends: $T_{1/2}$: the half-time of enzyme inactivation; T_{50}: temperature of 50% enzyme inactivation (in 10 minutes); wt: wild-type; patients' K_M values are results of three measurements (normal K_M values were evaluated from three healthy controls); for DNA numbering, the initiation codon is 1; GPI human gene: NM_001184722.1 and NP_001171651.1.

Table 4: Parameters of iron status and erythropoietic activity.

Parameters	Family 1			Family 2			
	Patient 1	Father	Mother	Patient 2	Patient 3	Father	Mother
Serum Fe (µmol/L)	51	-	-	20.4	33.1	-	-
TSAT (%)	86	-	-	39	84	-	-
Ferritin (µg/L)	327	32	68	143	1006	144	11
Hepcidin (µg/L)	28.88	15.74	95.99	5.6	7.9	75.8	2.8
Hepcidin/ferritin ratio	0.089	0.492	1.412	0.039	0.001	0.526	0.255
sTfR (mg/L)	17	-	-	24.5	21.6	-	-
GDF15 (ng/L)	465	-	-	989	777	-	-
EPO (IU/L)	34.1	-	-	63.8	147	-	-

Legends: Serum Fe (10.6-28.3 µmol/L); TSAT - transferrin saturation (21-48%); Ferritin (<200 female, <300 male and <70 µg/L children); Hepcidin (13.1-104.8 µg/L); Hepcidin/ferritin ratio (0.2-2.2) [24]; sTfR: Soluble transferrin receptor (1.9-4.4 mg/L); GDF15: Growth differentiation factor-15 (166-344 ng/L) [24]; EPO: Serum erythropoietin (4.3-29 IU/L).

Legends to figures

Fig. 1. Bone marrow abnormalities in GPI-deficient Patient 1. Erythroid hyperplasia with dysplastic changes in erythroid precursors: binuclearity and cytoplasmic bridging were detected in Patient 1. Bone marrow of Patient 2 and 3 showed similar features (see the description in Patients and Methods).

Fig. 2. Ribbon plot of the 3D GPI-mutant structures suggested putative changes caused by the mutations. A) Ser159 (shown in purple) is a part of the phosphate-binding site (blue) responsible for the potential hydrogen bond interaction with phosphate group of the GPI substrate (the sulfate was used to mimic the substrate phosphate group). and B) Ser159Pro substitution likely results in a disruption of the hydrogen bond (a black dotted line) between the residue at position 159 and the substrate phosphate group and thus destabilizes the substrate-enzyme complex; C) Arg471 is located at the edge of the large external loop (LEL, 438-468 residues) directly interacting (hydrogen bonding) with neighboring Gly469 residue and with Ile394 of the second monomer, and thus D) disruption of these interactions by Arg471Cys mutation leads to conformational changes within the LEL, serving as a dimer stabilizing hook, and thus to generation of a more labile GPI-dimer. The amino acid numbering in the figures begins with alanine as residue 1 instead of initial methionine. The structure illustrations were created with PyMOL program based on Protein data bank ID 1IAT. Black dot lines represent hydrogen bonds.

Fig. 3. Differentiation of different disease categories based on the plot of hepcidin/ferritin ratio against sTfR (A) and (hepcidin/ferritin)/sTfR index (B). Patients with GPI deficiency (GPI def., n=3) and PK deficiency (PK def., n=8) are shifted to the right and top in the graph compared to healthy controls (n=10) and show reduced (hepcidin/ferritin)/sTfR index due to hepcidin suppression by stimulated/ineffective erythropoiesis. The difference between controls and GPI deficiency in (B) is not statistically significant because of low number of GPI-deficient patients. The combination of PK- and GPI-deficient patients clearly shows that (hepcidin/ferritin)/sTfR index is significantly reduced in glycolytic enzymopathies (enzyme def.) and distinguishes them from healthy population. Also transfusion dependent DBA patients (DBA_TD, n=7) differ from healthy controls, but they are shifted to the left in the graph and show increased (hepcidin/ferritin)/sTfR index due to the absence of erythroid precursors in the bone marrow, which makes hepcidin level appropriate to increased iron stores.

Supplementary methods

Molecular analysis

Genomic DNA was isolated from peripheral blood samples collected into EDTA using a QIAamp DNA blood maxi kit (Qiagen, Valencia, CA). The PCR primers and conditions used to amplify all exons of *GPI* (NM_001184722.1), *CDAN1* (NM_138477.2), *SEC23B* (NM_001172745.1) and *KLF1* (NM_006563.4) genes are available upon request (some of the *SEC23B* primers were already previously published by Bianchi et al.). PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed with a BigDye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Restriction analyses were performed on newly amplified PCR-DNA products using the same set of primers as for the mutational analysis. The gDNA sequences of c.478T>C (exon 5) and c.1414C>G (exon 16) mutations were confirmed by *Hae*III and *Mlu*CI digestion, respectively, and the digests were analyzed by electrophoresis through 1.5% agarose gel in SB buffer. The restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Reference: P. Bianchi, E. Fermo, C. Vercellati, et al., Congenital dyserythropoietic anemia type II (CDAII) is caused by mutations in the SEC23B gene, Hum. Mutat. 30 (2009) 1292-1298.

Supplementary figures

Supplementary Figure S1. DNA sequencing of the *GPI* gene. Left panels: Patient 1 (Family 1) was a compound heterozygote for c.478T>C (exon 5) and c.1414C>T (exon 16) mutation in the *GPI* gene. His father was a heterozygote for the c.478T>C and his mother was a heterozygote for the c.1414C>T mutation; Right panels: Patients 2 and 3 (Family 2) were homozygotes for the c.1414C>T mutation. Both parents were carriers of this mutation.

Supplementary Figure S2. Restriction enzyme-based detection of two mutations in the amplified DNA samples from the patients and their parents.

The 550-bp PCR amplified product from the wild-type allele contains five recognition sites for *Hae*III that yields fragments of 279, 142, 68, 50, 7 and 4 bp (the last two small fragments are not visible on the gel). The mutation creates another recognition sequence for this enzyme resulting in additional fragments of 160 bp and 119 bp. Heterozygosity for the c.478T>C mutation was confirmed in Patient 1 (PAT 1, family 1) and also detected in his father (F_1), whereas it is absent in the patient's mother (M_1). As father is a carrier of heterozygous c.489A>G (SNP, p.Gly163Gly, rs1801015) mutation, which curiously generates another restriction site, another fragment (100 bp) is produced after digestion of the father's mutant allele.

Mutation c.1414C>G creates a new restriction site for *Mlu*CI and consequently 431 bp and 198 bp fragments are produced after digestion of the mutant allele, whereas 629 bp fragment is the result of digestion of the wild-type allele with this restriction enzyme. Heterozygosity for this mutation was confirmed in Patient 1 (PAT1, family 1) and his mother (M_1) and both parents (M_2 and F_2) of Patient 2 and 3 (PAT2 and PAT3, family 2), whereas both Patients 2 and 3 (PAT2 and PAT3) are homozygotes for c.1414C>G mutation. C, healthy control.

Figure 1

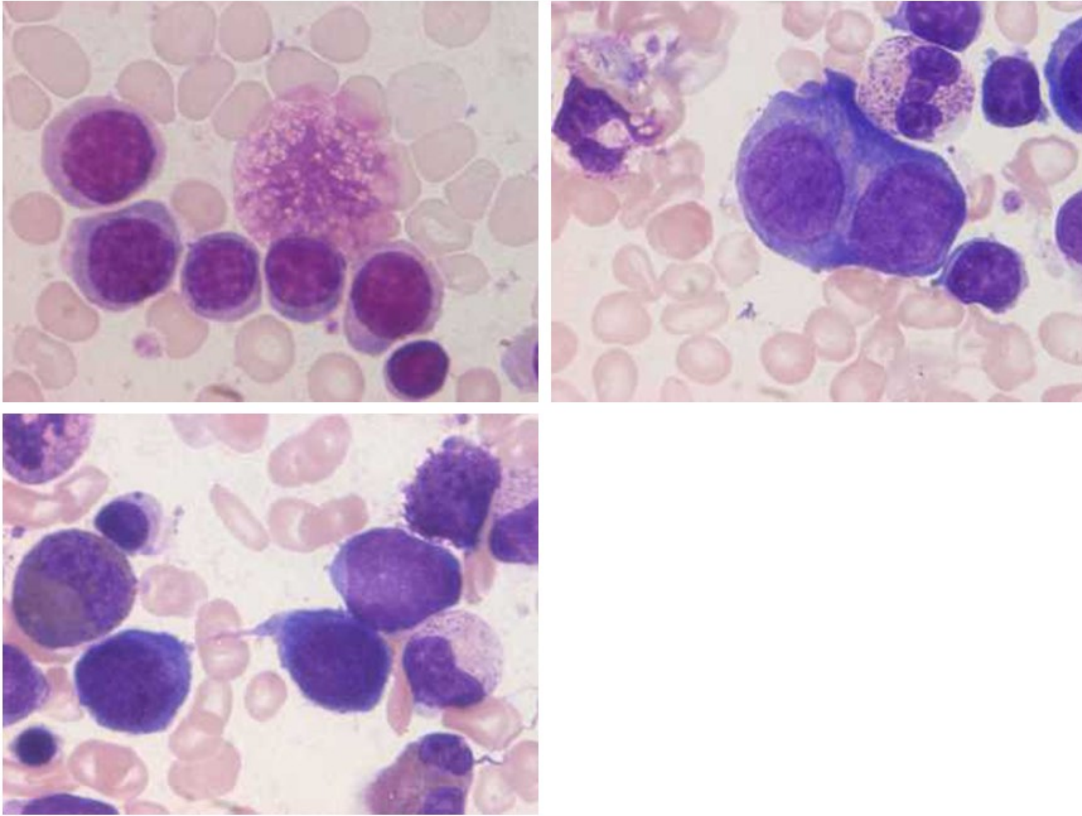


Figure 2

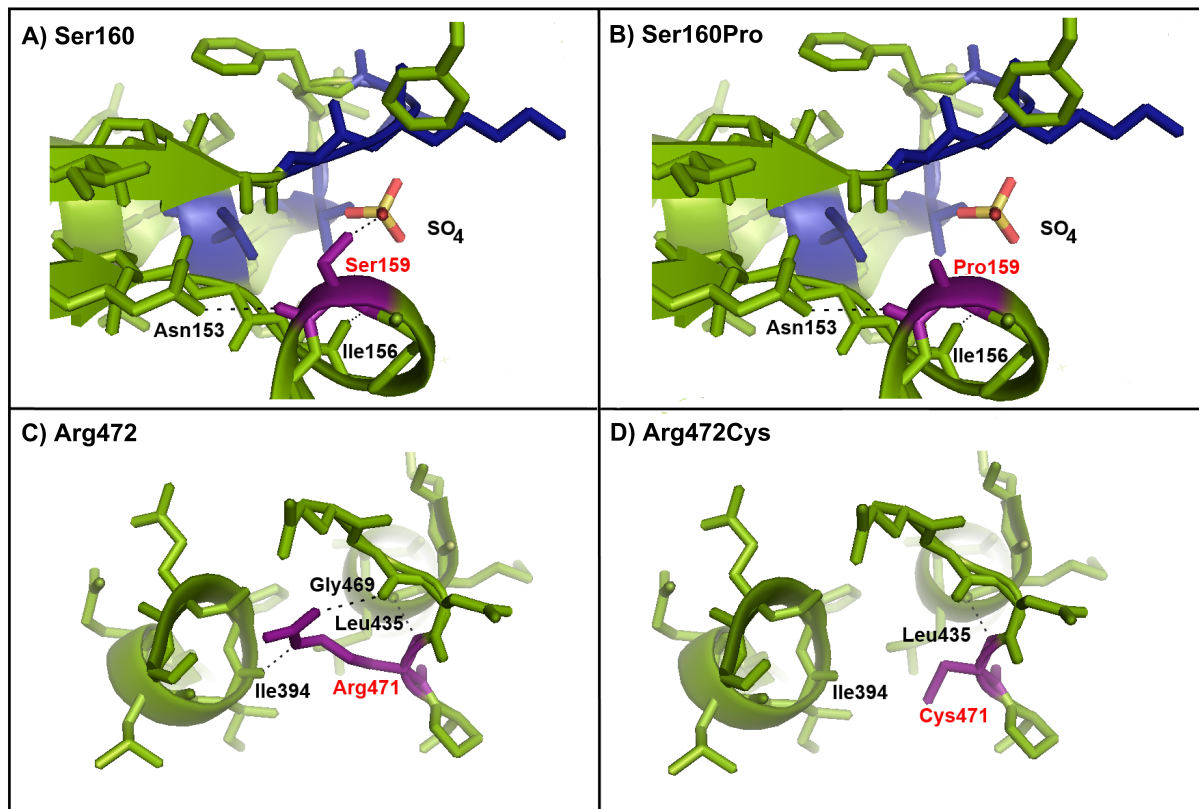


Figure 3

A)

controls
DBA_TD
GPI def.
PK def.

hepcidin/ferritin

0.01
0.1
1

0
5
10
15
20
25
30

sTfR [mg/L]

B)

P>0.005

1.0
0.5
0.0

(hepcidin/ferritin)/sTfR

P>0.2
P>0.02
P>0.006

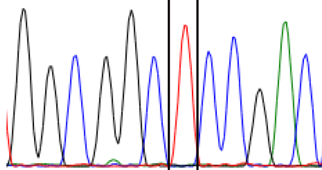
cotrols
DBA-TD
GPI def.
PK def.
enzyme def.

Figure S1

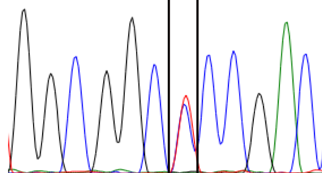
Family 1

478T>C

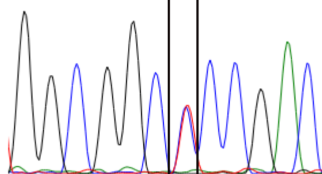
GGC GGC T CCGAC
Wild type



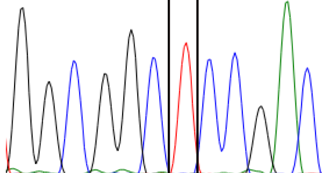
GGC GGC N CCGAC
Patient 1



GGC GGC N CCGAC
Father

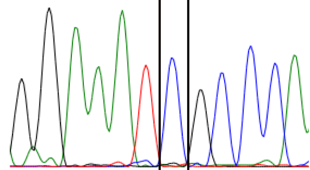


GGC GGC T CCGAC
Mother

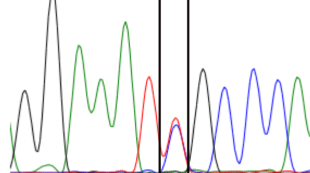


1414C>T

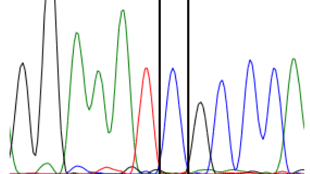
GGAAAT C GC CCA
Wild type



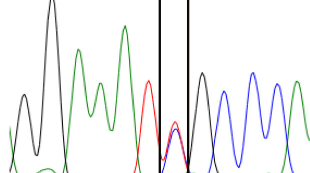
GGAAAT N GC CCA
Patient 1



GGAAAT C GC CCA
Father



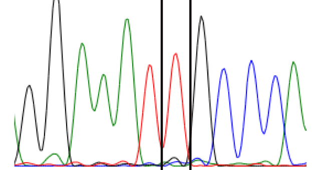
GGAAAT N GC CCA
Mother



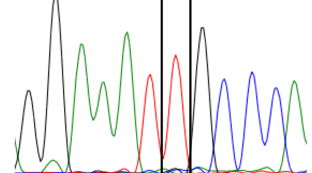
Family 2

1414C>T

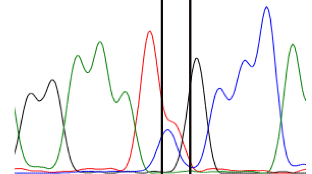
GGAAAT T GC CCA
Patient 2



GGAAAT T GC CCA
Patient 3



GGAAAT N GC CCA
Father



GGAAAT N GC CCA
Mother

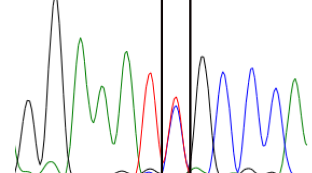
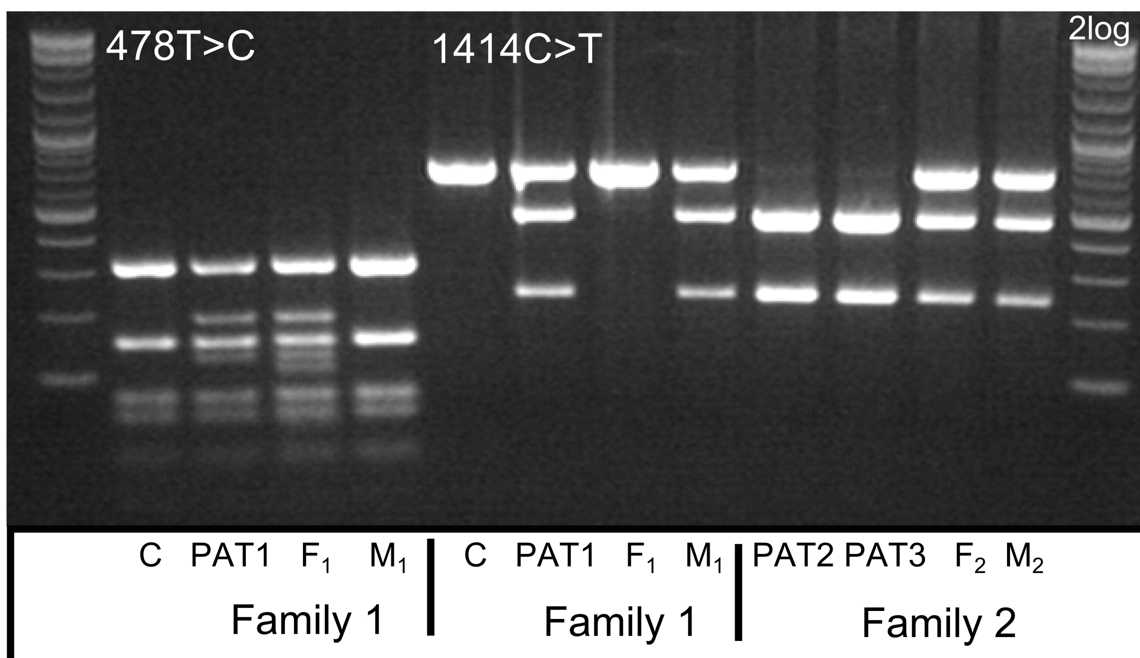


Figure S2



APPENDICES 4.3

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF VERY RARE HEXOKINASE MUTANTS CAUSING HNSHA

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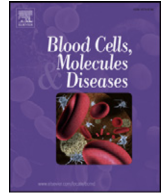

Molecular characterization of six new cases of red blood cell hexokinase deficiency yields four novel mutations in *HK1*

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A B S T R A C T

Hexokinase (HK) is a key enzyme of glycolysis, the only metabolic pathway able to provide the red blood cell with ATP. HK deficiency is a very rare hereditary disorder with severe chronic nonspherocytic hemolytic anemia (HNSHA) as a major clinical feature. To date, only 24 patients with HK deficiency have been identified. Here, we report the molecular analysis of six new cases of HK deficiency. A total of six different mutations were detected in *HK1*, four of them described here for the first time: c.2599C > T p.(His867Tyr), c.1799C > T p.(Thr600Met), c.873-2A > G and c.493-1G > A. The pathogenic nature of the identified missense mutations was confirmed by biochemical and 3-dimensional structural analysis. The effects of the novel splice site mutation c.873-2A > G were studied at the level of pre-mRNA processing, and confirmed at the protein level. All together, these results provide a better insight into the pathogenesis of this rare red cell disorder, and contribute to a better understanding of the genotype-phenotype correlation in HK deficiency.

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1. Introduction

Hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) is a key regulatory enzyme for the Embden-Meyerhof pathway (or glycolysis). Hexokinase (HK) catalyzes the irreversible transfer of high-energy phosphate from ATP to glucose, on which red blood cells' (RBCs) energy metabolism is fully dependent [1].

Red blood cells contain two HK isozymes – HK-1 and HK-R. Both isozymes are encoded by the same *HK1* gene located on chromosome 10 and their expression is regulated by the use of alternative promoters. The HK-R isozyme is exclusively expressed in erythroid cells, whereas HK-1 is present in various mammalian tissues [2].

HK deficiency is a very rare autosomal recessive disorder belonging to a group of very rare red cell enzyme defects (erythroenzymopathies). Generally, HK deficiency is associated with chronic nonspherocytic hemolytic anemia (HNSHA) [3]. Affected individuals exhibit mild to severe lifelong hemolytic anemia (HA) as the major clinical hallmark. In exceptional cases, however, multiple malformations including psychomotor retardation have been also reported [4–6]. A complete loss of *HK1* expression leads to intrauterine fetal death [7].

The first case of HK deficiency was described by Valentine et al. in 1967 [8]. Up to now, only 24 cases of HK deficiency have ever been reported [9]. This apparently low prevalence may indicate that in many cases HK deficiency is incompatible with life, perhaps because of the enzyme's crucial role of in glycolysis. Alternatively, many (mild) cases may remain undiagnosed due to the complicated clinical and laboratory diagnosis, *i.e.* the lack of appropriate diagnostic tools (only a few laboratories perform HK enzymatic assays). Only 4 cases of HK deficiency have been characterized at the molecular level [9], and very little is known about the genotype-to-phenotype correlation in HK deficiency. Six distinct mutations have been identified thus far: three missense mutations, two deletions, and one mutation located in the erythroid-specific promotor [10]. The first patient characterized at the molecular level was a compound heterozygote for a missense mutation c.1583T > C p.(Leu528Ser), in *trans* to a 96-bp deletion (HK – Melzo)

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[11]. The third reported mutation concerned a large deletion, c.493-481_1028 + 454del in *HK1*, found in the homozygous state and associated with severe HA and fetal death [7]. In another patient with HK deficiency and mild HA [12], a homozygous missense mutation c.2036C > G p.(Thr679Ser) (HK – Utrecht) was found to be the underlying cause [13]. The last patient characterized at the molecular level showed a mutation in the erythroid specific promotor −193A > G, in *trans* to a missense mutation c.278A > G p.(Arg93Gln). The latter mutation mainly exerted its effect by affecting pre-mRNA processing [14]. We describe six new cases of HK deficiency here. All of them were studied at the molecular level, and the causative mutations were identified and studied at different levels to establish the pathogenic nature. One additional patient was a G6PD deficient female with heterozygosity for *HK1* gene mutation.

2. Materials and methods

2.1. Patients

This study was performed according to the Helsinki international standards; informed consent was obtained from all patients and family members. We analyzed seven patients from 6 unrelated families with a different country of origin – Ireland, the Czech Republic, Denmark, the Netherlands and the Slovak Republic. The selected laboratory parameters are summarized in Table 1.

There was no HA family history in any of the families concerned. The majority of patients exhibited common signs of chronic hemolysis. Red cell membrane defects and hemoglobinopathies were excluded as a possible cause of HA. All but one of the patients were pediatric patients at the time of analysis. Patient 1 underwent transfusion and chelation therapy until age 3. However, his brother, patient 2, exhibited compensated HA with no clinical symptoms. Patient 3 was born with a massive reticulocytosis (99%) after an uneventful pregnancy from consanguineous parents (first cousins). Further examination of the newborn revealed a central nervous system bleeding (CNS), likely as a result of hypoxia. Severe hemolysis was also observed in patient 4. Both patients 3 and 4 required regular blood transfusions from birth and underwent chelation therapy. Patients 5 and 6 were first diagnosed with Gilbert syndrome. Because of an accompanying mild hemolysis with reticulocytosis, additional examinations were performed. Patient 7 exhibited only mild methemoglobinemia (7–11%) with no signs of hemolysis.

2.2. Red blood cell enzyme activities

A standard panel of enzyme activity assays (including HK, glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate isomerase (GPI) and pyruvate kinase (PK)) was performed in leukocyte/platelet-free erythrocyte lysates according to standard methods [15]. The PK/HK ratio was used to evaluate the influence of high reticulocyte numbers on total enzymatic activity.

2.3. Molecular analysis of HK1

DNA was isolated from peripheral blood using standard procedures. HK-R transcript (accession number: NM_033496.2) encompassing the specific erythroid promoter region and erythroid-specific exon 1 were amplified using PCR, according to the previously described conditions [13]. DNA sequencing analysis was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

2.4. Hexokinase

2.4.1. Kinetic properties and stability assays

To determine K_M values for glucose and ATP, different concentrations of glucose: 0.0125–2.0 mM and/or ATP 0.25–10 mM were used in a reaction mixture containing: 0.1 M Tris-HCL/0.5 mM EDTA, pH = 8.0; 0.01 M $MgCl_2$; 0.2 mM $NADP^+$; 0.1 IU/mL G6PD and 50 μL of RBC lysate (1:20). After 10 min incubation at 37 °C, changes in absorbance at 340 nm were recorded at 20 s intervals for 30 min. The thermal stability assays of HK mutants involve determining the half-life ($T_{1/2}$) and the half-inactivation temperature (T_{50}). Both thermal stability assays were performed in the previously described mixture, routinely containing 2 mM glucose and 10 mM ATP. For the $T_{1/2}$ assay, RBC lysate was pre-heated to 40 °C for 5–40 min followed by cooling on ice (2 min). After the pre-heating step, 50 μL of the RBC lysate was used in the reaction mixture as described above. $T_{1/2}$ was defined as the time needed to reach a 50% reduction in the initial activity [16]. For the T_{50} assay, RBC lysate was pre-incubated at different temperatures (37–44 °C) for 5 min, followed by a cooling period (2 min) on ice. The T_{50} was defined as the temperature at which HK maintains 50% of its activity. The pH stability assay was carried out in a 0.1 M Tris-HCL/0.5 mM EDTA buffer of different pH values (6.0–10.0) [12,15]. Data to determine K_M were analyzed with non-linear regression using GraphPad® Prism.

2.4.2. Cell culture of human nucleated erythroid cells

Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated from a heterozygous individual carrying the splice site mutation c.873-2A > G and a healthy control using Ficoll-Paque density gradient centrifugation (GE Healthcare). Cells were grown in StemSpan SFEM (Stemcell Technology), supplemented with human stem cell factor (100 ng/mL; Amgen), erythropoietin (2 U/mL; Eprex), interleukin-3 (1 ng/mL; Sigma-Aldrich), dexamethasone (1 μmol/L; Sigma-Aldrich) and SyntheChol™ NS0 Supplement (200-times diluted; Sigma-Aldrich). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. The Casy Cell Counter and Analyzer (Roche Life Science) was used to monitor cell viability and proliferation. After 10 days of erythroid proliferation, cells (mainly pro-erythroblasts) were harvested to isolate total RNA and carry out a Western blot analysis.

Table 1
Hematological data of the studied patients.

Origin	Patient 1[S]	Patient 2[S]	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
	Ireland	Ireland	Czech Republic	Denmark	Netherlands	Czech Republic	Slovak Republic
Sex	M	M	M	M	M	F	F
Age [year]	2	4	8 m	2	22	12	12
RBCs [$\times 10^{12}$]	2.57; (*--)	2.76; (*--)	2.40; (*--)	2.90; (*--)	4.40; (*--)	4.25; (-*-)	4.58; (-*-)
Hb [g/L]	79; (*--)	95; (*--)	74; (*--)	86; (*--)	160; (-*-)	135; (-*-)	137; (-*-)
Ret [%]	8.8; (--*)	6.2; (--*)	6.3; (--*)	4.7; (--*)	6.0; (--*)	3.1; (--*)	1.9; (--*)
Bili [μmol/L]	54; (--*)	62; (--*)	120; (--*)	124; (--*)	24; (--*)	100; (--*)	17.6; (-*-)
Transfusion therapy	Yes[+]	No	Yes	Yes	No	No	No
Other diagnosis	ND	ND	ND	ND	GS	GS	G6PD Gond

S – siblings; Age – age at diagnosis of HK deficiency, m – months; RBCs – red blood cells; Hb – hemoglobin, M – male; F – female; ND – not determined, GS – Gilbert syndrome; G6PD – glucose-6-phosphate deficiency, variant – GOND; [+] Transfusion therapy has been stopped at age 3; comparison of hematological data with a reference range: (*–) – low; (-*-) – normal; (–*) – high.

2.5. RNA studies

Total RNA was isolated using TRIzol® Reagent (Life Technologies) according to the manufacturer's protocol. RT-PCR was performed using GeneAmp RNA PCR Core Kit (Applied Biosystems). cDNA was obtained using 2 μg of total RNA and random hexamer primers. RT-PCR, was carried out using specific primers encompassing exons 7 to 9 of HK-R transcript: 5′-TGCCAACATCGTAGCTGTGG-3′; 5′-TGGAAATGAG CCAGGGTCTC-3′. The conditions applied were: 105 s 95 °C, 35 cycles: [30 s 95 °C, 30 s 60 °C], 7 min 72 °C.

Quantitative PCR (qPCR) was performed using FastStart SYBR Green Master (Roche) and ROX Passive Reference (BioRad) as an internal reference dye. Each reaction contained 0.5 μg of cDNA and a pair of specific primers spanning exons 7 and 8 (5′-AGTGGGCACCATGATGACCT-3′, 5′-TCGATGGCTGACACATCACTG-3′) to avoid the contribution of aberrantly spliced RT-PCR variants in the tested samples. qPCR specificity was verified by melting curve analysis. Beta-actin (ACTB) was used as an internal standard. Real-time analysis was performed using ViiA™ 7 Real-Time PCR System (Life Technologies) using the following cycling conditions: 10 min 95 °C, 40 cycles: [30 s 94 °C, 30 s 63 °C, 45 s 72 °C]. Relative quantification was evaluated based on the comparative C_T method ($2^{-\Delta\Delta C_T}$).

2.6. Western blot analysis

Pro-erythroblasts were treated with RIPA buffer (Teknova) and with protease inhibitors (100×; Thermo Scientific) to get a final protein concentration of 2.5 μg/μL. Total cell lysates (23 μg of proteins) were loaded onto a 8% precast SDS-PAGE gel (Bolt® Bis-Tris Plus gel, Life Technologies) and subsequently transferred to a PVDF membrane (Immobilion – FL, Millipore). HK1 was detected using antibody HK-1 (1:250; Abcam). Actin served as a loading control (anti-actin diluted 1:10,000; Merck Millipore). Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit (1:7500; Invitrogen) were used as secondary antibodies. Blots were imaged with an Odyssey Infrared Imaging System.

2.7. In silico analysis, 3D structural analysis, multiple sequence alignment

In silico analysis and multiple sequence alignment of mutations (p.His867Tyr and p.Thr600Met) were generated by PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/); Human Splicing Finder (http://www.umd.be/HSF3/) was used to assess the impact of novel splice mutations c.873-2A > G and c.493-1G > A. To investigate the structural and functional consequences of two novel missense mutations (p.His867Tyr and p.Thr600Met), we performed 3-dimensional (3D) structural modeling of the HK mutants (PDB entry 1DGK, [17]) with the PyMOL program (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.).

3. Results

All but one patient showed laboratory evidence suggesting HK deficiency as the underlying cause of HNSHA (Table 2). In general, HK activity was below 50% of the control range and/or the PK/HK ratio was elevated. Only patient 4 exhibited a normal HK activity as well as a normal PK/HK ratio. However, examination of the patient's parent showed a slightly increased PK/HK ratio (data not shown), prompting us to analyze *HK1* in this patient.

3.1. Genetic analysis

Molecular analysis identified six mutations in the *HK1* gene (Table 2). Four of these were novel: c.2599C > T p.(His867Tyr), c.1799C > T p.(Thr600Met), c.873-2A > G and c.493-1G > A. Patients 1 and 2 (siblings) were found to be compound heterozygotes for mutations c.−193A > G and c.873-2A > G. The maternally inherited −193A > G mutation is located in the promotor *HK1* region, and the paternally inherited splice site mutation c.873-2A > G is located in intron 7. Mutation c.278A > G p.(Arg93Gln) has previously been reported but is found here for the first time in the homozygous state (patient 3). Patient 7 is heterozygous for the same mutation. Patient 4 is homozygous for the novel missense mutation c.2599C > T p.(His867Tyr), located in exon 17. Finally, a novel missense mutation c.1799C > T p.(Thr600Met) and the novel splice site mutation c.493-1G > A were identified in the heterozygous state in patients 5 and 6, respectively.

3.2. Missense mutations: SIFT, PolyPhen, 3D results

In silico analysis by PolyPhen and SIFT confirmed the pathogenic nature of the p.(Thr600Met) mutation. Further evaluation using a 3D HK-1 model shows that Thr600 is located in a highly conserved region and close to the ADP-ligated active site, facilitating interactions within the ADP/glucose-complex. No changes in polar bonds were observed upon the substitution of Thr600 by Met. However, Thr600 directly interacts with Leu533, participating in a loop (residues 532–539) which binds Glc-6-P (Fig. 1, panel B). This loop undergoes conformational changes which influence a productive binding of the phosphoryl moiety of Mg^{2+}-ATP. Thus, the substitution of a fairly reactive threonine by an almost non-reactive methionine likely affects the mechanism of phosphate transfer. In turn, this likely affects the enzyme catalysis mechanism in a negative manner [17,18].

With regard to amino acid substitution p.(His867Tyr) PolyPhen and SIFT gave conflicting results (PolyPhen: damaging, SIFT: tolerated), however, like Thr600, His867 is located in a highly conserved region of the C-terminal half of the enzyme, and close to the ADP-ligated active site. The substitution of histidine by tyrosine at position 867 leads to appreciable changes in hydrogen bonds with residues Glu791, His870 and

Table 2
Biochemical and molecular data of patients with mutations in *HK1*.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
HK	0.64[¥]	0.58[¥]	0.71[*]	1.14[+]	0.65[¥]	0.37[*]	0.33[*]
PK	12.3[¥]	12.6[¥]	3.25[*]	12.5[+]	12.2[¥]	7.16[*]	6.2[*]
PK/HK	19.2[¥]	21.7[¥]	9.5[*]	10.90[+]	18.7[¥]	19.3[*]	18.7[*]
HK-R mutations							
Allele 1	c.−193A > G	c.−193A > G	c.278A > G p.(Arg93Gln)	**c.2599C > T p.(His867Tyr)**	**c.1799C > T p.(Thr600Met)**	**c.493-1G > A**	c.278A > G p.(Arg93Gln)
Localization	Erythroid promoter	Erythroid promoter	Exon 3	Exon 17	Exon 12	Intron 4	Exon 3
Allele 2	**c.873-2A > G**	**c.873-2A > G**	c.278A > G p.(Arg93Gln)	**c.2599C > T p.(His867Tyr)**	Normal	Normal	Normal
Localization	Intron 7	Intron 7	Exon 3	Exon 17	–	–	–

Mutations are numbered according erythroid specific transcript HK-R (NCBI reference sequence: NM_033496.2).
Novel mutations in bold.
¥ HK [0.8–1.5 U/g Hb], PK[6.1–12.3 U/g Hb], PK/HK [4.8–11.9].
* HK [0.8–1.65], PK[5.12–5.78], PK/HK [3.60–6.30].
\+ HK [0.62–1.26], PK [8.40–15.2], PK/HK [7.2–15.6].

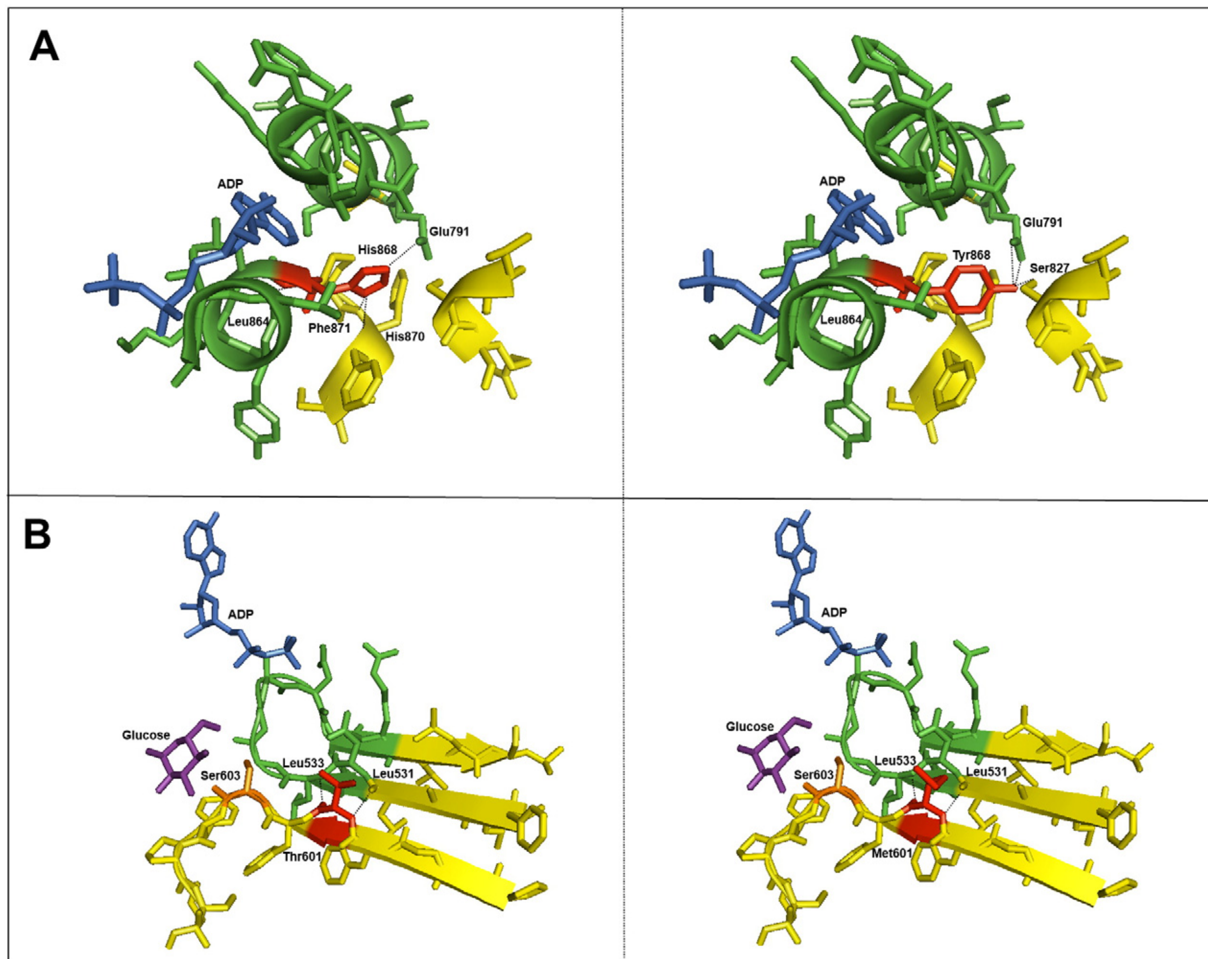


Fig. 1. 3D crystal structure of HK-R mutants – His867Tyr and Thr600Met (PDB 1DGK – protein databank). A) His867 (corresponding to His868 in HK-1, shown in red) is located between ADP-ligated active sites (green). No direct interaction between ADP (blue) and His has been described. However, His substitution with Tyr probably leads to a disruption of multiple hydrogen bonds (black dotted lines) among residues Phe871, His870 and a formation of new hydrogen bonds with Glu791 and Ser827. The changes in hydrogen bonding likely affect electrostatic interactions through the ADP-ligated active site, leading to interaction impairments within the ADP/glucose-complex. B) Met601 (corresponding to Met600 in HK-1, shown in red) is in close proximity of loop 532–539 (green) which is involved in productive binding of ADP (blue) and Ser603 (orange) directly interacting with a molecule of glucose (purple). Although no changes in hydrogen bonding were observed, the Met substitution with Thr in this position may impair electrostatic interactions within an enzyme catalysis site affecting the mechanism of phosphate transfer.

Phe871 (Fig. 1, panel A). This likely affects the mechanism of interaction within the active site. We therefore consider this mutation to be pathogenic.

3.3. Splicing mutations: HFS results

Human Splicing Finder (HFS) predicted that the A > G substitution at position c.873-2 as well as G > A on position c.496-1 disrupt the splice acceptor sites of intron 7 and 4, respectively. In addition to this, a cryptic acceptor site within intron 4 is activated by the c.493-1G > A mutation.

3.4. Kinetic studies of selected HK mutants

In order to determine the effects of the missense substitutions p.(Arg93Gln) and p.(His867Tyr) on HK function, we determined kinetic properties using RBCs from the patients homozygous for these substitutions (patients 3 and 4). HK harboring the p.(Arg93Gln) substitution showed a slightly higher affinity for ATP and a 2-times lower affinity for glucose (Table 3). Furthermore, its thermal stability was drastically decreased; $T_{1/2}$ was < 1.5 min, T_{50} < 37 °C. Interestingly, enzymatic activity was totally eliminated after a 10-min incubation at 40 °C.

Table 3
Kinetic properties of selected HK mutants.

Mutation	Family 1			Patient 3	Patient 4
	Patient 1	Patient 2	Father		
Allele 1	c.−193A > G	c.−193A > G	c.873-2A > G	p.Arg93Gln	p.His867Tyr
Allele 2	c.873-2A > G	c.873-2A > G	Normal	p.Arg93Gln	p.His867Tyr
Km Mg.ATP [1.286 ± 0.3000]	1.110 ± 0.1715	1.472 ± 0.1253	1.186 ± 0.1704	0.939 ± 0.1689	4.224 ± 0.3459
Km glucose [0.107 ± 0.0201]	0.070 ± 0.0052	0.058 ± 0.0048	0.052 ± 0.0089	0.256 ± 0.0520	0.151 ± 0.0120
$T_{1/2}$ at 40 °C [7.0–22.0 min]	10.2	8.5	8.0	<1.5	ND
T_{50} [42–44 °C]	40.5	39.6	39.9	<37.0	ND
pH stability	Normal	Normal	Normal	Normal	ND

ND – not determined.

The p.(His867Tyr) HK mutant showed a 3-times lower ATP affinity and a slightly lower glucose affinity. Unfortunately, thermal stability assays for this HK mutant could not be performed due to an insufficient sample amount.

HK from the compound heterozygous patients 1 and 2 was characterized by a normal ATP affinity and a slightly increased glucose affinity. $T_{1/2}$ was normal, whereas T_{50} was lower than the controls (Table 3).

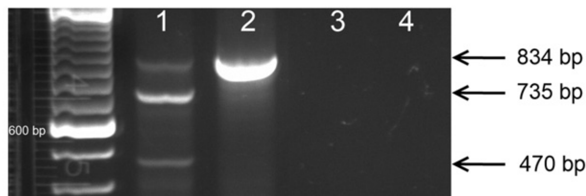
3.5. RNA studies of splice site mutation c.873-2A > G

To study the effect of the c.873-2A > G mutation on pre-mRNA processing, we isolated total erythroid RNA from *ex vivo* cultured pro-erythroblasts of a heterozygous carrier of this mutation (the father of patients 1 and 2). Agarose gel electrophoresis of RT-PCR products spanning exons 7 to 9 of the HK-R transcript revealed the presence of a RT-PCR product of normal size and, in addition, two RT-PCR products of alternate size (Fig. 2). Sequencing of both alternate RT-PCR products demonstrated that the c.873-2A > G change causes either skipping of exon 8 or both exons 8 and 9. Furthermore, qPCR results demonstrated that the normally spliced transcript was downregulated 3-times compared to the control subject. Subsequent Western blot analysis using *ex vivo* cultured pro-erythroblast showed a markedly decreased amount of HK, thereby confirming this decreased HK expression also at the protein level (Fig. 3).

4. Discussion

HK deficiency is a very rare hereditary disorder manifesting as HNSHA. Up to now, 24 cases of HK deficiency associated with HNSHA have been identified [9] and only 6 mutations associated with HK deficiency have been reported in literature [7,13,14,19]. Previously identified mutations have been numbered according to the HK-1 transcript. We propose renumbering mutations according to the erythroid specific HK-R transcript. An accordingly revised mutation overview is presented in Table 4.

Here, we report on six new cases of HK deficiency associated with HSNHA; one additional patient was a G6PD deficient female with heterozygosity for a *HK1* gene mutation. A total of six different mutations



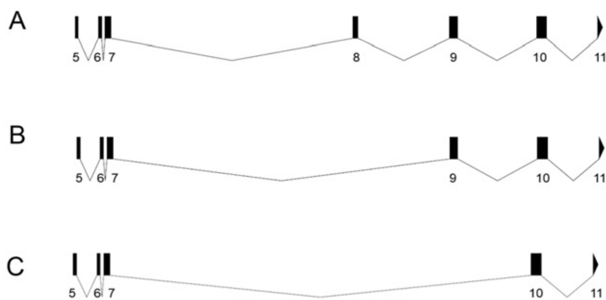


Fig. 2. Agarose electrophoresis of RT-PCR products of a heterozygous c.873-2A > G mutation carrier and a control subject. A region spanning exons 7 to 9 of HK-R transcript was amplified. A presence of two additional fragments was observed in the heterozygous carrier (line 1) compared to the control subject (line 2). Lines 3 and 4 were negative controls for reverse transcription and PCR. Below: a schematic drawing of all created HK-R variants. The variant A corresponds to a normally spliced variant (band 834 bp). Aberrantly spliced variants B and C lack exon 8 (band 735 bp) and/or both exons 8 and 9 (band 470 bp).

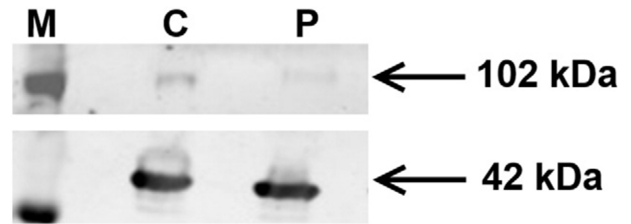


Fig. 3. Western blot analysis of *ex-vivo* cultured pro-erythroblasts from a heterozygous carrier of the mutation c.873-2A > G and a control subject. Line: M – marker; C – the control subject; P – the heterozygous carrier of the mutation c.873-2A > G. HK protein was visualized in position 102 kDa; actin 42 kDa.

in the *HK1* gene were identified. Four of them are described here for the first time: missense mutations c.2599C > T p.(His867Tyr) and c.1799C > T p.(Thr600Met), and splice site mutations c.873-2A > G and c.493-1G > A.

All but one patient showed a reduced HK activity and/or an elevated PK/HK ratio. Patients 1 and 2 (siblings) were found to be compound heterozygous for mutations c.−193A > G and c.873-2A > G. Interestingly, only patient 1 exhibited severe hemolysis requiring transfusion therapy. Previous investigations of the mutation c.−193A > G, maternally inherited in these patients, showed a strong down-regulation of the erythroid specific promoter activity [14]. Therefore, patients 1 and 2 could be regarded as pseudo homozygous for the c.873-2A > G splice site mutation. In agreement with this, the biochemical characteristics of mutant HK from patients 1 and 2 was comparable with HK from the father who is heterozygous for the c.873-2A > G mutation. Mutant HK was characterized by normal ATP affinity and a slightly increased glucose affinity. $T_{1/2}$ was normal, whereas T_{50} was lower than the controls (Table 3). It remains to be determined how this can be correlated to the two aberrant transcripts resulting from the splice site mutation. The mutation was found to induce skipping of exon 8 or both exons 8 and 9. If translated, the truncated HK protein would lack either 52 amino acids or 130 amino acids from the HK protein's regulatory part, encompassing either the substrate binding site or the substrate binding site and the G6P binding site [17,20]. This is expected to lead to a loss of catalytic function, perhaps to a larger extend than actually measured in patient cells. QPCR analysis revealed that the expression levels of normally spliced pre-mRNA were 3-times down-regulated compared to control subjects. Pre-mRNA reduction correlates with HK's down-regulation at protein level and may represent the most important cause for HK deficiency in patients 1 and 2.

Patient 3 was homozygous for the p.(Arg93Gln) mutation. This mutation has been reported once before in the heterozygous state [14]. The mutation in our patient was inherited from both parents who are first cousins. At age three, in addition to severe transfusion dependent HA, psychomotor retardation and secondary epilepsy was seen. Although psychomotor retardation has been described in HK deficient patients [4,5,21], in this case the malformations were likely caused by CNS bleeding as a result of hypoxia during delivery. De Vooght et al. postulated that p.(Arg93Gln) exerts its effect mainly by affecting pre-mRNA processing, leading to the formation of severely truncated HK which is very likely inactive and unstable [14]. Mutant red cell HK from our patient's cells indeed showed strongly decreased thermal stability. On the other hand, increased ATP affinity as well as a 2-times lower glucose affinity was also observed. These relatively mildly altered kinetic properties lead us to conclude that some normal pre-mRNA processing probably does occur from the c.278A > G mutation, leading to the formation of normally spliced mRNA harboring an Arg > Gln substitution at position 93.

Interestingly, the c.278A > G p.(Arg93Gln) mutation was also found in the heterozygous state in patient 7. This female patient was also homozygous for a mutation in G6PD: c.477G > C p.(Met159Ile). Despite a G6PD deficiency and a partial HK deficiency, no hemolysis was observed. Slight methemoglobinemia (7–11%) was the only clinical

Table 4
Overview of mutations associated with HK deficiency.

Accession number	Transcript HK-1 NM_000188.2		Transcript HK-R NM_033496.2		Localization	References
	Mutation	AA substitution	Mutation	AA substitution		
CR099859	–		−193A>G	–	Erythroid promoter	[14]
CS099858	c.291A > G	p.(Arg94Gln)	c.278A>G	p.(Arg93Gln)	Exon 3	[14]
CG024909	c.496-481_1031 + 454del	–	c.493-481_1028 + 454del	–	Intron 4–8	[7]
CG952319	c.496_del592	p.(166-197)	c.493_589del	p.(165-196del)	Exon 5	[11]
CM950628	1586T > C	p.(Leu529Ser)	c.1583T > C	p.(Leu528Ser)	Exon 11	[11]
CM030044	c.2039C > G	p.(Thr680Ser)	c.2036C > G	p.(Thr679Ser)	Exon 15	[13]
	c.496-1G > A	–	c.493-1G > A	–	Intron 4	This study
	c.876-2A > G	–	c.873-2A > G	–	Intron 7	This study
	c.1802C > T	p.(Thr601Met)	c.1799C > T	p.(Thr600Met)	Exon 12	This study
	c.2602C > T	p.(His868Tyr)	c.2599C>T	p.(His867Tyr)	Exon 17	This study

symptom present in this patient. A G6PD deficiency is mostly asymptomatic, however, mild methemoglobinemia has been described in several G6PD deficiency cases [22]. Although G6PD and partial HK deficiencies are well tolerated in this patient, potential negative factors as infections, drug administration or aging could induce hemolysis later in life.

The novel c.2599C > T p.(His867Tyr) mutation in patient 4 was associated with severe transfusion-dependent HA. Based on 3D structural modeling of this HK mutant, we found that His867 is located in the proximity of the ADP-ligated active site and, thus may impair the catalytic process of high-energy phosphate's transfer to glucose. This was supported by a strongly increased Michaelis-Menten constant for ATP (Table 3) as calculated from mutant HK from this patient's red cells. Patient 4′s severe phenotype illustrates the residue 867′s importance in proper HK function. The same catalytic process is likely affected in patient 5 heterozygous for the p.(Thr600Met) mutation. The substituted threonine, also located near the ADP-ligated active site, directly affects Leu533 residue in a loop (residues 532–539), which binds Glc-6-P [17]. Although no changes in polar bonds were observed, the substitution of fairly reactive threonine to almost non-reactive methionine likely affects enzyme catalysis as described for the His867Tyr change. The decreased HK activity in this patient did not cause hemolysis, and was an accidental finding during the diagnostic analysis for unexplained jaundice, later attributed to Gilbert syndrome.

Gilbert syndrome was also confirmed in patient 6. However, persistent mild hemolysis, reticulocytosis and low HK activity lead us to identify heterozygosity for the c.493-1G > A mutation. *In silico* analysis predicted disruption of the splice acceptor site of intron 4, causing activation of a cryptic site in intron 4. Although we conclude that aberrantly spliced pre-mRNA probably represents the major mechanism by which this mutation leads to deficient enzyme function, additional investigations of this mutation at the RNA and protein level are required. Also, at this moment it is unclear whether or not heterozygosity for this specific mutation is sufficient to cause hemolysis in this patient.

Taken together, we have established the molecular basis for decreased HK activity and hemolytic anemia due to HK deficiency in a relatively large number of patients suffering from this very rare disorder in red blood cell metabolism. These results contribute to a better understanding of the pathophysiology of HK deficiency.

Acknowledgment

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Two novel homozygous mutations (Arg94Gln and His868Tyr) in *HK1* gene associated with hereditary non-spherocytic anemia described in Spain and Czech patients

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INTRODUCTION

Hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1; HK) is initial enzyme of Embden-Meyerhof pathway which catalyzes the irreversible transfer of a high-energy phosphate from ATP to glucose (Figure 1). Hexokinase deficiency is a very rare autosomal recessive disorder which is associated with hereditary non-spherocytic hemolytic anemia. To date, only 20 cases of hexokinase deficiency have been described[1].

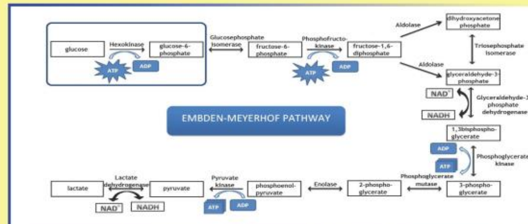

FIGURE 1: EMBDEN - MEYERHOF PATHWAY. Anaerobic glycolysis is a metabolic process in which glucose is broken down to produce lactate. It is the only source of energy (ATP) for red blood cells because they lack mitochondrias. Hexokinase, the first step in the glycolysis pathway, catalyzes phosphorylation of glucose to glucose-6-phosphate (blue border). This reaction is irreversible and it is considered to be one of the three regulatory points in the glycolysis.

PATIENTS AND METHODS

1. patient: 2.5-years old boy from Spain who has suffered severe hemolytic anemia since birth. Regular blood transfusions normalized his hemoglobin level to above 86 g/L. 2. patient: 1.5-years old boy from Czech Republic who also presented with transfusion-dependent hemolytic anemia since birth. Hemoglobin level is around 90 g/L (Table 1). In both cases, there was no family history of hemolytic anemia.

Enzyme activities (HK,G6PD,PK) were determined according to Beutler[2]. Genomic DNA was isolated according standardized procedures and it was used for sequencing analysis which was performed using ABI 310 Genetic Analyzer (Applied Biosystems). Biochemical characterization of HK mutants included Michaelis-Menten constant (Km) determination, thermal and pH stability assays. Km values for glucose and ATP were measured with different concentrations of glucose (2 - 0.0125 mM) and/or ATP (10 - 0.25 mM) in Tris/EDTA, $MgCl_2$, NADP and glucose-6-phosphate dehydrogenase mixture. Thermal stability was determined in hemolysate in Tris/EDTA buffer and incubated at 40 °C for 5 min. A range of Tris/EDTA buffer of different pH values (6.0 - 10.0) was used for assessment of effect on mutant pH stability. The models of the mutant forms of HK were obtained by side-chain substitution using program Pymol.

TABLE 1: **SELECTED HEMATOLOGICAL DATA OF BOTH PATIENTS**

	Patient 1	Patient 2	Reference range
Hemoglobin (g/L)	86	90	112 - 143
RBC ($x10^{12}$/L)	2.9	3.0	4.23 - 5.03
MCV (fL)	92	93	80 - 96
Reticulocytes ($x10^9$/L)	136	120	30 - 110

Legends: RBC - red blood cells, MCV - mean corpuscular volume

RESULTS

TABLE 2: **BIOCHEMICAL DATA OF THE HK-DEFICIENT PATIENTS**

	Patient 1	Patient 2	Controls
HK [IU/g Hb]	1.14	0.71	0.62 - 1.26/1.09 - 1.65
G6PD [IU/g Hb]	6.7	4.5	5.7 - 9.9/5.36 - 7.04
PK [IU/g Hb]	12.5	3.25	8.4 - 15.2/5.12 - 5.78
PK/HK	10.9	4.6	7.2 - 15.6/3.6 - 6.3
K_m Mg.ATP [mM]	4.13	0.94	1.66 - 1.93
K_m glucose [mM]	0.14	0.32	0.10 - 0.13
Thermal stability (40°C)	ND	Heat labile	Normal
pH stability	ND	Normal	Normal

Legends: HK - hexokinase, G6PD - glucose-6-phosphate dehydrogenase, PK - pyruvate kinase, ND - not determined

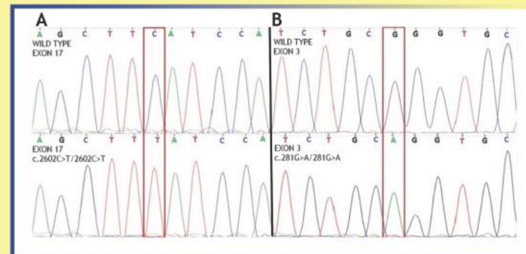

Figure 2: **DETECTION OF *HK1* GENE MUTATATIONS BY SEQUENCING OF AMPLIFIED DNA.** A) Patient 1 with homozygous mutation c.2602C>T in exon 17 (p.His868Tyr); B) Patient 2 with homozygous mutation c.281G>A in exon 3 (p.Arg94Gln).

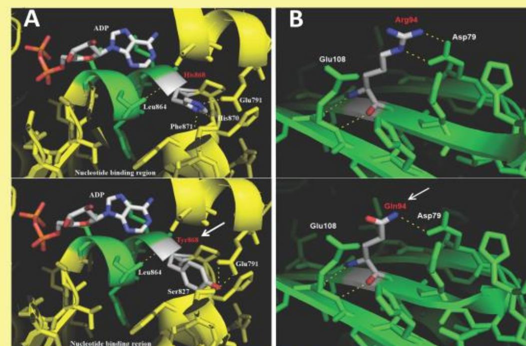

Figure 3: **3D STRUCTURE OF NORMAL AND MUTATED HK** (pdb code1DKG – protein data bank).
A) Patient 1 (His868 HK mutant): His868 is located nearby nucleotide - binding region (green). It is an important part of the catalytic region (yellow) of HK1 which provides interaction with ATP/ADP. His868Tyr substitution leads to appreciable changes in multiple hydrogen bonds. B) Patient 2 (Arg94 HK mutant): Mutation Arg94Gln is located in regulatory region of HK1 (green) and straightly affects hydrogen bond Asp79. (atom coloring : carbon - white; nitrogen - blue; oxygen - pink; phosphor - orange; hydrogen bond - yellow dotted lines).

SUMMARY AND CONCLUSION

The first patient was found to be homozygous for a novel c.2602C>T mutation (p.His868Tyr) in exon 17 of *HK1* (Figure 2). Biochemical characterization of mutant HK showed altered kinetic properties; the Km for ATP was 2.3-times increased compared to controls and Km for glucose was slightly increased (Table 2). Structural analysis of this mutation revealed that it is located near nucleotide-binding region (Figure 3). Residual HK activity of the second patient was around 50% of controls. Molecular analysis cofirmed homozygous mutation c.281G>A (p.Arg94Gln) in exon 3 of *HK1* (Figure 2). Kinetic studies showed reduced affinity for glucose (2.7-times) and increased affinity for ATP (1.9-times). Thermal stability assay revealed greatly decreased stability of mutant HK; pH optimum was unaffected (Table 2). Mutation is located in regulatory region of hexokinase (Figure 3).

Our results obtained from functional studies of both mutations (p.His868Tyr and p.Arg94Gln) suggested a good relationship between mutations and their effects on the enzyme structure and function; the altered kinetic properties of the mutants likely account for the severity of both patients' phenotypes.

These cases are the first described HK mutations in Spain and Czech Republic.

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FIVE NEW CASES OF HEXOKINASE DEFICIENCY: BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A NOVEL SPLICE SITE MUTATION AND 2 NOVEL MISSENSE MUTATIONS IN *HK1*

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BACKGROUND

Hexokinase (HK) deficiency is a very rare cause of hereditary nonspherocytic hemolytic anemia (HNSHA). Hexokinase is one of the key regulatory enzymes of glycolysis, on which the red blood cell is totally dependent the production of ATP (Figure 1). Deficiency of HK disrupts cellular metabolism, which ultimately results in HNSHA.
To date, 24 cases of hexokinase deficiency have been described. Only six of them have been characterized on the DNA level. We here describe five new cases and 3 novel mutations in *HK1*.

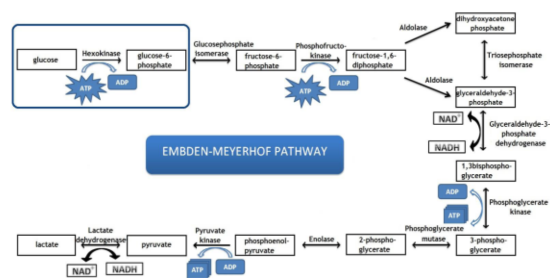

Figure 1: EMBDEN MEYERHOF PATHWAY. Anaerobic glycolysis is a metabolic process in which glucose is broken down to produce lactate. It is the only source of energy (ATP) for red blood cells because they lack mitochondrias. Hexokinase, the first step in the glycolysis pathway, catalyzes phosphorylation of glucose to glucose-6-phosphate (red border). This reaction is irreversible and it is considered to be one of the three regulatory points in the glycolysis.

AIMS

Characterize a genetic defect in patients who were found to be HK-deficient by spectrophotometrically determined red blood cell HK and pyruvate kinase (PK) enzymatic activities.

METHODS

Enzyme activities were determined according to Beutler. PK/HK ratio was applied to evaluate the effect of age-related increases in enzymatic activity. To confirm the diagnosis, DNA sequence analysis of HK1 was performed by Sanger sequencing. Novel mutations were characterized by biochemical methods (Km for glucose and Mg.ATP, pH stability and thermolability) and molecular studies (RT-PCR, quantitative RT-PCR, and Western Blot analysis on ex vivo cultured patient erythroblasts).

RESULTS

DNA sequence analysis of HK1 of 5 patients with suspected HK deficiency revealed a total of 5 different mutations in HK1. Three of them were novel (Table, novel mutations in bold). In *silico* analysis of mutations p.His868Tyr and p.Thr601Met by Polyphen-2 and SIFT predict that both substitutions are not tolerated. Both are located in the catalytic region, and kinetic properties are likely to be impaired upon mutation. Kinetic studies of the p.His868Tyr HK mutant showed that its affinity for ATP was indeed markedly decreased (3.2-times), whereas the affinity for glucose was slightly increased (Table). The molecular effects of the novel splice site mutation c.876-2A>G in intron 7 was studied on mRNA isolated from *ex vivo* cultured erythroblasts from the patients' father (patients 1,2), who was heterozygous for this mutation.
RT-PCR analysis showed the presence of normal as well as two aberrant mRNAs species. The aberrantly spliced transcripts lacked either exon 8 or both exons 8 and 9. Quantitative RT-PCR analysis showed that expression levels of the normally spliced mRNA variant were down-regulated 3-times compared to normal. These findings were confirmed on the protein level by Western blot analysis of HK from nucleated erythroid cells (Figure 2).

RESULTS

Table: Biochemical and molecular data of HK-deficient patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
HK [0.8 – 1.5]	0.64	0.58	0.71*	1.14+	0.65
PK [6.1 – 12.3]	12.3	12.6	3.25*	12.5+	12.2
PK/HK ratio [4.8 – 11.9]	19.2	21.7	9.50*	10.90+	18.7
Amino acid substitution	c.[-193A>G];**[876-2A>G]**	c.[-193A>G];**[876-2A>G]**	c. [281A>G];[281A>G] p.[Arg94Gln];[Arg94Gln]	**c.[2602C>T];[2602C>T] p.[His868Tyr];[His868Tyr]**	**c.[1802C>T] p.[Thr601Met]**
Km Mg.ATP [0.99-1.59]	1.11	1.42	0.94	4.22	ND
Km glucose [0.09 - 0.13]	0.07	0.06	0.26	0.15	ND
Thermal stability	Slightly decreased	Slightly decreased	Heat labile	ND	ND
pH stability	Normal	Normal	Normal	ND	ND

*HK [1.09 – 1.65], PK[5.12 – 5,78], PK/HK [3.60 – 6.30]; +HK [0.62 – 1.26], PK [8.40 – 15.2], PK/HK [7.2 – 15.6]; ND- not determined

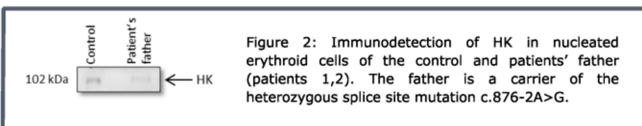

Figure 2: Immunodetection of HK in nucleated erythroid cells of the control and patients' father (patients 1,2). The father is a carrier of the heterozygous splice site mutation c.876-2A>G.

SUMMARY/CONCLUSION

We report 5 mutations in HK1 in 4 unrelated families. Three of them, c.876-2A>G, p.Thr601Met, and p.His868Tyr have not been previously reported. The pathogenic nature of novel mutations was confirmed by molecular and biochemical studies. Our results contribute to a better understanding of the genotype-to-phenotype correlation of HK deficiency, a very rare enzyme disorder of the red blood cell.

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APPENDICES 4.4

MOLECULAR CHARACTERIZATION A NOVEL NON-HEMOLYTIC ANEMIA
VARIANT OF PGK

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Case report

Recurrent episodes of myoglobinuria, mental retardation and seizures but no hemolysis in two brothers with phosphoglycerate kinase deficiency

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Abstract

We report two brothers with mild intellectual deficiency, exercise intolerance, rhabdomyolysis, seizures and no hemolysis. Phosphoglycerate kinase (PGK) activity was strongly decreased in their red blood cells. Subsequent molecular analysis of PGK1 revealed hemizygosity for a novel mutation c.756 + 3A > G, in intron 7. Analysis of the effect of this mutation on pre-mRNA processing demonstrated markedly decreased levels of normal PGK1 mRNA. In addition, the c.756 + 3A > G change resulted in abnormally spliced transcripts. If translated, these transcripts mostly encode for C-terminally truncated proteins. The consequences of the c.756 + 3A > G mutation is discussed, as well as the genotype-to-phenotype correlation with regard to previously described mutations (PGK Fukuroi and PGK Antwerp), which also result in C-terminal truncated proteins.
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Keywords: Phosphoglycerate kinase; Metabolic myopathy; PGK

1. Introduction

Phosphoglycerate kinase (PGK) is a key glycolytic enzyme directly involved in cellular generation of ATP. PGK deficiency is a very rare X-linked disorder associated with a triad of clinical manifestations involving different cell types: muscle (exercise intolerance, rhabdomyolysis), red blood cells (hemolytic anemia), and the central nervous system (seizures, mental retardation, parkinsonism) [1,2]. In general, mutations of PGK resulting in marked protein instability and mild impairment of kinetic properties are usually associated with neurological disorders and hemolysis, but not with myopathy [3]. On rare occasions, affected individuals show the full spectrum of clinical features. Thus, wide clinical heterogeneity is seen in patients with PGK deficiency [2].

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2. Case report

Two brothers, aged 14 and 16, born after an uneventful pregnancy, presented with slightly delayed walking (18 months). They subsequently developed mild intellectual deficiency (total IQ: 60 at WPPSI-III for the oldest brother), accompanied by recurrent episodes of generalized weakness following effort from the age of 4. At the age of 8, after spending twenty minutes playing in a swimming pool, the younger brother experienced an episode of weakness, together with a painful swelling of the left thigh and myoglobinuria (max CK 540.000 UI/l). He was hospitalized on the intensive care unit and treated with intravenous fluids and loop diuretics. At the age of 13, he presented with refractory status epilepticus, complicated by rhabdomyolysis and acute renal failure (max CK 710.000 UI/l). Status epilepticus required the administration of several doses of lorazepam, phenytoin and continuous intravenous midazolam. He had no recurrence of seizures under levetiracetam monotherapy. Currently, both brothers still complain of muscle pain and sometimes weakness after moderate exercise. They still avoid over-exertion to lessen the pain. Muscle strength

is completely normal between episodes of rhabdomyolysis or acute weakness. Their level of creatine kinase (CK) is approximately 500 UI/l (N < 308 UI/l) between episodes of rhabdomyolysis.

An electroencephalogram (EEG) showed an excess of delta slow waves but no epileptiform discharges in the younger brother. EEG of the older brother showed generalized spike-wave discharges, but no clinical seizure was observed. Complete blood count of both brothers showed no evidence of hemolysis. (younger brother: RBCs: 4.46×10^{-12}/l Hb:13.3 g/dl Htc: 39.8% MCH: 29.8 pg MCV : 89.2 fl; older brother: RBCs: 5.64×10^{-12}/l, 16.1 g/dl Htc: 47.1% MCH: 28.5 pg MCV: 83.6 fl). Serum urate level was normal (4.2 mg/dl).

Brain MRI was normal in both brothers. In the younger brother a muscle MRI was performed during the first rhabdomyolysis episode and showed T2 signal hyperintensities, compatible with edema, mainly in the anterior compartment of the thigh and in the semi-tendinosus, with the left thigh being much more affected than the right. Muscle MRI was normal in the older brother.

2.1. Histopathology, biochemical and molecular genetic analyses

Muscle-biopsy (Quadriceps), was done four months after an episode of rhabdomyolysis. Routine stains and most histochemical techniques (including HE, PAS, modified Gomori-trichrome, ATP-ase, and Acid phosphatase), were unremarkable.

Oxidative-enzymes staining (COX, SDH, combined COX/SDH, NADH-TR) showed no defective histochemical reactivity (and no evidence for mitochondriopathy). In addition, spectrophotometry showed normal mitochondrial respiratory-complexes activity.

The only salient feature was a slight increase in intra-cytoplasmic content of neutral lipids as detected by ORO stain.

Electron-microscopy showed foci of vacuolization; vacuoles were partly lipidic in origin but many were of undetermined nature.

Altogether, histo-morphological findings (Fig. 1) suggested a metabolic vacuolar myopathy with discrete to moderate lipid overload.

Lactate levels determined on plasma and cerebrospinal fluid as well as total free carnitine were normal. Urinary organic acids, acylcarnitines profiles and fatty acid oxidation studies on lymphocytes were unremarkable. Sequencing of *CPT2* gene encoding carnitine palmitoyltransferase 2 revealed no abnormalities.

Determination of red blood cell PGK enzyme activity revealed a markedly decreased PGK activity (<15% of controls). PGK activity of the patient's mother was only slightly decreased; the activity of father was normal (Table 1).

Subsequent DNA analysis of the *PGK1* gene by Sanger sequencing demonstrated a hemizygous A > G single base substitution in intron 7 (c.756 + 3A > G) in both brothers. This mutation was inherited from the patient's mother who was found to be heterozygous for this mutation. The mutation was not detected in a control population of >100 normal alleles (53 control subjects). Because the mutation alters the consensus sequence of the donor splice site of intron 7, we investigated the effect of the mutation on *PGK1* pre-mRNA processing. For this, total RNA was isolated from *ex vivo* cultured erythroblasts of both patients and a control subject, and reverse-transcribed according to standardized procedures. A region encompassing exons 7 and 8 of *PGK1* was amplified from cDNA. One single-size transcript was present in the control subject and both patients (Fig. 2), whilst additional RT-PCR products, representing splice variants, were amplified from patient-specific RNA. RT-PCR products were subcloned into pCR™4-TOPO vector (TOPO® TA Cloning® Kit for Sequencing, Invitrogen). Positive colonies were screened by colony PCR. Plasmid DNA was isolated and DNA sequencing analysis was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). This procedure identified a number of aberrantly spliced transcript variants (Fig. 2). The two most abundant transcripts were characterized by either the insertion of 52 bp of intron 7, or the complete sequence of intron 7. Two other, less abundant, transcripts lacked either 18 bp of exon 7 or skipping of complete exon 7 (Fig. 2).

In order to quantify transcript levels of the normally processed *PGK1* pre-mRNA, quantitative RT-PCR (qPCR) analysis was applied to both patient-specific and normal control RNA. This revealed a marked down-regulation (8.5-times) of the normally spliced pre-mRNA in patients compared to the control subject.

3. Discussion

The patients reported here exhibited mild intellectual deficiency, exercise intolerance, rhabdomyolysis, seizures, and no evidence of hemolysis. The clinical phenotype of the patients raised the suspicion of PGK deficiency as the underlying cause. This was confirmed by the finding of decreased red cell PGK activity in both patients and their mother, as well as the identification of a novel splice site consensus sequence change (c.756 + 3A > G) in intron 7 of *PGK1*. *In silico* analysis (Human Splicing Finder) [4] predicts that the A > G substitution abolishes the intron 7 donor site. To confirm this, RNA studies were conducted, which revealed the presence of four different abnormally spliced pre-mRNA transcripts. We find that the c.756 + 3A > G causes: a) the retention of the first 52 bp of intron 7, b) the retention of intron 7, c) the deletion of 18 bp from the 3′-end of exon 7, and d) skipping of exon 7 (Fig. 2).

Table 1
Enzyme analysis and *PGK1* genotypes of the family.

	Son 1	Son 2	Mother	Father
PGK [188–302 U/g Hb]	34	35	181	212
HK [0.5–1.5 U/g Hb]	1.0	1.3	1.6	1.0
Nucleotide substitution	c.756 + 3A > G	c.756 + 3A > G	c.756 + 3A > G	–

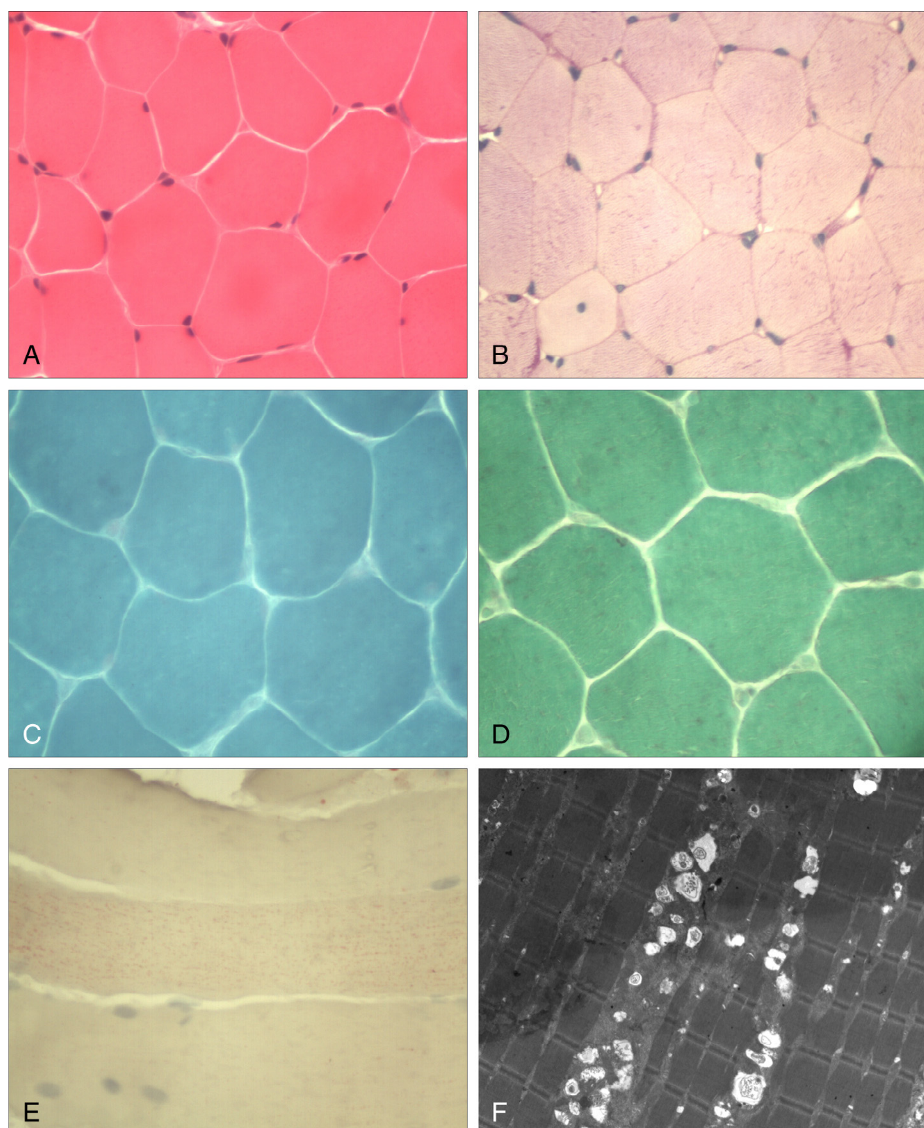

Fig. 1. Muscle Biopsy Observations. Histomorphological findings with "routine" stainings, namely, Hematoxylin-eosin (A), Periodic acid Schiff (B), modified Gomori (C), Acid phosphatase (D), and Oil-Red-O staining (E) were generally unremarkable. Transmission electron microscopy (F) showed foci of vacuolization in many fibers. Original magnification: ×320 (A); ×400 (B, C, D, E); and ×4400 (F).

The retention of 52 bp of intron 7, as well as the retention of intron 7, both result in a shift of the reading frame, leading to the formation of a premature stop codon. Consequently, if translated, the resulting protein is predicted to lack the C-terminal part (165 amino acids), which is responsible for nucleotide binding. The loss of 18 bp from the 3′-end of exon 7 encodes the deletion of six amino acids (residues 247–252). The skipping of exon 7 also disrupts the reading frame, causing a premature stop at amino acid position 269. Thus, the resulting predicted protein here, too, would lack the C-terminal end. RNA analysis also revealed that normal splicing is not completely abolished. However, qPCR analysis showed that normally processed mRNA levels were markedly down-regulated (8.5-times) compared to the control subject. It is likely this will result in markedly decreased PGK enzyme levels.

It has been hypothesized that different clinical manifestations of PGK1 deficiency can be correlated with the type of alterations caused by mutations in the PGK1 gene, highlighting the need for determination of the molecular properties of PGK variants to assist in prognosis and genetic counseling [5].

Aberrantly processed pre-mRNA transcripts containing the first 52 bp of intron 7 have been reported previously in patients with PGK variants Fukuroi (c.756 + 5G > A) and PGK Antwerp (c.755A > C, p.(Glu252Ala)). In both cases, no other aberrant mRNAs were identified. Phenotypically, both patients displayed recurrent episodes of myoglobinuria with rhabdomyolysis, without signs of hemolysis. This resembles the phenotype of our two patients. The patient with PGK Fukuroi, in addition, displayed intellectual deficiency and abnormal EEG. In both cases qualitative changes to muscle fiber were minimal and no accumulation of glycogen was observed [6,7].

A phenotype similar to that of our patients (myopathy, mental retardation and no hemolysis) has also been described in the patient with PGK Hamamatsu (c.758T > C, p.(Ile253Thr))

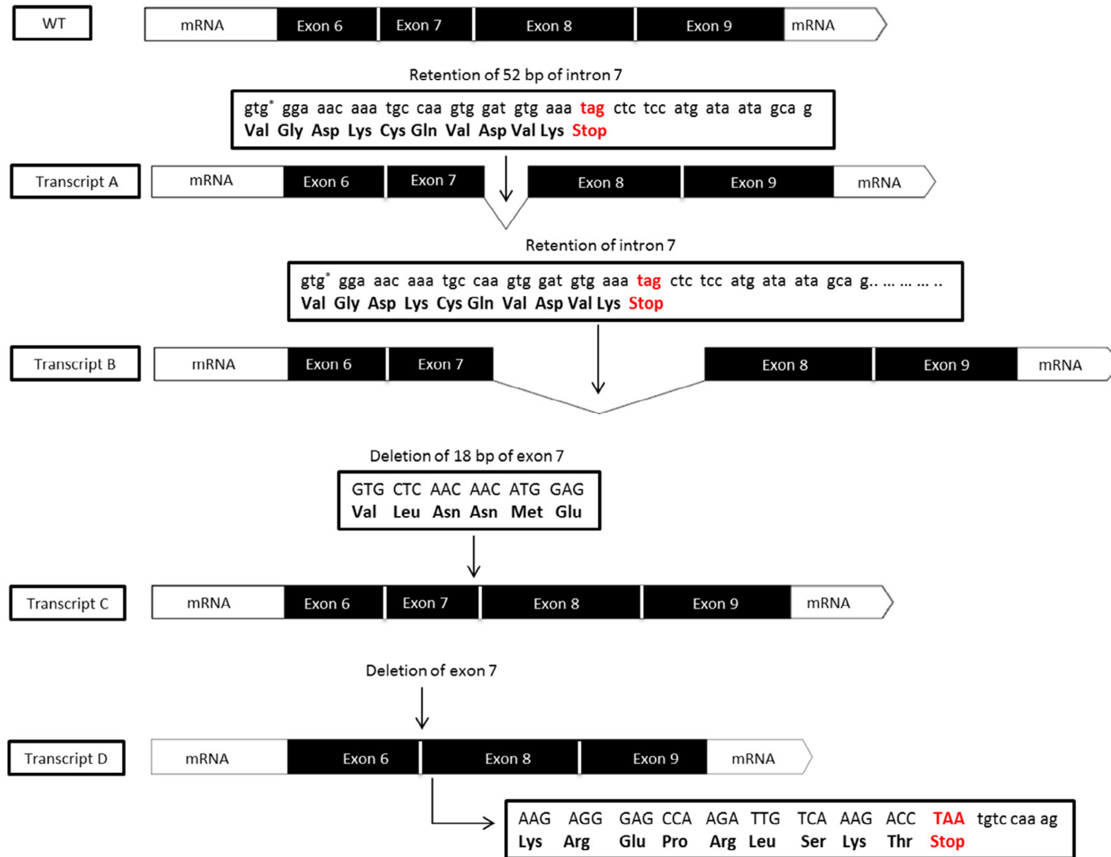

Fig. 2. A schematic drawing of the aberrantly spliced transcript variants arising due to the mutation c.756 + 3A > G. The wild type (WT) variant is normally processed mRNA. Arrows indicate the retention or the deletion of the particular sequence of *PGK1*. The stop codons are in bold and colored in red.

[8,9]. This mutation affects the same region that is predicted to be deleted by at least part of the aberrant transcripts identified in our patients.

These observed similarities may point to a correlation between the genotype and phenotype, but definite conclusions cannot be drawn at this moment. It seems reasonable to assume, however, that additional, yet-unknown factors, are likely to contribute to the phenotypic expression of PGK deficiency.

In conclusion, phosphoglycerate kinase (PGK) deficiency, a rare defect in glycolysis pathway, has its place in the differential diagnosis of metabolic myopathies. The association of exercise intolerance and recurrent episodes of myoglobinuria with hemolytic anemia, intellectual deficiency and other neurological disorders is highly suggestive of the diagnosis.

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APPENDICES 4.5

IN SILICO MODELLING OF A NOVEL MUTATION OF *PFKM* GENE ASSOCIATED WITH PFK DEFICIENCY

frontiers in PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
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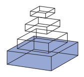

First description of phosphofructokinase deficiency in spain: identification of a novel homozygous missense mutation in the PFKM gene

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Phosphofructokinase deficiency is a very rare autosomal recessive disorder, which belongs to group of rare inborn errors of metabolism called glycogen storage disease. Here we report on a new mutation in the phosphofructokinase (PFK) gene *PFKM* identified in a 65-years-old woman who suffered from lifelong intermittent muscle weakness and painful spasms of random occurrence, episodic dark urines, and slight haemolytic anemia. After ruling out the most common causes of chronic haemolytic anemia, the study of a panel of 24 enzyme activities showed a markedly decreased PFK activity in red blood cells (RBCs) from the patient. DNA sequence analysis of the *PFKM* gene subsequently revealed a novel homozygous mutation: c.926A>G; p.Asp309Gly. This mutation is predicted to severely affect enzyme catalysis thereby accounting for the observed enzyme deficiency. This case represents a prime example of classical PFK deficiency and is the first reported case of this very rare red blood cell disorder in Spain.

Keywords: phosphofructokinase deficiency, glycogen storage disease, PFKM gene, missense mutation, enzyme catalysis

INTRODUCTION

Phosphofructokinase (ATP: D- fructose-6-phosphate-1-phosphotransferase; EC 2.7.1.11; PFK) is a key regulatory enzyme of the glycolytic cycle and catalyses the conversion of fructose-6-phosphate to fructose-1,6-diphosphate (**Figure 1**). Human PFK is composed of three isoenzymes, muscle (M), liver (L), and platelet (P) (Vora, 1983; Nakajima et al., 2002). The P type is also known as Fibroblast type (F). Mammalian PFK is a tetrameric enzyme that is subjected to allosteric regulation. Tissue isozymes randomly aggregate to form homotetramers or heterotetramers depending on the relative abundance of the subunits in a particular tissue. PFK-M is the sole subunit in muscle cells whereas red blood cells (RBCs) contain both L and M subunits and form their hybrids (M4, L4, M3L, M2L2, and ML3).

Phosphofructokinase deficiency (OMIM 171 850) is a very rare autosomal recessive condition with heterogeneous clinical symptoms, mainly characterized by myopathy and/or haemolysis (Hirano and Di Mauro, 1999). Myopathy is caused by the accumulation of glycogen in muscle tissue due to the metabolic defect and is also known as glycogenosis type VII or Tarui disease. It is characterized by muscle pain, exercise-induced fatigue, cramps, and myoglobinuria. The observed clinical symptoms reflect lack of muscle PFK activity and partial reduction of enzymatic activity in erythrocytes. The latter usually is associated with mild haemolysis.

Up to now, only about 100 patients with PFK deficiency have been reported worldwide and 22 PFK-deficient *PFKM* alleles have been characterized. The gene encoding the M subunit (*PFKM*) has been assigned to chromosome 12q13.3 and spans 30 kb. It contains 24 exons and at least 3 promoter regions (Elson et al., 1990; Yamada et al., 2004). Among the detected mutations are mostly missense mutations and splicing defects.

We now describe here a Spanish patient with a clinical history of anemia, haemolysis, and intermittent muscle weakness, who was found to be homozygous for a novel mutation in the *PFKM* gene (c.926A>G). This mutation encodes the substitution of aspartic acid by glycine at residue 309 (p.Asp309Gly). This is the first description of PFK deficiency in Spain.

CASE REPORT

A 65-years-old woman with long standing hypertension and type 2 diabetes was referred to our Unit because of intolerance to exercise and chronic fatigue. From youth she suffered from spasms of random occurrence associated with muscle weakness, painful intolerance to small efforts, and intermittent dark urines, especially after exercise due to myoglobinuria, as revealed by urinalysis. Physical examination showed no weakness or muscle atrophy and only a moderate splenomegaly without hepatomegaly or lymphadenopathy. Parents were unavailable for study and there was no known consanguinity. The same clinical picture, however, was present in a non-smoker brother whereas the three patient's daughters were normal. Patient's clinical condition has remained stable in follow-up.

Complete blood count (CBC) showed moderate anemia (Hb: 115 g/L), with slight macrocytosis (105 fl) and increased

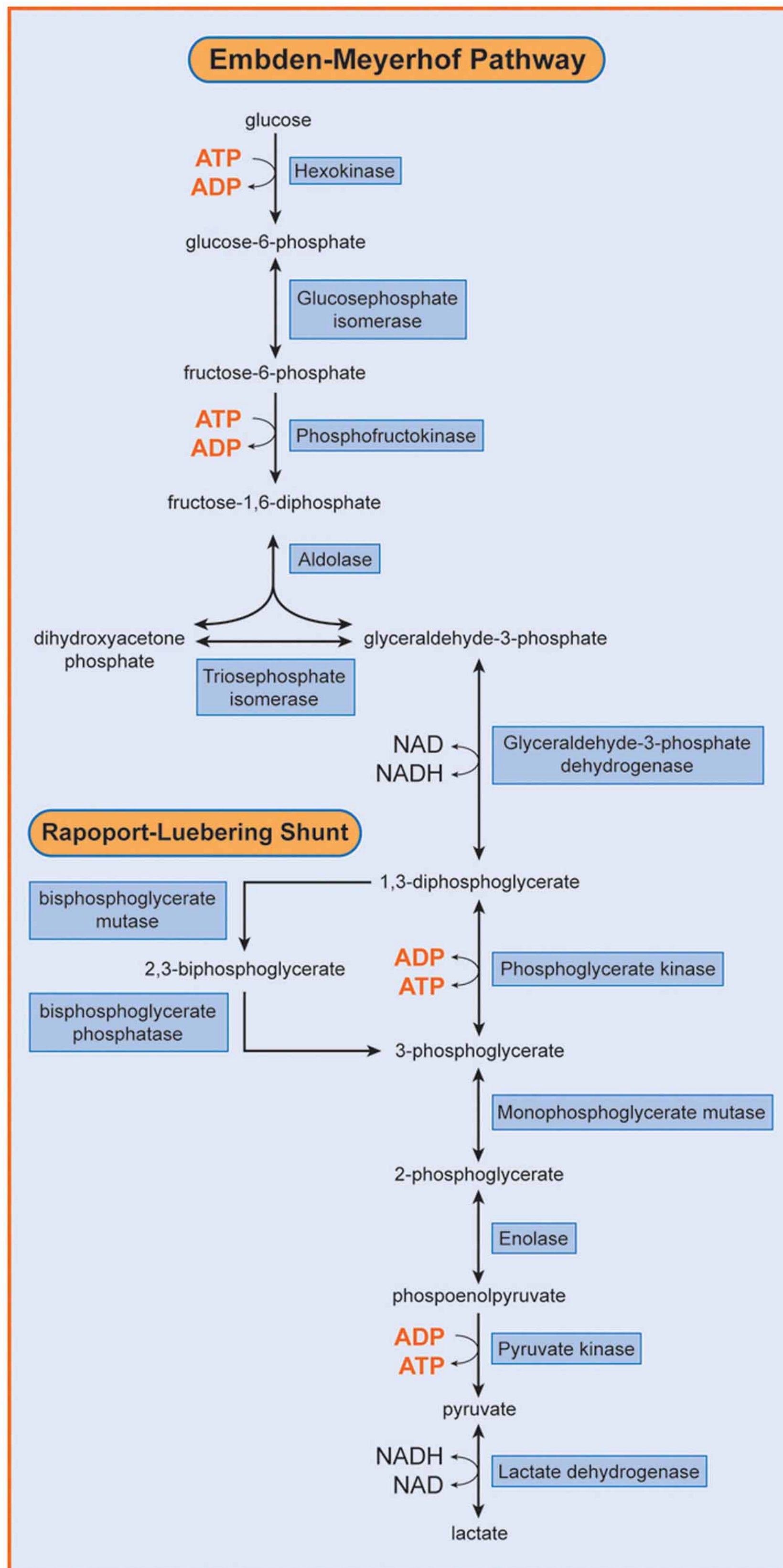

FIGURE 1 | Embden Meyerhof Pathway of RBC metabolism. Phosphofructokinase (PFK) catalyzes the transformation of fructose 6-phosphate into fructose 1,6 diphosphate. [Reproduced with permission from Van Wijk and van Solinge (2005)]

number of circulating reticulocytes (110×10^9/L). Leukocyte and platelet counts, as well as general serum biochemical analysis, were within normal range, except for a moderate rise in non-conjugated bilirubin, lactate dehydrogenase (LDH) and uric acid (hyperuricemia). Biological signs of diabetes mellitus type 2 were also present. The studies performed to rule out the origin of the anemia, discarded nutritional deficiencies (serum iron tests, cobalamin and serum folate were all normal), haemoglobinopathies (HPLC and thermal stability), and paroxysmal nocturnal haemoglobinuria (normal flow cytometry measurement of CD45 and CD49 in leukocytes and RBCs). Hereditary RBC membrane defects were ruled out by morphological observation of May-Grünwald-Giemas stained blood smears, and a normal osmotic fragility test. Extensive study of RBC enzyme activity measurements demonstrated a marked decrease (<30% of normal) in PFK activity (**Table 1**). DNA sequence analysis of individual exons of *PFKM*, including flanking splice sites, revealed that the patient was homozygous for a missense mutation in exon 11: c.929A>G. This mutation, that has not been previously reported in the literature, encodes the substitution of aspartic acid by glycine at residue 309 (p.Asp309Gly). Mutation prediction programs PolyPhen-2 (Adzhubei et al., 2010) and SIFT (Kumar et al., 2009) predict this mutation to be pathogenic (i.e., disease causing).

The complete lack of PFK activity in muscle was confirmed on both histological preparations and muscle extracts. Muscle abnormality was also confirmed by electromyography (EMG), that showed mild myopathic changes and by the forearm test, characterized by a plane lactate curve with normal increase of ammonium. As usual in patients with glycogenosis, a painful spasm occurred at the end of the forearm test. Muscle biopsy showed slight amounts of polysaccharide (PAS) not digested by diastase and abnormalities in NADH-TR reaction.

DISCUSSION

In this report, we describe, for the first time, the occurrence of PFK deficiency in Spain in an adult woman of 65 years of age. Her moderate haemolysis was associated with the accumulation of muscle glycogen comparable to that of Tarui disease. Muscle pain and exercise-induced fatigue and weakness associated with dark urines were the most relevant clinical manifestations. She was found to be homozygous for a novel missense mutation in *PFKM*, the gene that encodes the PFK-M subunit. On the amino acid level, this mutation (c.929A>G) causes the substitution of aspartic acid by glycine at residue 309. Asp309 is part of an α-helix, and replacement with glycine could disrupt this α-helix. Furthermore, structural analysis using the 3D molecular model of rabbit muscle type PFK (PDB entry 3O8L) showed that the p.Asp309Gly substitution is located in direct vicinity of the nucleotide (ADP) binding site in the center of the PFKM subunit (**Figure 2A**). PFK is allosterically activated by ADP, and the ADP binding site has been recently identified (Banaszak et al., 2011). Asp309 does not directly interact with ADP, however, substitution of Asp309 by glycine is likely to disrupt multiple hydrogen bonds with Gly177, Gly179, Ser 180, and Ser306 (**Figure 2B**). This loss of hydrogen bonds and the change of polarity and electrostatic interactions upon mutation of Asp309 will likely affect correct positioning of the side-chains of amino acids directly involved in ADP binding (**Figure 2B**), in particular neighboring residue Phe308, which makes stacking interaction with the adenine ring of ADP. This hypothesis is further supported by recent functional studies of the Asp543Ala change in PFKM. This mutation is associated with Tarui disease, and substitution of Asp543 by Ala was shown to disrupt hydrogen bonds with ADP (Brüser et al., 2012). We therefore postulate that decreased binding of the allosteric activator ADP will inhibit PFK enzymatic activity, in particular under low energy levels. These findings support the physiological importance of Asp390 for the recently identified ADP binding site.

The missense mutation is thus predicted to lead to a less functional PFK-M subunit. In accordance with this, the complete lack of PFK activity in muscle was confirmed on both histological preparations and muscle extracts. The muscle abnormality was also confirmed by electromyography, ischemic exercise testing, histochemistry and electron microscopy. Furthermore, partial red blood cell PFK deficiency was reflected by the moderately decreased enzymatic activity in red blood cells, leading to mild haemolytic anemia

PFK is a key regulatory enzyme for glycolysis (Van Wijk and van Solinge, 2005) and catalyzes the irreversible transfer of phosphoryl from ATP to fructose-6-phosphate, and converts it to fructose-1,6-bisphosphate. Thus, tissues deficient in PFK cannot use free or glycogen-derived glucose as a fuel source and accumulate glycogen (glycogenosis). PFK deficiency (Tarui disease) was the first disorder recognized to directly affect glycolysis (Tarui et al., 1965). Since this first description of the disease, a wide range of biochemical, physiological and molecular studies have greatly contributed to our knowledge concerning not only PFK function in normal muscle, but also on the general control of glycolysis and glycogen metabolism. So far, more than one 100 patients have been described with prominent clinical symptoms characterized by muscle cramps, exercise intolerance, rhabdomyolysis and myoglobinuria, often associated with haemolytic anemia and hyperuricemia. In classic Tarui disease, the genetic defect involves the M isoform, resulting in the absence of enzymatic activity in the muscle. Erythrocytes lack the M4 and hybrid isozymes and only express the L4 homotetramers, resulting in about 50% of normal PFK activity (**Figure 3**). Thus, haemolysis is a result of partial erythrocyte PFK deficiency.

Despite PFK deficiency is a very rare autosomal recessive disease; its true incidence may be higher due to lack of recognition, as symptoms may be quite mild. In fact, our case highlights the latter, because the mild clinical presentation led her to be diagnosed,

Table 1 | Red blood cell enzyme activity measurements.

	Patient	Reference value
G6PD activity (IU/g Hb)	7.0	6.2–9.9
HK activity (IU/g Hb)	1.2	0.6–1.3
PGI activity (IU/g Hb)	61.9	46.0–66.0
PK activity (IU/g Hb)	15.3	9.5–15.6
PFK activity (IU/g Hb)	3.07	6.4–13.9

Glucose-6-phosphate dehydrogenase; HK, Hexokinase; PGI, Phosphoglucose isomerase; PK, Pyruvate kinase; PFK, Phosphofructokinase.

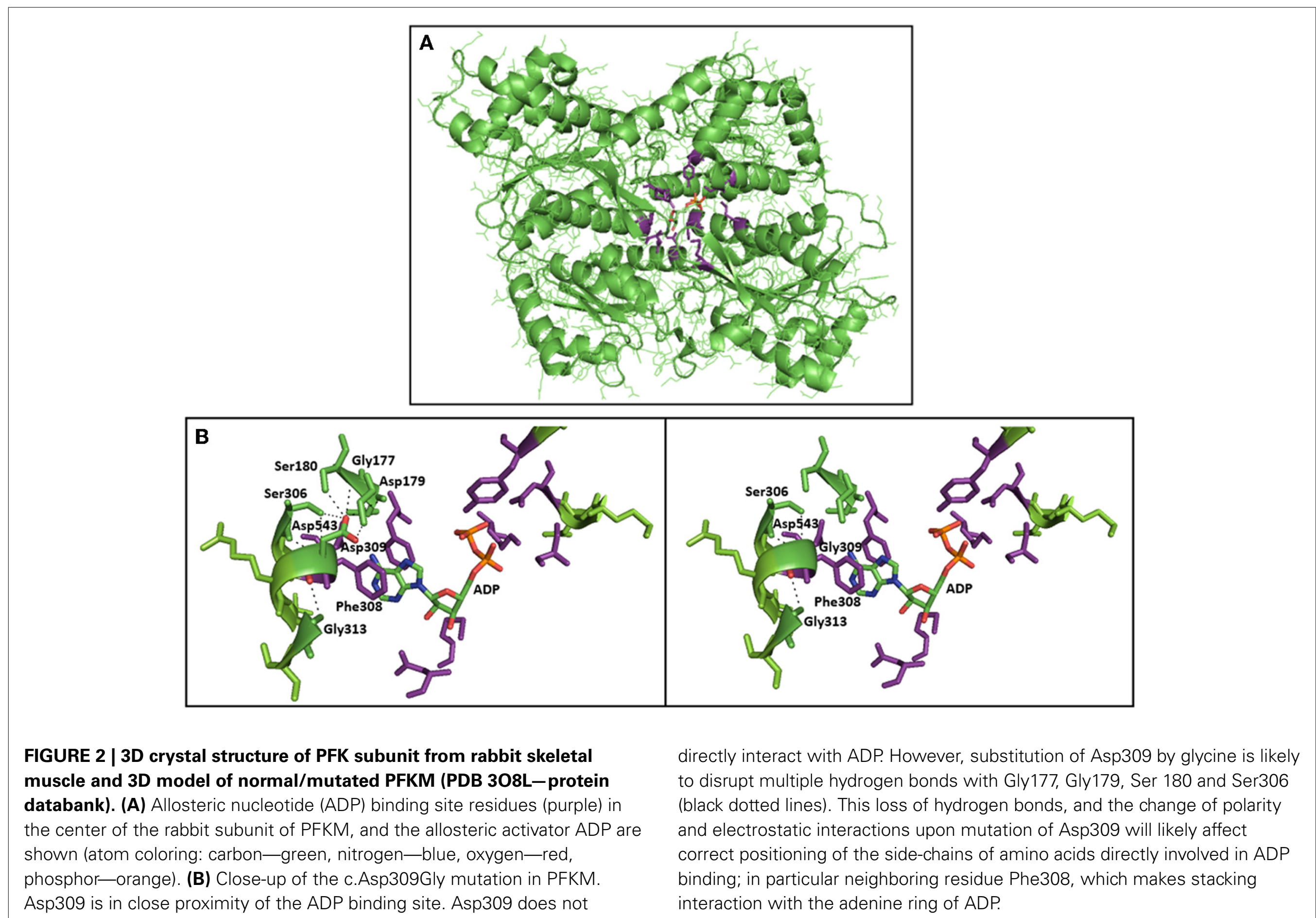

FIGURE 2 | 3D crystal structure of PFK subunit from rabbit skeletal muscle and 3D model of normal/mutated PFKM (PDB 3O8L—protein databank). (A) Allosteric nucleotide (ADP) binding site residues (purple) in the center of the rabbit subunit of PFKM, and the allosteric activator ADP are shown (atom coloring: carbon—green, nitrogen—blue, oxygen—red, phosphor—orange). **(B)** Close-up of the c.Asp309Gly mutation in PFKM. Asp309 is in close proximity of the ADP binding site. Asp309 does not directly interact with ADP. However, substitution of Asp309 by glycine is likely to disrupt multiple hydrogen bonds with Gly177, Gly179, Ser 180 and Ser306 (black dotted lines). This loss of hydrogen bonds, and the change of polarity and electrostatic interactions upon mutation of Asp309 will likely affect correct positioning of the side-chains of amino acids directly involved in ADP binding; in particular neighboring residue Phe308, which makes stacking interaction with the adenine ring of ADP.

for many years, as chronic fatigue, until the consideration of dark urines ultimately led to the study of chronic haemolysis. In our case the combination of RBC enzyme activity measurements and muscle biopsy analysis allowed for the correct diagnosis even at this late stage of life.

Generally, PFK deficiency presents in childhood. Clinical history however, defines 4 main subtypes: (1) classic, (2) infantile onset, (3) late onset, and (4) haemolytic. Most of the reported cases belong to the classic form, characterized by exercise intolerance, fatigue, muscle cramps with pain and myoglobinuria (Hirano and Di Mauro, 1999). A compensated haemolysis with jaundice, increased serum creatine kinase (CK) and hyperuricemia is also commonly present. Sometimes, nausea and vomiting appear after intense physical efforts Patients with the infantile onset may manifest as "floppy babies" that die within the first year of life. Symptoms include myopathy, psychomotor retardation, cataracts, joint contractures, and death during early childhood. They can also show evidence of arthrogryposis and mental retardation. Patients with the late-onset form may present in adulthood with progressive muscle weakness, cramps and myalgias in later life. Exercise ability, however, is low already in childhood, and a mild muscle weakness may appear in the 5th decade leading to severe disability. Diagnosis depends on patient history, physical examination and the findings from muscle biopsy, electromyography, ischemic forearm testing, CK testing. RBC enzyme activity measurement is, however, the easiest way to establish the definitive diagnosis. Fatal infantile type and late-onset forms of PFK clinical expression are very rare, with only several reported cases. The haemolytic form presents with hereditary non-spherocytic haemolytic anemia without muscle manifestations (Fujii and Miwa, 2000). Due to the molecular genetic heterogeneity, a clear-cut genotype-phenotype correlation has not been recognized in patients with PFK deficiency (Toscano and Musumeci, 2007). Unfortunately, no specific treatment or cure of enzyme deficiency exists. Although diet therapy may be highly effective at reducing clinical manifestations, Tarui disease resolves with rest. Fortunately, the condition does not progress to severe disability. Because the liver and kidneys express only the L isoform, these organs are spared.

About 100 cases of PFK deficiency have been reported in patients with Tarui disease from Europe, USA,and Japan with some predominance in Ashkenazi Jews (Fujii and Miwa, 2000). Up to know, 22 different mutations of PFK have been described. Missense and splicing mutations are the most frequently occurring mutations in *PFKM*. Intriguingly, PFK-deficient Ashkenazi Jews share 2 common mutations in the gene. A splicing defect caused by the G>A base change at the first nucleotide in intron

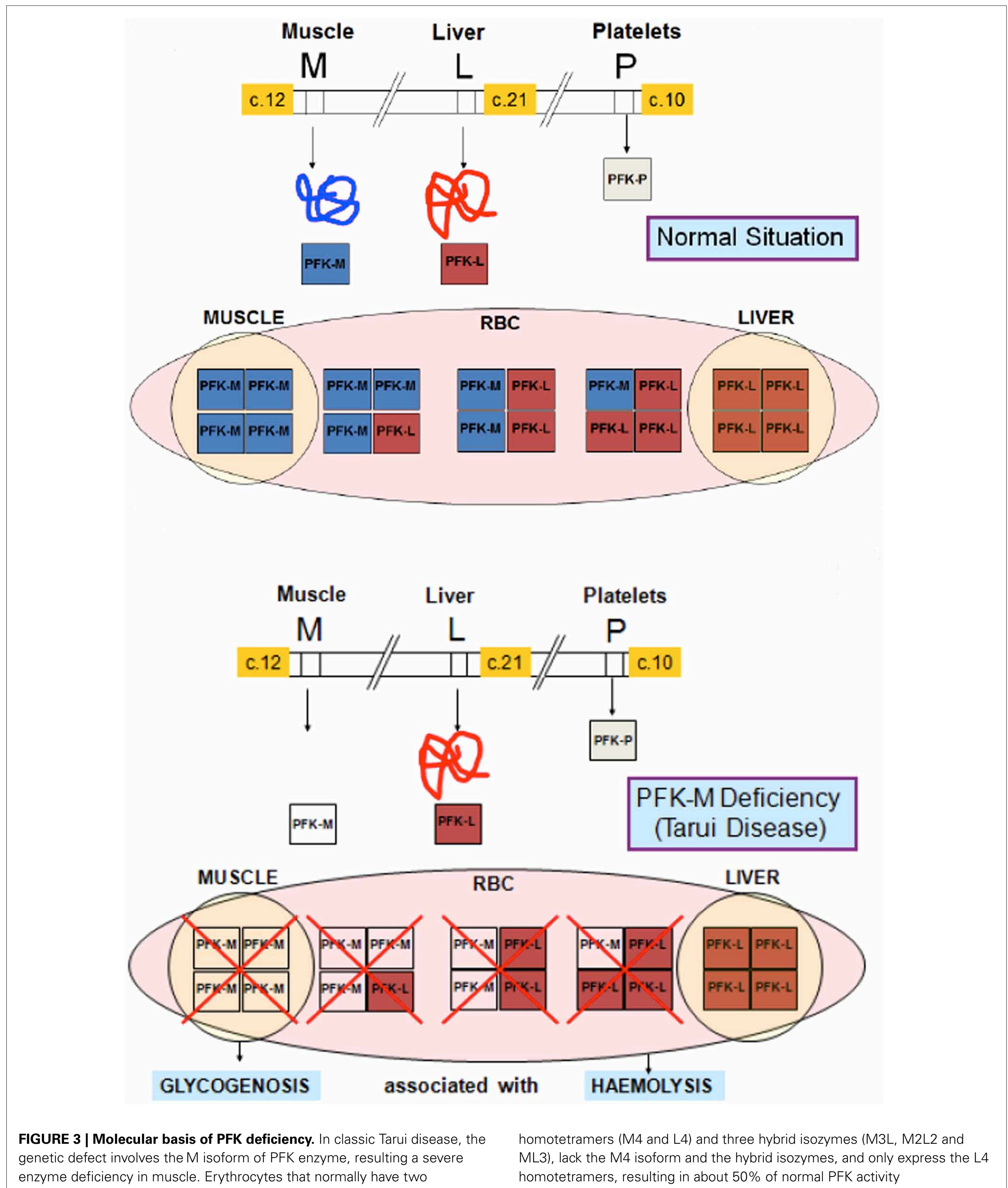

FIGURE 3 | Molecular basis of PFK deficiency. In classic Tarui disease, the genetic defect involves the M isoform of PFK enzyme, resulting a severe enzyme deficiency in muscle. Erythrocytes that normally have two homotetramers (M4 and L4) and three hybrid isozymes (M3L, M2L2 and ML3), lack the M4 isoform and the hybrid isozymes, and only express the L4 homotetramers, resulting in about 50% of normal PFK activity

5 (c.237+1G>A) accounts for 68% of mutant Ashkenazi alleles, and a single base deletion in exon 22 (c.2003delC) accounts for about 27% of mutant Ashkenazi alleles (Raben and Sherman, 1995). The here described homozygous patient is the first Spanish case described to be affected by this very rare disease. The identification of a novel homozygous missense mutation further extends the repertoire of PFK deficiency-associated mutations in *PFKM*.

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