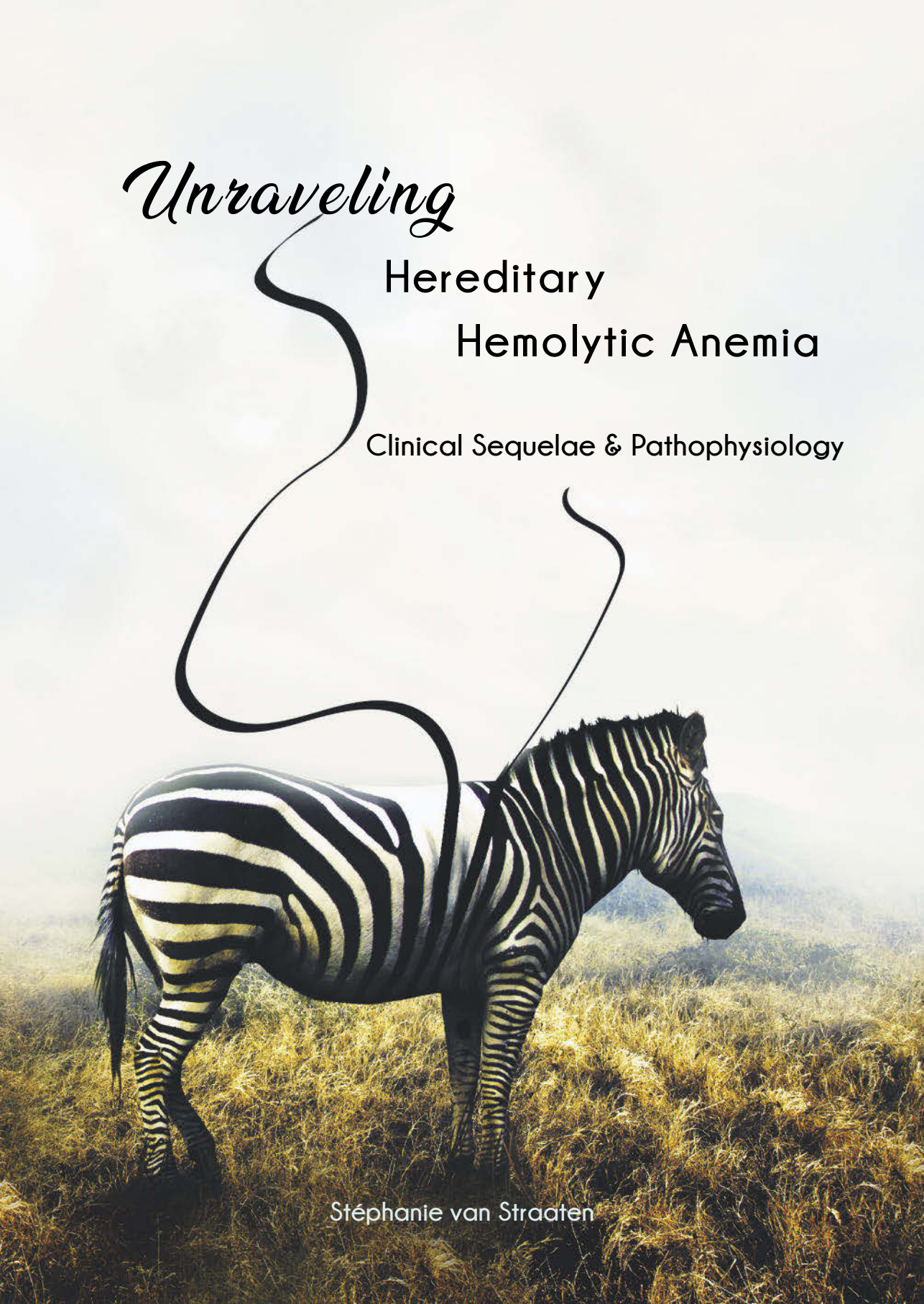
Unraveling

Hereditary Hemolytic Anemia

Clinical Sequelae & Pathophysiology



Stéphanie van Straaten

UNRAVELING HEREDITARY HEMOLYTIC ANEMIA: CLINICAL SEQUELAE AND PATHOPHYSIOLOGY

Stéphanie van Straaten

Unraveling hereditary hemolytic anemia: clinical sequelae and pathophysiology

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UNRAVELING HEREDITARY HEMOLYTIC ANEMIA: CLINICAL SEQUELAE AND PATHOPHYSIOLOGY

ERFELIJKE HEMOLYTISCHE ANEMIE: KLINISCHE GEVOLGEN EN PATHOFYSIOLOGIE

(met een samenvatting in het Nederlands)

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1 GENERAL INTRODUCTION

CLINICAL RELEVANCE

Hereditary hemolytic anemia (HHA) is globally one of the top causes of years lived with disability and due to better survival of patients it is a growing health problem.(1-3)

HHA encompasses all genetic diseases characterized by premature destruction of red blood cells. (4, 5)

The destruction of red blood cells is caused by various intrinsic defects. These defects can be classified in three main categories: hemoglobin disorders, red blood cell enzyme disorders and red blood cell membrane and hydration disorders. In this chapter we will explain the main categories, and expand on the intrinsic defects within every category that are most relevant for this thesis.

Hemoglobin disorders

Hemoglobin disorders encompass the genetic diseases affecting hemoglobin. The clinically most common and relevant forms are sickle cell disease (SCD) and α- and β-thalassemia. Among hereditary anemias, hemoglobin disorders are the most common genetic defects, with an estimate of 269 million carriers worldwide. Currently SCD already accounts for 300,000 newly diagnosed infants each year globally, and this number is expected to increase to 400,000 in 2050.(6)

Sickle cell disease

SCD is named after the distorted shape of part of the red blood cells of affected patients. In SCD there is an abnormality in the amino acid sequence of the β-globin chain. This leads to an altered globin chain called hemoglobin-S (HbS). HbS has the tendency to form long polymers upon deoxygenation. They distort the shape and flexibility of the red blood cell, producing a stiff, sickled red blood cell that is easily destroyed. Red blood cell sickling is the most important cause of intravascular hemolysis in SCD and eventually results in progressive organ damage.(7, 8) Polymerization of HbS and thus sickling happens during deoxygenation of the red blood cells.(9, 10) The higher the degree of deoxygenation, the higher the degree of sickling.(11)

β-Thalassemia

In β-thalassemia a mutation on the globin gene, present on chromosome 11, leads to a quantitative reduction of the β-globin chains that, together with α-globin, make up the hemoglobin molecule. This leads to an α- to β-globin ratio imbalance. The unbound excess α-chains and free heme are unstable and cause oxidative damage to the red cell precursors. This leads to massive destruction of these cells and hence hemolysis and ineffective erythropoiesis.(12, 13) There are over 200 disease causing mutations described in the β-globin gene. Their effect ranges from a silent mutation (silent β) to a relative reduction of β-globin chain (β+), to complete absence

of β-globin chain synthesis (β-0). Based on globin chain imbalance, severity of anemia and clinical picture at presentation, patients can be categorized as β-thalassemia major, intermediate and minor. In clinical practice, patients are often categorized based on their transfusion requirements and patients are addressed as transfusion dependent or independent respectively.

Red blood cell enzyme disorders

Red blood cell enzyme disorders are genetic diseases that disturb red blood cell metabolism, leading to decreased red blood cell life span. Red blood cell metabolism is vital for correct cellular functioning. Because mature red blood cells lack nuclei and mitochondria, they depend on anaerobic glycolysis through the Embden Meyerhof pathway for energy (Figure 1). Red blood cells have a unique bypass in the Embden Meyerhof pathway, called the Rapoport-Luebering shunt. Through the Rapoport-Luebering shunt, the red blood cell can bypass the phosphoglycerate kinase step in the Embden Meyerhof pathway. This bypass step of glycolysis serves mainly the production of 2,3-diphosphoglycerate, which regulates the affinity of oxygen to hemoglobin and can serve as an energy buffer.(14) In red cells, energy metabolism either proceeds through the Embden Meyerhof pathway, or through the hexose monophosphate shunt (also called the pentose phosphate pathway).(15-17) The main goal of the hexose monophosphate pathway is to create NADPH, which is required to maintain sufficient amounts of reduced glutathione (GSH). GSH is the red cell's major defense mechanism against oxidative stress. Mutations of enzymes involved in the Embden-Meyerhof pathway or hexose monophosphate shunt can lead to disturbed cell integrity and shortened cell survival.(18)

Pyruvate kinase deficiency

Pyruvate kinase (PK) deficiency is the most common red cell glycolytic enzyme defect causing HHA, with a prevalence estimated between 1:20,000 to 1:300,000 in the Caucasian population (19-22). The prevalence is higher in certain subpopulations, e.g. the Pennsylvania Amish, due to a founder effect. It is also likely higher in malaria endemic regions, where PK deficiency may confer a protective advantage (23-29). PK is the last and rate limiting step of the Embden Meyerhof pathway. Once the PK enzyme is deficient, the capacity to generate energy will drop and cell viability will be severely affected. In PK deficiency, 2,3-DPG increases as a result of retrograde accumulation of glycolysis products. As a result, the two major abnormalities in PK deficiency are a reduction in ATP and an increase of 2,3-DPG. 2,3-DPG lowers the oxygen affinity of red blood cells, meaning that the oxygen is more readily transferred to the tissue. In PK deficiency this is believed to ameliorate the anemia and improve the exertional tolerance. (18) This way, anemia in PK deficiency could be better tolerated than in other conditions, because of a shift in the oxygen dissociation curve, favoring the unloading

of oxygen to the tissues.(30) However studies on fatigue and self-assessed quality of life of patients with PK deficiency are lacking. The role of the pyruvate kinase enzyme in the pathophysiology of other forms of HHA is described in **chapter 6.**

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

G6PD deficiency is the most common red blood cell enzyme disorder, affecting near 400 million people worldwide.(31) This X-linked disease was discovered in 1956, when researcher recognized a unique hemolytic sensitivity of some persons to certain oxidant drugs.(32, 33) G6PD is involved in the hexose monophosphate shunt, where it functions to reduce NADP while oxidizing glucose-6-phosphate. In healthy red blood cells, G6PD only functions at 1-2% of its maximum potential.(34) There is a large reserve of reductive potential, which is decreased in patients with G6PD deficiency. Hence, G6PD provides the only means to generate NADPH, a deficiency makes the red blood cell particularly vulnerable for oxidative damage.(33). A total deficiency of G6PD is incompatible with life.(35)

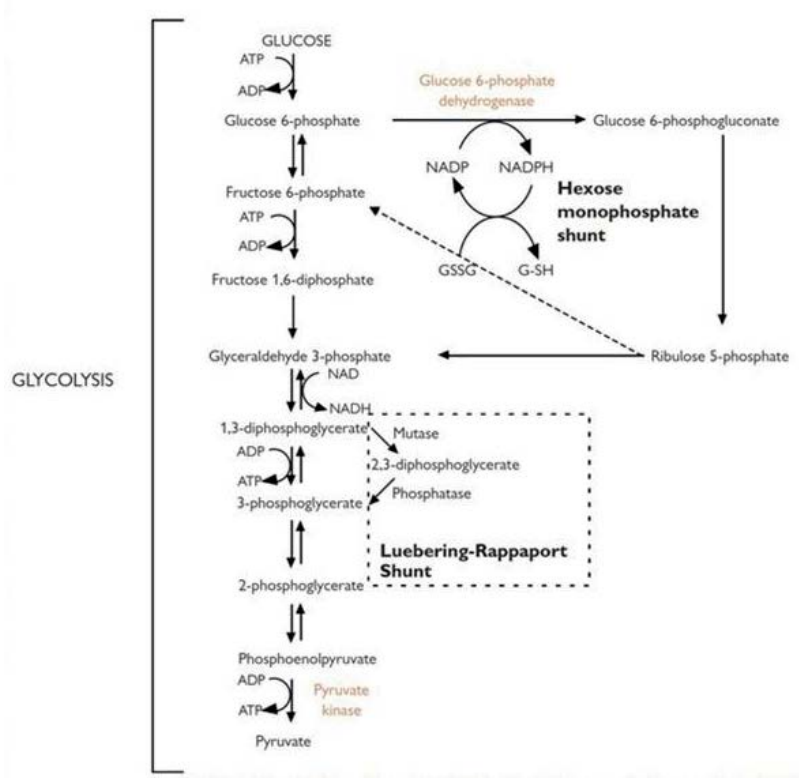

Figure 1. Embden Meyerhof pathway, Oxford Handbook of Clinical Haematology, 2nd edition, 2004

Red blood cell membrane and hydration disorders

Mature red blood cells do not have mitochondria or a nucleus, making red blood cells the only human cells in which the plasma membrane and cytoskeleton account for all antigenic, transport and mechanical characteristics. Because of its unique membrane and shape, a healthy red blood cell is able to undergo large passive deformations during passage through the microvascular system. Red blood cell membrane disorders are genetic diseases that cause defects of membrane or cytoskeletal proteins or altered membrane permeability. This leads to decreased red cell deformability, red cell lifespan and, eventually, hemolytic anemia.(36)

Hereditary spherocytosis

Hereditary spherocytosis is one of the most common forms of HHA, especially in people of northern European descent (1 out of 2000/3000).(36, 38) In hereditary spherocytosis, the red blood cell loses membrane due to diminished membrane cohesion by reduced vertical linkage between the lipid bilayer and the spectrin-based

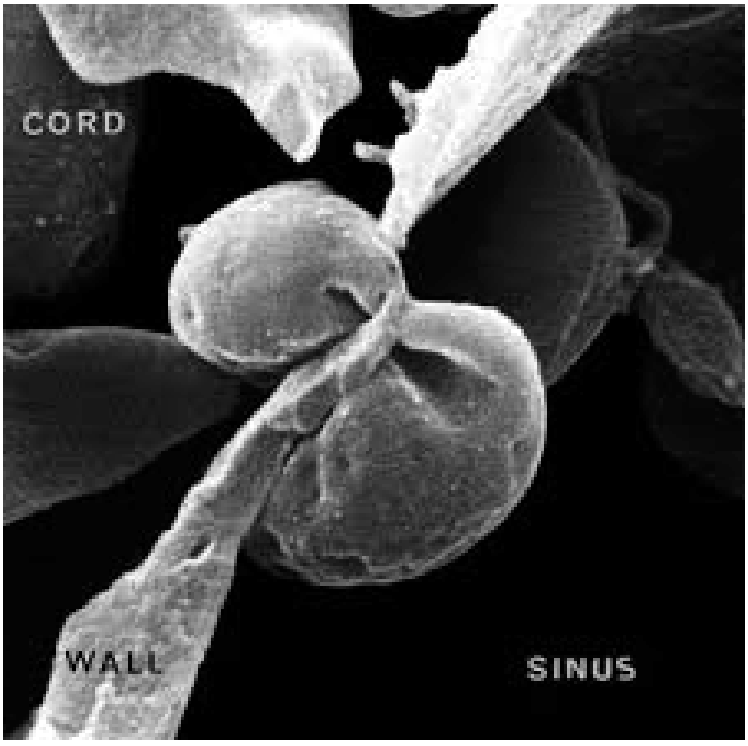

Figure 2. A reticulocyte traversing from the splenic cord to splenic sinus. Original magnification ·15 000.
An X, Mohandas N. Disorders of red cell membrane. British journal of haematology. 2008 May;141(3):367-75. (37)

membrane skeleton.(39) Because of loss of cell surface area, the mutation leads to a decreased cell surface area-to-volume ratio, increased hemoglobin concentration and consequent increased cell sphericity.(36) The decreased surface area also decreases red blood cell deformability and therefore the possibility of the red blood cell to effectively traverse the spleen (Figure 2). As a consequence spherocytes are effectively removed from the circulation.(37) The severity of the disease depends on the extent of surface area lost and ranges from asymptomatic or very mild forms to severe cases requiring extensive red cell transfusions. (38, 40)

Hereditary xerocytosis

Hereditary xerocytosis, also called dehydrated hereditary stomacytosis is characterized by red cell dehydration, resulting in increased mean corpuscular hemoglobin concentration and decreased osmotic fragility.(41) Normally, cellular water content is regulated by a large number of membrane ion transporters and channels. In hereditary xerocytosis there is a membrane transport defect. A net decrease of cation content, accompanied by a decrease of cellular water leads to increased hemoglobin concentration and cytoplasmic viscosity. This in turn leads to decreased red cell deformability and therefore reduced cellular survival. The primary cause of hereditary xerocytosis is a mutations in the PIEZO1 gene, coding for the pore-forming membrane channel PIEZO1.(42)

CLINICAL PICTURE

HHA generally causes a baseline level of mild to moderate hemolysis, which can be completely or partially compensated by increased erythropoiesis. The anemia may worsen by several triggers like inflammation, pregnancy or certain medication, leading to increased hemolysis or transient bone marrow aplasia due to viral infections.(4)

Despite the growing knowledge on the etiology of HHA, information on disease burden and health related quality of life (HR-QoL) of patients with HHA is scarce. Quality of life in HHA will be discussed in **chapter 2.**

Apart from stem cell transplantation, there is currently no curative treatment available for HHA. Stem cell transplantation in PKD is discussed in **chapter 9.** Current treatment is mainly supportive, consisting of regular red cell transfusions, and in severe cases splenectomy is performed. Medication can be subscribed to ameliorate anemia symptoms.

COMPLICATIONS AND TREATMENT STRATEGIES

Iron overload

As mentioned, the main supportive treatment strategy in severe HHA is red blood cell transfusions.(5) However, with every unit of packed red blood cells approximately 200-250mg of iron is transferred to the patient.(43) To add to that, HHA also results in

increased erythropoiesis in order to compensate for red cell destruction. Via induction of erythroferrone this leads to suppression of hepcidin, which in turn also results in increased uptake of dietary iron (Figure 3).(44) Because the human body does not have a mechanism to actively remove excess iron, this causes iron overload. Without treatment, iron overload can be fatal in the second decade of life. (45-47)

Currently most guidelines for the treatment of iron overload in HHA are based on experience in β-thalassemia. (48-51). Information about iron overload in other forms of HHA is limited. Iron overload in HHA is discussed in **chapter 7 and 8.**

Other complications

Gall stone formation due to increased breakdown of hemoglobin is one of the most common complications of HHA. Another common complication is thrombosis. Etiology of a hypercoagulable state in patients with HHA is suggested to involve inflammation and splenectomy, but remains largely unclear. Circulating cell derived, membrane enclosed extracellular vesicles (EVs), may contribute to the hypercoagulable state in HHA and other diseases. (52-57) Possibly, in HHA splenectomy reduces the efficacy of clearance of the phosphatidylserine (PS) exposing EVs. Exposure of negatively charged PS serves as a cofactor for coagulation by acting as a procoagulant surface that acts to orient coagulation proteases.(58, 59). The possible role of PS-exposing EVs in hypercoagulable state in patients with HHA is discussed in **chapter 5.**

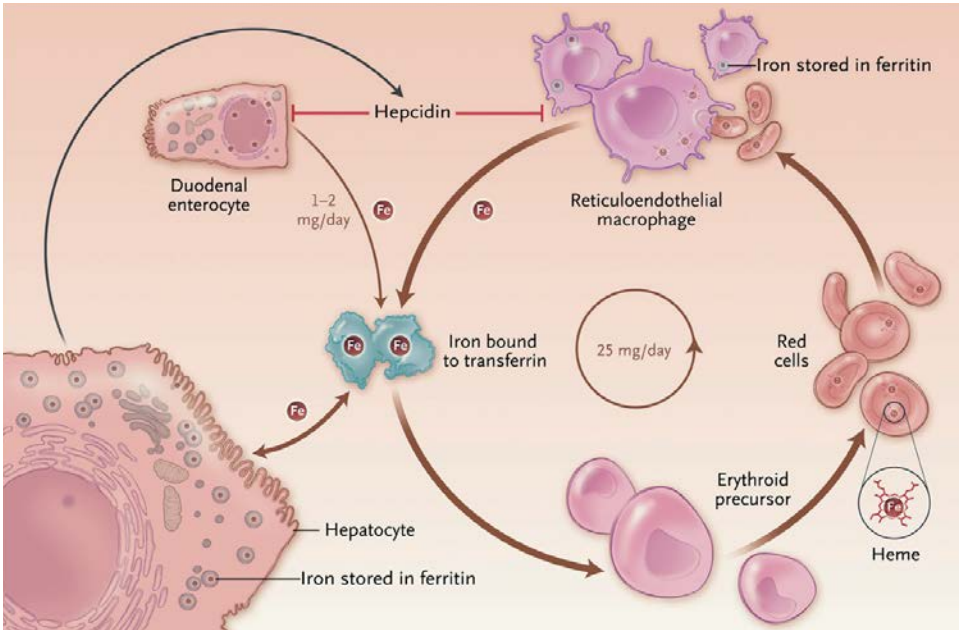

Figure 3. Iron Cycle. Fleming RE, Ponka P. N Engl J Med 2012;366:348-359

Splenectomy also increases the risk of infectious complications, especially with encapsulated bacteria. A rare, but very dangerous complication of HHA is pulmonary hypertension, which is discussed in **chapter 4**. An analysis of all forms of disease related organ involvement is described in **chapter 3**.

THESIS RATIONALE

The diseases included in the "umbrella term" HHA, although each unique, have many elements of disease symptoms, pathophysiology and treatment strategies in common. However, because of the rarity of the diseases, much information is still missing, and usually guidelines established for one disease (often β-thalassemia) are simply applied to all the others.

This thesis is designed to contribute to unraveling various aspects of hereditary hemolytic anemia (HHA), and to outline the first steps towards creating an evidence based framework for future guidelines regarding diagnosis and treatment of HHA. The aims of this thesis are:

1. to improve understanding of the disease burden of hereditary HHA, by studying both shared and distinct features of the diseases involved

2. to study pathophysiology of HHA

3. to evaluate effectiveness of current treatment strategies

The thesis is built up in three parts to answer the abovementioned three questions. Every part contains several research questions:

Part 1: From diagnosis to clinical symptoms

- What is the quality of life of patients with HHA?

- Do patients with rare forms of HHA suffer from organ involvement to the same extend as patients with SCD and thalassemia, and do we need guidelines for screening?

- Is the 6-minute walk test a useful tool to diagnose pulmonary hypertension in HHA?

Part 2: From clinical symptoms to pathophysiology

- Do cell derived extracellular vesicles play a role in creating a hypercoagulable state after splenectomy in patients with HHA?

- Is acquired decreased stability of red cell PK a common pathophysiological feature of SCD and other hemoglobin disorders, and does this affect clinical symptoms and disease severity?

Part 3: From pathophysiology to treatment strategies

- Do all patients with HHA suffer from iron overload, even patients who never received red cell transfusions? Can the currently used guidelines for screening for iron overload, based on experience in β-thalassemia, safely be applied in rare HHAs?

- Is stem cell transplantation a successful treatment option in pyruvate kinase deficiency?

OUTLINE OF THIS THESIS

Part 1: From diagnosis to clinical symptoms:

The first part of this thesis focuses on the clinical picture of HHA. In **chapter 2** we describe the self-reported quality of life in patients with HHA. We make both within group comparisons as well as comparisons to healthy peers, and formulate considerations regarding quality of life in patients with HHA in clinical practice.

In **chapter 3** we focus on disease related organ involvement in HHA. In this chapter we make an inventory of the occurrence and severity of organ involvement in HHA. This encompasses (early markers of future) organ damage, markers of altered organ function (e.g. endocrine changes) and other non-hematological symptoms known to influence morbidity and prognosis in chronically ill patients (e.g. inflammation). We formulate suggestions on which tests could be included in future organ involvement screening protocols for patients with HHA.

In **chapter 4** we focus on pulmonary hypertension, a very rare but possibly life threatening complication of HHA. We analyze occurrence of pulmonary hypertension in our population of HHA patients and analyze the occurrence of increased cardiac triscuspid regurgitant jet flow which in sickle cell disease is not only associated with pulmonary hypertension but also with increased mortality risk.(60) We make suggestions regarding the necessity of screening for pulmonary hypertension in patients with HHA.

Part 2: From clinical symptoms to pathophysiology

In HHA, especially after splenectomy, patients have an increased risk of thrombosis. The pathophysiology behind this is insufficiently established. In **chapter 5** we study the role of phosphatidylserine exposing cell-derived extracellular vesicles in hypercoagulability after splenectomy in HHA patients.

Oxidative stress is a common feature in various forms of HHA. The enzyme pyruvate kinase is very sensitive to oxidative stress and essential for red blood cell energy supply. In **chapter 6** we explore the role of decreased stability of pyruvate kinase in sickle cell disease, and study the link between instability of pyruvate kinase and oxidative stress.

Part 3: From pathophysiology to treatment strategies

One of the most important complications of HHA is iron overload. In **chapter 7** we describe a cross-sectional analysis of 130 adult patients with HHA who underwent MRI liver iron content measurement. We describe whether iron overload occurs in all forms of HHA and whether it can also occur in patients who never received transfusion therapy. Lastly, we evaluate current guidelines and give recommendations for adaptation.

Chapter 8 also focuses on iron overload. Here we describe an international cohort of pyruvate kinase patients of all ages. Again we focus on iron overload in never transfused patients, and study iron overload in children.

Currently the only curative strategy for HHA is stem cell transplantation. However, especially in the more rare forms of HHA, not much is known about the outcome of stem cell transplantation. In **chapter 9** we describe all cases of pyruvate kinase deficiency known to be transplanted, and the outcome after transplantation. We make suggestions regarding the role of stem cell transplantation in the current treatment strategy of patients with pyruvate kinase deficiency.

Lastly **chapter 10** summarizes the research described in this thesis, discusses the outcome and gives future perspectives for diagnosis and treatment of HHA.

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I

FROM DIAGNOSIS TO CLINICAL SYMPTOMS

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QUALITY OF LIFE IN PATIENTS WITH HEREDITARY HEMOLYTIC ANEMIA IS ASSOCIATED WITH ORGAN INVOLVEMENT AND SOCIAL WELL-BEING

ABSTRACT

Background

Last decades, life expectancy of patients with hereditary hemolytic anemia (HHA) has improved. Whether this is accompanied by a good health-related quality of life (HR-QoL) is not well known.

Our aim was to analyze HR-QoL in patients with HHA and to evaluate what patient characteristics are associated with impaired HR-QoL.

Methods

This is a cross-sectional, observational study of 85 patients with HHA. Patients filled out two validated questionnaires (EQ5D5L and FACT-An). Scores were evaluated by use of norm data and provided time-trade-off tools.

Results

The total patient population did not score a clinically meaningful difference on general HR-QoL compared to normal population. Transfusion dependent patients had a lower general HR-QoL.

HR-QoL did not correlate with hemolysis parameters, but physical, emotional and anemia related HR-QoL did correlate with the 6-minute walk distance. All domains, except emotional well-being correlated with hemolysis associated organ involvement.

Conclusion

Especially patients with hemoglobin disorders, and transfusion dependent patients have a lower HR-QoL and social well-being compared to other patients with HHA and HR-QoL correlated to hemolysis-associated organ involvement. Therefore, we suggest that improving social support and adequately treating organ involvement could be an interesting focus to increase HR-QoL in these patients.

INTRODUCTION

Over the past few decades, life expectancy of patients with hereditary hemolytic anemia (HHA) has shown remarkable improvement. The availability of blood transfusions, chelation therapy and other supportive treatment options has substantially improved life expectancy well into adulthood.(1, 2) Despite this improvement, HHA is still the number one cause of anemia burden in the high income Western World and due to better survival of patients it's a growing health problem.(3) For example: sickle cell disease (SCD) already accounts for 300.000 newly diagnosed infants each year globally, and this number is expected to increase to 400.000 in 2050.(4)

In HHA, especially in pyruvate kinase deficiency (PKD), hemoglobin level is not directly correlated to clinical severity of the disease, and neither is disease genotype. (5) Complex interaction of additional factors and compensatory mechanisms, like 2,3-diphosphoglycerate (2,3-DPG) levels, make for a wide variety of clinical phenotypes.(6, 7)

Information on disease burden and health related quality of life (HR-QoL) of patients with HHA is scarce, despite the growing knowledge on the etiology of HHA. As an example there is only one very recently published report available on HR-QoL in PKD and in hereditary spherocytosis only a few reports are available. (8-10) No data is available for G6PD-deficiency, or hexokinase deficiency.

The aim of this study was to evaluate patient reported HR-QoL by use of standardized questionnaires, and to analyze possible correlations with clinical or laboratory parameters. The results of this study might provide an essential first step towards pinpointing ways to improve HR-QoL of life in these patients.

PATIENTS AND METHODS

This is a cross-sectional, observational study on patients with HHA. Patients were all participants of the ZEbRA-study (NTR5337), a monocenter study conducted in the University Medical Center Utrecht in Utrecht, the Netherlands, focusing on the clinical sequelae and pathophysiology of HHA. The study required a single visit, a 6-minute walk test, two questionnaires, one blood donation and consent to a chart review. All adult patients with HHA were eligible to enrol. Patients with and without transfusion history were able to participate in this study. Patients were divided into three main categories: hemoglobin disorders (e.g. SCD, β-thalassemia), red cell enzyme disorders (e.g. PKD) and red cell membrane or hydration disorders (e.g. hereditary spherocytosis). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

All patients completed FACT-An, a validated questionnaire developed within the Functional Assessment of Chronic Illness Therapy (FACIT) system.(11) The questionnaire consists of five subdomains. The first four domains: physical, social/

family, emotional and functional well-being (PWB, SWB, EWB, FWB) are combined to create a general HR-QoL-scale (FACT-G) that can be compared to available normative data provided by FACIT, based on a sample of the general United States (US) adult population. The last domain (FACT-AnS) measures additional symptoms of anemia, including fatigue-related and non-fatigue anemia symptoms.(12) To analyze clinically meaningful differences between patients and normative data we used established "Minimally Important Difference" (MID), based on anchor- and distribution-based methods.(13) For Fact-G general HR-QoL total score the difference was five points, for individual subdomains two points. For FACT-AnS anemia subscale, because no reference data was available, MID could not be computed.

Patients also completed EQ5D5L, a generic five-dimensional questionnaire from the EuroQoL Research Foundation.(14) EQ5D5L aims to measure HR-QoL on five dimensions of health: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Every dimension has five answer categories (no problems, some problems, moderate problems, severe problems and extreme problems/unable to perform) and therefore this questionnaire can describe 5^5 (3125) health states. Next to the descriptive system it also entails a visual analogue scale (EQvas) for quality of life.

A single weighted utility score was created by comparing the data to the published Dutch Tariff based on composite time trade off (cTTO), creating societal valuation of the respondent's health state.(15, 16) cTTO is an extensively used method for health state evaluation. The value of a health state is derived by finding the amount of time in full health *x*, that is considered equal to a given amount of time in a suboptimal health state *t*. The difference between cTTO and regular TTO is that once respondents consider the health state under valuation so poor that they'd rather die immediately than have to live with this health state for a certain period of time (score of 0), they are switched to a lead time TTO task, meaning that the health state under valuation still would last for *t* years, but it would be preceded by a period of time *l* in full health to express negative values. cTTO is presented on a scale from -1 to 1.0 (full health). For more detailed information the Dutch Tariff and cTTO we refer to the references. (15, 16)

All patients who were willing and able also performed a 6-minute walk test. The 6-minute walk test was performed according to the practical guidelines of the American Thoracic Society statement.(17) For calculation of percentage of predicted distance (6MWD), we used the equation of Tveter et al (2014).[20]

Disease related organ involvement was calculated only for patients who had more than ten organ involvement scoring items available and was calculated as number of positive items as a percentage of the total amount of scored items.(18)

Definitions and statistical analysis

Statistical analysis was carried out using non-parametric tests because of the abnormal distribution of some of the data. Nominal data was compared using Mann Whitney U or

Kruskall Wallis tests when appropriate. Categorical data was compared using Fisher's exact or Fisher-Freeman-Halton tests for binomial tables. Statistical significance was considered if P≤0.05. Post hoc correction was not applied.

RESULTS

Baseline characteristics

Between 2016 and 2017, 100 patients were enrolled. Eighty five patients completed the questionnaires and were enrolled in the analysis. At baseline, patients had a median age of 43 years (range 18-84) and a median Hb of 7.1 mmol/L (range 4.0-10.2 mmol/L). Forty two patients (49%) were female. Twenty three patients had a hemoglobin disorder (ten SCD, eight β-thalassemia, five other hemoglobin disorders), 30 patients had a red cell enzyme disorder (21 PKD, six glucose-6-phosphate dehydrogenase (G6PD) deficiency, two hexokinase deficiency and one glutamate cysteine ligase deficiency), and 32 had a membrane or hydration disorder (22 hereditary spherocytosis, eight hereditary xerocytosis, two hereditary elliptocytosis). Baseline characteristics per disease category are presented in Table 1.

There was a good convergent validity between FACT-An and EQ5D5L utility scores and VAS-scores (R =0.615-0.745, p=<0.001 in all domains).

Quality of life in hereditary hemolytic anemia according to EQ5D5L

Twenty eight patients (33%) reported no problems (score of 11111) on the EQ5D5L questionnaire. Of the patients who reported problems, most complained of pain (47 patients, 55%), followed by problems with activity (39 patients, 46%), anxiety

Table 1. baseline characteristics per disease category

	Hemoglobin disorders		Enzyme disorders		Membrane disorders	
Female	12/23	(52%)	13/30	(43%)	17/32	(53%)
Age (years)	30	(18-79)	45	(20-70)	47	(18-84)
Hb (mmol/L)	5.8	(4.5-8.5)	6.8	(4.0-10.1)	8.6	(6.1-10.2)
MCV (fL)	83	(43-126)	103	(88-123)	92	(81-109)
Retics (*10^9/L)	143	(36-382)	297	(61-1096)	303	(27-1015)
Ferritin (ug/L)	540	(50-3584)	506	(37-3586)	160	(33-694)
Liver iron content (mg/g DW)	11.8	(2.2-19.6)	5.6	(0.3-19.6)	5.3	(2.4-10.1)
Transfusion						
Never transfused	2/22	(9%)	14/28	(50%)	15/28	(54%)
Transfusion independent	5/22	(23%)	6/28	(21%)	12/28	(43%)
Transfusion dependent	15/22	(69%)	8/28	(29%)	1/28	(4%)
Organ involvement	20/22	(91%)	26/28	(93%)	23/28	(82%)
6MWD (median % of predicted)	83	(63-106)	87	(69-104)	84	(64-131)

Numbers are medians(range) or number/total(percentage)

(27 patients, 32%), mobility (22 patients, 26%) and selfcare (six patients, 7%). There was a strong correlation between activity and mobility (ρ=0.625, and activity and pain (ρ=0.652). The lowest correlation was between anxiety and pain (ρ=0.298) and anxiety and selfcare (ρ=0.303, Supplemental table 1a).

Between the disease categories, there was a significant difference in percentage of patients that reported no health problems (Supplemental table 2). Three out of 23 (13%) patients with hemoglobin disorders reported no health problems, versus 10/30 (33%) patients with enzyme deficiencies and 15/32 (47%) with membrane or hydration disorders (p=0.027). Between the transfusion categories, there was no significant difference. Twelve out of 31 never transfused patients (39%) reported no health problems, versus 9/23 (39%) of sporadically transfused patients and 3/24 (13%) of transfusion dependent patients (p=0.057)

In the subdomains (mobility, selfcare, usual activity, pain and anxiety) there was no significant difference in reported problems between the different disease categories. There was a significant difference in the reported social problems between the different transfusion categories (Figure 1).

We compared EQ5D5L cTTO utility scores between patient groups and between transfusion groups. Patients with hemoglobin disorders had lower utility scores than patients with enzyme or membrane disorders (respectively 0.82 vs 0.89 and 0.89), but the difference between the three groups was not significant (p=0.081, Figure 2). Transfusion dependent patients also had lower utility scores than patients that were transfusion independent or never transfused (respectively 0.82 versus 0.89 and 0.89), but this difference was also not significant (p=0.093). Two patients scored a utility score lower than zero. Both patients were diagnosed with β-thalassemia and both were transfusion dependent.

Quality of life according to FACT-An

Forty eight patients (57%) had a FACT-An general health score (FACT-G) that was higher than the general US population median. Thirty five patients (42%) had a general health score that was lower. Fourteen patients (17%) had a general health score lower than the 25th percentile of the general US population. Of the patients that scored lower than the 25th percentile, eight were diagnosed with hemoglobin disorders, two with enzyme disorders and four with membrane or hydration disorders.

The total patient population did not score a clinically meaningful difference (CMD) on general HR-QoL (Fact-G) compared to the general US population (difference 2.1 CMD=5, Table 2) However, patients with enzyme or membrane disorders and patients who had never received a transfusion had a clinically meaningful higher general health score compared to normative data. Patients who were transfusion dependent had a lower general health score. On the individual subscale scores, patients had a clinically meaningful higher score for social well-being (SWB) compared to normative data. Comparative data for the anemia subscale (FACT-AnS) was not available.

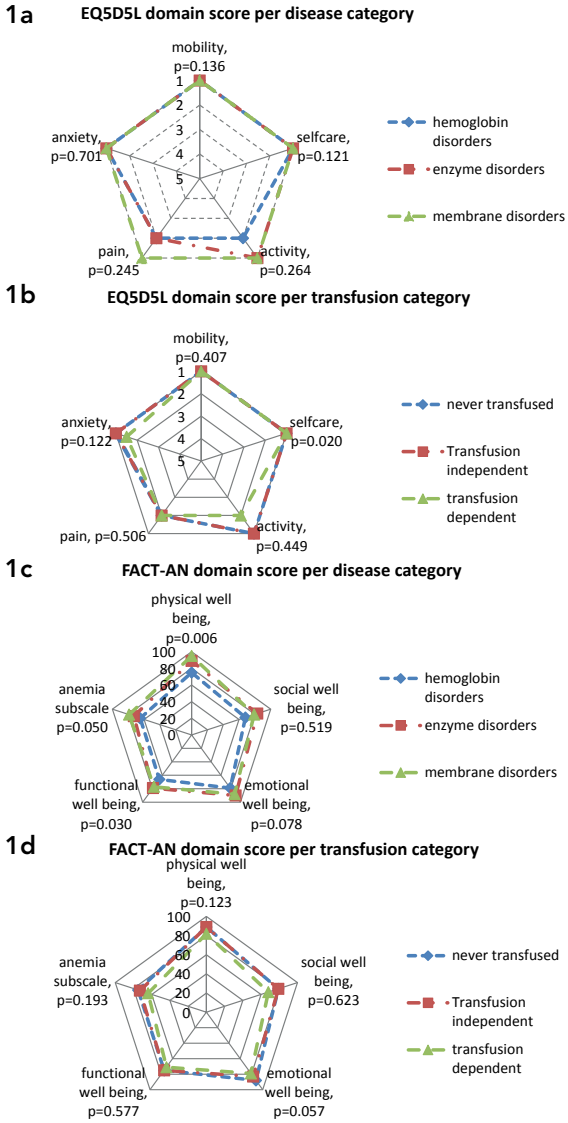

Figure 1. EQ5D5L and FACT-AN domain scores

Figure 1a. Median HR-QoL visualized on an ordinal scale per disease category EQ5D5L scores, from 1 to 5, where 1 indicates no problems and 5 indicates severe problems. P-values represent fisher's exact testing for no problems (score of 1) versus problems (score 2-5)

Figure 1b. Median HR-QoL visualized on an ordinal scale per transfusion category EQ5D5L scores, from 1 to 5, where 1 indicates no problems and 5 indicates severe problems. P-values represent fisher's exact testing for no problems (score of 1) versus problems (score 2-5)

Figure 1c. Median HR-QoL visualized on an ordinal scale per disease category FACT-An scores, represented as percentage from maximum score. P-values represent kruskall-wallis testing.

Figure 1d. Median HR-QoL visualized on an ordinal scale per transfusion category FACT-An scores, represented as percentage from maximum score. P-values represent kruskall-wallis testing.

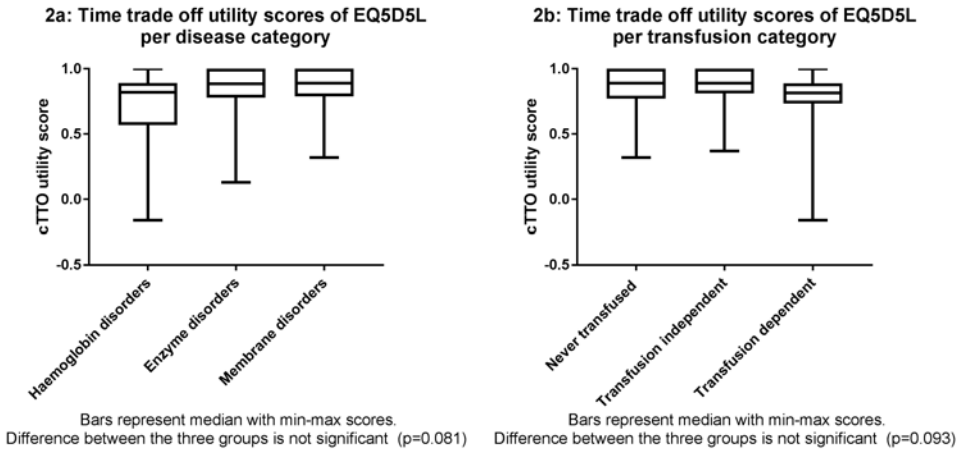

Figure 2. EQ5D5L cTTO utility scores

Table 2. Clinically meaningful differences in FACT-AN scores compared to the general US population

Medians	Hemoglobin disorders	Red cell enzyme disorders	Red cell membrane disorders	Transfusion dependent patients	Transfusion independent patients	Never transfused	All patients
PWB	**-3.5**	0.5	**2.0**	-1.5	0.5	0.5	0.5
SWB	-0.6	**3.6**	**2.6**	-0.6	**2.6**	**2.57**	**2.4**
EWB	**-2.0**	0.5	0.0	**-2.0**	0.0	0.0	-1.0
FWB	-1.1	**2.4**	1.9	0.4	1.4	1.4	1.4
Fact-G	-3.2	**5.6**	**6.1**	**-5.9**	2.1	**5.1**	2.1
FACT-AnS	*52.0*	*58.5*	*63.5*	*51.0*	*60.0*	*60.0*	*59.0*

Differences in scores compared to normative data of normal population. Clinically meaningful differences are marked bold. FACT-AnS: no normative data available, numbers reported are actual FACT-AnS scores.

There was a strong correlation between physical well-being and anemia subscale score (ρ=0.866). The lowest correlation was between social well-being and physical well-being (ρ=0.271) and social well-being and emotional well-being (ρ=0.336, Supplemental table 1b).

Patients who were diagnosed with red cell enzyme disorders, and red cell membrane disorders, but not patients with hemoglobin disorders had a higher social well-being compared to normative data. The same was seen for never transfused and transfusion independent patients, but not for transfusion dependent patients.

Correlation between quality of life and clinical parameters

We performed non-parametric correlation tests between HR-QoL and documented clinical and laboratory parameters, results of the 6-minute walk test (six-minute

walking distance (6MWD), expressed as percentage of predicted distance) and documented organ damage (calculated as number of positive items as a percentage of the total amount of scored items, Table 3). To minimize risk of confounders, patients with transfusion dependency or SCD were excluded from correlation analyses with hematological parameters. There was a negative correlation between SWB and age(ρ=-0.348, p=0.001). There was a positive correlation between the subscales of physical wellbeing (PWB) emotional wellbeing (EWB) and anemia (FACT-AnS) and 6MWD (as percentage of predicted distance, respectively 0.282, 0.236 and 0.257, all p<0.05). There was also a correlation between organ damage and all HR-QoL (sub) scales except emotional wellbeing (See Table 3). There was no correlation between quality of life and Hb, reticulocytes, lactate dehydrogenase, brain natriuretic peptide, ferritin or liver iron content by T2*MRI.

DISCUSSION

Generally, patients with HHA reported a HR-QoL similar to their healthy peers, however, there was a very large range. Patients with hemoglobin disorders, especially patients with β-thalassemia, and patients who were transfusion dependent on average reported a very low HR-QoL, both compared to their healthy peers and their peers with HHA.

In our study patients with HHA on average scored a similar HR-QoL compared to the general US population. Similar results were found in a population of mixed US-based cancer patients.(13, 19) Similar to our population, the cancer study population had a clinically meaningfully higher social domain score. It is conceivable that having a chronic illness simply causes a higher social support demand. However, it is also conceivable that living with a chronic disease leads to normalization of the condition, therefore underestimating the true impact of the disease.(8)

Table 3. correlation between HR-QoL (sub)scales and clinical parameters

Spearman correlation	Age	6 minute walk distance	Organ involvement
PWB	0,09	**0,28***	**-0,23***
SWB	**-0 ,35****	-0,01	**-0,28***
EWB	-0,00	**0,24***	-0,16
FWB	-0,09	0,22	**-0,31****
Fact-G	-0,10	0,22	**-0,30****
FACT-AnS	0,09	**0,26***	**-0,27***
EQ Vas	0,06	0,19	**-0,29***
EQ Utility score	-0,07	0,15	**-0,30****

**p<0,05, **p<0,01, 6 minute walk test: as percentage of predicted, organ damage: as a percentage of the total amount of scored items*

When patients were divided into disease categories, our study shows a slightly worse HR-QoL in patients with hemoglobin disorders compared to the general population, although this did not reach a clinically meaningful difference. Two patients with hemoglobin disorders even scored a EQ5D5L cTTO score of zero or lower, meaning that the Dutch reference population would rather die immediately than having to experience the patient's current quality of life state. This is in contrast with patients with enzyme or membrane disorders, who actually had a clinically meaningful higher score for general quality of life. However, the differences between various disease categories might be influenced by the high number of patients that were transfusion dependent in the hemoglobin disorders group. Also, based on the baseline laboratory parameters depicted in Table 1, one could argue that the membrane disorders group has a relatively mild form of hemolytic anemia.

Patients with hemoglobin disorders, as opposed to patients with enzyme or membrane disorders, did not have a clinically meaningful higher social domain score, compared to the US population. Earlier published research in children with SCD showed a significant association between higher levels of (parent) social support and decreased depressive symptoms and better quality of life.(20) Reports on β-thalassemia patients showed that perceived social support is an important factor influencing reduction of depression and anxiety.(20-22) Therefore, increasing social support in patients with hemoglobin disorders groups might serve as an interesting tool to improve quality of life.

Earlier published research also showed that, although patients with hemoglobin disorders scored lower in all domains, lower quality of life was most pronounced in the physical functioning domain and mainly linked to bodily pain.(23-28) Indeed our hemoglobin disorder patients showed a clinically meaningful lower score in the domain pain of FACT-An, again both compared to the healthy population and their peers with other forms of HHA. Although in EQ5D5L we did not see a significant difference for pain between the groups, the category of hemoglobin disorders did have the highest percentage of patients scoring severe pain (22% versus 3% in the other groups).

Our study identifies a worse quality of life in patients who are transfusion dependent. This may be a reflection of the overrepresentation of patients with hemoglobin disorders in this group or could be merely a reflection of disease burden. Unfortunately, our study was too small to correct for this. However, earlier published research in children with β-thalassemia also showed decreasing quality of life with increasing transfusion frequency.(29) It is conceivable that regular hospital visits could easily interfere with daily life. On the other hand, a study in children with sickle cell anemia found an increase in HR-QoL, due to increased physical health functioning and better overall health, in children randomly assigned to receive regular transfusion compared to children who were assigned to the observation group. (30)

Based on the baseline transfusion and laboratory parameters depicted in Table one, one could argue that the difference in HR-QoL between the hemoglobin disorder

group and the other groups is based, at least partially, on a difference in anemia and therefore disease severity. However, our study does not show a correlation between laboratory parameters and HR-QoL. It does show an interesting correlation between 6MWD and physical, emotional and anemia-related subdomain scores. This suggests that, although a patients' perception of anemia is not always reflected in hemoglobin levels, it does manifest in a patient's functional exercise capacity. Although the correlation between 6MWD and HR-QoL, to our knowledge, is not studied in the general population, similar results are found in patients with pulmonary arterial hypertension.(31, 32) In patients with pulmonary arterial hypertension, 6MWD reflected daily quality of life better than standard hemodynamic parameters.

Our study also shows a correlation between organ involvement and HR-QoL. Several forms of organ involvement have very good treatment options. Possibly, treating the organ involvement might also result in an improvement HR-QoL.

In conclusion, generally HR-QoL in patients with HHA is perceived as similar to their healthy peers. However, especially patients with hemoglobin disorders, patients with hemolysis associated organ involvement and patients that are transfusion dependent have a lower HR-QoL.

This study also confirms that hemoglobin and other laboratory values of hemolysis levels are not necessarily associated with patient perceived HR-QoL in HHA.

Finally, our study clearly shows the importance of social well-being for HR-QoL of patients with HHA. We therefore suggest that treating organ involvement and improving social support could be an interesting future focus to increase HR-QoL, especially transfusion dependent patients or patients with hemoglobin disorders.

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SUPPLEMENTAL DATA

Supplemental table 1a. Correlation between EQ5D5L subscales

Spearman's rho	Mobility	Selfcare	Activity	Pain	Anxiety
Mobility	X	0,54**	0,63**	0,55**	0,46**
Selfcare	0,54**	X	0,45**	0,45**	0,30**
Activity	0,63**	0,45**	X	0,65**	0,42**
Pain	0,52**	0,45**	0,65**	X	0,30**
Anxiety	0,46**	0,30**	0,42**	0,30**	X

***Correlation is significant at the 0.01 level (2-tailed)*

Supplemental table 1b. Correlation between FACT-An questionnaire subscales

Spearman's rho	Physical	Social	Emotional	Functional	Anemia
Physical	X	0,27*	0,67**	0,71**	0,87**
Social	0,27*	X	0,34**	0,59**	0,28**
Emotional	0,67**	0,34**	X	0,60**	0,68**
Functional	0,71**	0,59**	0,60**	X	0,73**
Anemia	0,87**	0,28**	0,70**	0,73**	X

**. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).*

Supplemental table 2. individual EQ5D5L scores per category

	Hemoglobin disorders N=23	enzyme disorders N=30	membrane disorders N=32	p-value#	never transfused N=31	transfusion independent N=23	transfusion dependent N=24	p-value#
Mobility								
No problems	15 (65.2%)	26 (86.7%)	22 (68.8%)	0.14	24 (77.4%)	18 (78.3%)	15 (62.5%)	0.41
Slight problems	4 (17.4%)	3 (10.0%)	7 (21.9%)		6 (19.4%)	3 (13.0%)	5 (20.8%)	
moderate problems	0 (0%)	0 (0%)	3 (9.4%)		1 (3.2%)	1 (4.3%)	0 (0%)	
severe problems	4 (17.4%)	1 (3.3%)	0 (0%)		0 (0%)	1 (4,3%)	4 (16.7%)	
Selfcare								
No problems	19 (82.6%)	29 (96.7%)	31 (96.9%)	0.12	30 (96.8%)	23 (100.0%)	19 (79.2%)	0.02
Slight problems	1 (4.3%)	1 (3.3%)	0 (0%)		0 (0%)	0 (0%)	2 (8.3%)	
moderate problems	1 (4.3%)	0 (0%)	1 (3.1%)		1 (3.2%)	0 (0%)	1 (4.2%)	
severe problems	2 (8.7%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	2 (8.3%)	
Activities								
No problems	9 (39.1%)	18 (60.0%)	19 (59.4%)	0.26	18 (58.1%)	13 (56.5%)	10 (41.7%)	0.45
Slight problems	10 (43.5%)	6 (20.0%)	7 (21.9%)		9 (29.0%)	7 (30.4%)	6 (25.0%)	
moderate problems	2 (8.7%)	5 (16.7%)	5 (15.6%)		4 (12.9%)	2 (8.7%)	5 (20.8%)	
severe problems	2 (8.7%)	1 (3.3%)	1 (3.1%)		0 (0%)	1 (4.3%)	3 (12.5%)	
Pain								
No problems	7 (30.4%)	14 (46.7%)	17 (53.1%)	0.25	15 (48.4%)	11 (47.8%)	8 (33.3%)	0.51
Slight problems	6 (26.1%)	13 (43.3%)	10 (31.3%)		11 (35.5%)	7 (30.4%)	9 (37.5%)	
moderate problems	5 (21.7%)	2 (6.7%)	4 (12.5%)		3 (9.7%)	4 (17.4%)	3 (12.5%)	
severe problems	5 (21.7%)	1 (3.3%)	1 (3.1%)		2 (6.5%)	1 (4.3%)	4 (16.7%)	
Anxiety								
No problems	14 (60.9%)	21 (70.0%)	23 (71.9%)	0.70	22 (71.0%)	18 (78.3%)	12 (50.0%)	0.12
Slight problems	7 (30.4%)	7 (23.3%)	6 (18.8%)		7 (22.6%)	2 (8.7%)	11 (45.8%)	
moderate problems	1 (4.3%)	2 (6.7%)	2 (6.3%)		2 (6.5%)	2 (8.7%)	1 (4.2%)	
severe problems	1 (4.3%)	0 (0%)	1 (3.1%)		0 (0%)	1 (4.3%)	0 (0%)	

Supplemental table 2. (continued)

	Hemoglobin disorders N=23	enzyme disorders N=30	membrane disorders N=32	p-value#	never transfused N=31	transfusion independent N=23	transfusion dependent N=24	p-value#
VAS-score	75(58-80)	75 (68-85)	80 (60-85)	0.57	75 (66-84)	80 (70-85)	75 (59-81)	0.68
Utility score	82(57-89)	89 (78-100)	89 (79-100)	0.08	89 (77-100)	89 (81-100)	82 (74-89)	0.09

p-value: test performed: fisher's exact: problems/no problems for the subscales and kruskal Wallis for the VAS-score and index score.

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3

ORGAN INVOLVEMENT OCCURS IN ALL FORMS OF HEREDITARY HEMOLYTIC ANEMIA

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SUMMARY

In this short report we analyzed organ involvement in 90 patients with hereditary hemolytic anemia. Organ involvement occurs in all forms of hemolytic anemia studied and occurs also in 77% of patients without transfusion history. The development of better screening guidelines is important to identify organ involvement in patients with hereditary hemolytic anemia at an early stage.

INTRODUCTION

Over the past few decades, life expectancy of patients with hereditary hemolytic anemia (HHA) has shown remarkable improvement. The availability of blood transfusions, chelation therapy and other supportive treatment options has substantially improved life expectancy well into adulthood. (1-4) HHA is the number one cause of anemia burden in the high income Western World and due to better survival of patients it is a growing health problem.(5) As an example: sickle cell disease (SCD) already accounts for 300.000 newly diagnosed infants each year globally, and this number is expected to increase to 400.000 in 2050.(6)

HHA encompasses all genetic diseases characterized by premature destruction of red blood cells. (7, 8)

The destruction of red blood cells is caused by various intrinsic defects. These defects can be classified in three main categories: hemoglobin disorders (e.g. β-thalassemia, SCD), red blood cell enzyme disorders (e.g. pyruvate kinase deficiency (PKD), glucose-6-phosphate dehydrogenase deficiency (G6PD)) and red blood cell membrane and hydration disorders (e.g. hereditary spherocytosis, hereditary xerocytosis). Now that patients are getting older, many of them are confronted with chronic organ involvement. In SCD and β-thalassemia the occurrence of disease-related organ involvement is an important determinant of morbidity and prognosis. (1, 2, 4, 9, 10)

However, for the other forms of HHA, not much is known about disease-related organ involvement, especially for non-transfusion dependent patients. The goal of this study was to make an inventory of the occurrence and severity of organ involvement in several forms of HHA. This encompasses (early markers of future) organ damage, markers of altered organ function (e.g. endocrine changes) and other non-hematological symptoms known to influence morbidity and prognosis in chronically ill patients (e.g. inflammation). Furthermore, we analyzed whether organ involvement does also occur in patients without transfusion history. This analysis could be a first step towards determining whether organ involvement screening protocols would be necessary for all patients with HHA.

METHODS

Patient selection and study design

This is a cross-sectional, observational study on patients with HHA. Patients were all participants of the ZEbRA-study (Netherlands Trial Registter (NTR) identifier, NTR5337), a monocenter study conducted in the University Medical Center Utrecht in Utrecht, the Netherlands, focusing on the clinical sequelae and pathophysiology of HHA. The study required a single visit, one blood donation and consent to a chart review. All adult patients with HHA were eligible to enrol. Both patients with and without transfusion history were able to participate in this study.

Definitions and statistical analysis

Data was collected through chart review. Medical history based parameters of organ involvement were scored according to diagnosis by the treating physician. They were regarded as present when mentioned and as absent when not mentioned in the medical history of the patient. (Bio)marker based organ involvement was based on most recent parameters unless otherwise stated (Supplemental table 1) and regarded as missing when absent. For a more detailed description of definitions of each parameter see Supplemental table 1. Only patients with more than 10 items available were included in the analysis.

Continuous variables were expressed as medians and range. Statistical analysis was carried out using non-parametric tests because of the abnormal distribution of some of the data. Nominal data was compared using Mann Whitney U or Kruskall Wallis tests when appropriate. Categorical data was compared using Fisher's exact or Fisher-Freeman-Halton for binomial tables. Statistical significance was considered if $P \leq 0.05$.

RESULTS

Between 2016 and 2017 a total of 100 patients were enrolled in the study. Three patients were excluded because their metabolic diagnosis was still pending at the time of the analysis. Ninety patients had information on organ involvement available (i.e.: more than 10 scoring items available) and were included in the analysis. At enrollment patients had a median age of 40 years (range 18-84) and a median hemoglobin concentration (Hb) of 6.9 mmol/l (range 4.0-10.2 mmol/L), 46 patients (51%) were female. Patients were divided into four disease categories: SCD (15 patients), other hemoglobin disorders (12 β-thalassemia, 4 unstable hemoglobins, 1 HbH-disease variant), red cell enzyme disorders (23 pyruvate kinase deficiency, 4 glucose-6-phosphate-dehydrogenase deficiency, 2 hexokinase deficiency and 1 glutamate cysteine ligase deficiency), and red cell membrane disorder (21 hereditary spherocytosis, 5 hereditary xerocytosis, 2 hereditary elliptocytosis). Baseline characteristics per category are described in Table 1.

Seventy-eight patients (87%) showed at least some form of organ involvement. Patients who did not show any organ involvement, also had significantly less clinical test results in total available than patients who did (median 17 vs 24, $p<0.001$). The most reported forms of organ involvement were iron overload (41/53, 77%) vitamin D deficiency (32/54, 59%) and cholecystectomy (39/90, 43%). Patients who did have organ involvement did not show significant differences in Hb (median 6.8 vs 7.8 mmol/L, $p=0.220$) or reticulocytes (median 236 vs 202 x10^9/L, $p=0.879$) compared to patients who did not have organ involvement.

Of the 31 patients that had never received a red cell transfusion (SCD excluded), 24 (77%) showed at least some form of organ involvement. Most reported forms of

Table 1. baseline characteristics

	sickle cell disease	other hemoglobin disorders	red cell enzyme disorders	red cell membrane disorders
Female	9/15 (60%)	7/17 (41%)	13/30 (43%)	17/28 (61%)
Age (years)	30 (21-60)	30 (18-67)	45 (20-70)	47 (18-84)
Splenectomy	na	10/17 (56%)	16/30 (53%)	10/28 (36%)
Hemoglobin (mmol/L)	6.1 (5.0-8.5)	5.7 (4.2-8.4)	7.0 (4.0-10.1)	8.5 (6.1-10.2)
MCV (fL)	92 (73-128)	80 (43-98)	102 (88-123)	91 (81-109)
Reticulocytes (*10^9/L)	178 (80-382)	143 (36-634)	408 (61-1096)	285 (27-717)
Ferritin (ug/mL)	562 (37-3584)	479 (101-6060)	485 (37-3586)	132 (33-694)
Never transfused	3/15 (20%)	1/16 (6%)	15/30 (50%)	15/28 (54%)
Not transfused last year	6/15 (40%)	5/17 (29%)	21/30 (70%)	27/28 (96%)

numbers are medians (range) (N), or number/total(percentage)
Not transfused last year: patients that did not receive transfusions in last year

organ involvement in never transfused patients were iron overload (65%), vitamin D deficiency (58%), and cholecystectomy (39%, Table 2).

Never-transfused patients who did have organ involvement did not show significant differences in Hb (median 8.2 vs 8.4 mmol/L p=0.755) or reticulocytes (256 vs 173 x10^9/, p=0.600) compared to never-transfused patients who did not have organ involvement.

Organ involvement occurred in all disease categories, but there was a significant difference in number of positive items (as a percentage of the total amount of scored items, p=0.031). Patients with SCD scored a median of 4 positive items out of a median of 24 scored items. For patients with other hemoglobin disorders this was 3/26, for enzyme deficiencies 3/23 and for membrane disorders 1/19 (Table 2).

DISCUSSION

Our study shows that organ involvement occurs in all forms of hereditary hemolytic anemia included in this study and is not exclusive to patient with SCD or β-thalassemia. Notably, many forms of organ involvement were not related to clinical symptoms. This is in line with previously observations in for instance SCD. (11, 12)

Our results emphasize that organ involvement is not limited to frequently transfused patients. We used a very strict cut-off for transfusion dependency, allowing only patients with complete medical history from birth to day of inclusion in the study, who never received a red cell transfusion to be designated as "never transfused". Even in this stringent defined group of patients, 77% showed organ involvement. This is relevant for clinical practice, given that these patients are often considered as mildly affected and might not regularly attend the clinic. SCD patients were excluded from analysis of organ involvement in this group because the distinctive pathophysiology of SCD and the fact that presence of organ damage often is the indication for blood transfusion.

The observational set up of the study makes it impossible to draw conclusions on causality. As an example, the high percentage of vitamin D deficiency identified in this cohort is similar to that found in the healthy Dutch population. (13) However, as our study also identifies osteoporosis in 26% of patients, evaluating vitamin D levels is very relevant in our patients. Further, our data shows that patients with any organ involvement have significantly more clinical test results, such as magnetic resonance imaging, cardiac ultrasound and bone densitometry available. This represents an information bias, but it also indicates that screening for organ involvement does not structurally occur in many patients with HHA. At the same time, our data shows no differences in hemoglobin level between patients with and without organ involvement. In SCD, although genetic and hematological modifiers of disease severity are well described on population level, no specific characteristics to identify a high risk profile for organ involvement has been identified at the patient level.(11, 14) Also

Table 2. organ involvement per disease category

		Dutch population*	Sickle cell disease	Other haemoglobin	Red cell enzyme	Membrane/ hydration	Never transfused (SCD excluded**)
Cardio	Cardiac iron overload	<1%	13% (1/8)	15% (2/13)	0% (0/20)	0% (0/7)	0% (0/13)
Vascular	Increased TRV	3%	0% (0/12)	36% (5/14)	17% (2/12)	0% (0/4)	0% (0/7)
	Increase BNP	5%	13% (2/15)	14% (2/14)	0% (0/22)	18% (2/11)	6% (1/17)
	Heart failure	1%	0% (0/15)	6% (1/17)	0% (0/29)	0% (0/28)	0% (0/31)
	Arrhythmia	2%	7% (1/15)	12% (2/17)	0% (0/29)	7% (2/28)	7% (2/31)
	Hypertension	31%	7% (1/15)	6% (1/17)	7% (2/29)	7% (2/28)	10% (3/31)
	Priapism in males	<1%	67% (4/6)	0% (0/10)	0% (0/16)	0% (0/11)	0% (0/17)
	Thrombotic event	<1%	13% (2/15)	18% (3/17)	10% (3/29)	14% (4/28)	10% (3/31)
Abdominal	Iron overload (liver)	<1%	75% (6/8)	83% (10/12)	68% (15/22)	91% (10/11)	65% (11/17)
	Microalbuminuria	7%	33% (5/15)	42% (5/12)	39% (7/18)	0% (0/8)	17% (2/12)
	Renal failure	4%	0% (0/15)	0% (0/15)	3% (1/26)	4% (1/23)	7% (2/27)
	Cholecystectomy	<1%	27% (4/15)	29% (5/17)	73% (22/30)	29% (8/28)	39% (12/31)
	Liver cirrhosis	<1%	0% (0/14)	6% (1/17)	0% (0/29)	0% (0/28)	0% (0/31)
Neurologic	*CNS hemorrhage*	3%	7% (1/15)	6% (1/17)	0% (0/29)	0% (0/28)	0% (0/31)
	CNS infarction		20% (3/15)	0% (0/17)	0% (0/29)	7% (2/28)	3% (1/31)
	Seizures	1%	7% (1/15)	0% (0/17)	0% (0/29)	0% (0/28)	0% (0/31)
Musculo-	Osteoporosis	3%	8% (1/13)	64% (7/11)	15% (2/13)	20% (1/5)	20% (2/10)
skeletal/ skin	*Fractures*	7%	0% (0/15)	6% (1/16)	0% (0/29)	4% (1/28)	3% (1/31)
	Leg ulceration	<1%	13% (2/15)	12% (2/17)	7% (2/29)	0% (0/28)	0% (0/31)
Endocrine	Low LH males	<1%	0% (0/4)	38% (3/8)	0% (0/8)	0% (0/4)	0% (0/6)
	Low FSH in males	<1%	0% (0/3)	25% (2/8)	0% (0/7)	0% (0/2)	0% (0/5)
	Low testosterone	2%	17% (1/6)	18% (2/11)	14% (1/7)	25% (1/4)	17% (1/6)
	Vitamin D deficiency	49%	67% (10/15)	64% (9/14)	50% (8/16)	56% (5/9)	58% (7/12)
	IGF>-2SD from normal	2%	22% (2/9)	33% (4/12)	43% (6/14)	33% (1/3)	25% (2/8)
	Thyroid disease	3%	8% (1/13)	0% (0/15)	0% (0/18)	12% (2/17)	5% (1/19)
	Diabetes (all types)	6%	7% (1/15)	16% (3/17)	0% (0/29)	4% (1/28)	3% (1/31)

Table 2. (continued)

		Dutch population*	Sickle cell disease	Other haemoglobin	Red cell enzyme	Membrane/ hydration	Never transfused (SCD excluded**)
Inflammatory	Increased CRP	1%	20% (3/15)	6% (1/17)	4% (1/26)	4% (1/23)	4% (1/25)
Audiovisual	Hearing loss	4%	0% (0/2)	13% (1/8)	0% (0/2)	na (0/0)	na (0/0)
	Retinopathy	<1%	46% (6/13)	0% (0/10)	0% (0/2)	0% (0/1)	0% (0/1)
Other	*Fibromyalgia*	2%	0% (0/15)	0% (0/17)	3% (1/29)	7% (2/28)	3% (1/31)
	Depression	3%	13% (2/15)	12% (2/17)	7% (2/29)	0% (0/28)	3% (1/31)

Medical history based parameters *(italic)*: regarded as present when mentioned or performed and as absent when not mentioned in the medical history. Biomarker based parameters (normal font): most recent values were used unless otherwise stated in supplemental table. TRV: tricuspid regurgitant velocity in meter per second, BNP: brain natriuretic peptide, LIC: liver iron content in milligram iron per gram dry weight, CNS: central nervous system, LH: luteinizing hormone, FSH: follicle stimulating hormone, IGF: insulin-like growth factor. CRP: C-reactive protein. * References and information about calculation of disease prevalence in the general Dutch population can be found in the supplemental table. ** SCD patients were excluded because the presence of organ damage often is an indication for blood transfusion.

in our study we were unable to identify a distinct clinical or hematological profile that discriminates patients with organ involvement from those without. Therefore it is important to implement systematic screening guidelines for organ involvement in patients with HHA.

The main goal of this study was to evaluate whether organ involvement occurs in various rare hemolytic anemias and whether it occurs in patients who were never transfused. Because of the design of the study exact prevalence and exclusion of certain types of organ damage in subgroups cannot be calculated. As an example, in our study priapism was only found in sickle cell patients, but there are case reports describing the phenomenon in β-thalassemia as well. (15, 16)

In summary, our study clearly shows that organ involvement is not limited to patients with SCD or β-thalassemia, but also occurs in other forms of HHA. Importantly, organ involvement also occurs in patients who never received blood transfusions.

Following the improvement in survival in hereditary hemolytic anemia, the unfavorable effect of organ involvement on morbidity, mortality and quality of life is an important new concern. Therefore, screening for organ involvement that has a high prevalence and has treatment options available seems prudent. Based on our report we suggest this could include screening for iron overload, osteoporosis, vitamin D deficiency, sex hormone deficiencies and microalbuminuria.

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Supplemental Table 1. definitions of organ involvement

			Estimated prevalence in Dutch population*
Cardiovascular	Cardiac iron overload	T2*MRI cardiac iron assessment of <20ms ((17, 18)	<1%(19, 20)
	Increased TRV	Elevated Tricuspid regurgitant jet flow velocity >2.5 m/s at rest measured with transthoracic ultrasound (9, 21)	2,6%(22)
	Increased BNP	Plasma Brain Natriuretic peptide >30pmol/L	4,5% (13)
	Heart failure	*Medical history based parameter*	1,3% (23)
	Arrhythmia	*Medical history based parameter*	2,4% (24)
	Hypertension	*Medical history based parameter*	31,4% (25)
	Priapism in males	*Medical history based parameter, males only*	<1%(26)
	Thrombotic event	*Medical history based parameter, includes thrombosis, sinus thrombosis, embolism*	<1% (27-29)
Abdominal	Iron overload (liver)	T2*MRI Liver iron content of >3mg Fe/g DW (18, 30-32)	<1% (33)
	Microalbuminuria	Ratio between urinary microalbumin (mg/l) to urinary creatinine (mmol/l) of >3.5 in one urine sample (11)	7% (34)
	Renal failure	Creatinine clearance if <60ml/min/1,73m2 calculated with CKD-EPI without adjustment for ethnicity(35, 36)	3,7% (37)
	Cholecystectomy	*Medical history based parameter*	0,6% (29)
	Liver cirrhosis	*Medical history based parameter*	0,4% (38)
Neurologic	*CNS haemorrhage*	*Medical history based parameter*	2,8% (39)
	CNS infarction	*Medical history based parameter*	
	Seizures	*Medical history based parameter (not related CNS infarction or haemorrhage)*	1% (29)
Musculo-skeletal/ dermatologic	Osteoporosis	(History of) T-score equal to or less than -2.5 in either hip or lumbar spine on dexa scan (17, 18)	2,5%(40)
	Hip or vertebra fracture	*Medical history based parameter*	7,1% (41)
	Leg ulceration	*Medical history based parameter*	<1%(42)
Endocrine	Low LH males	LH in blood in IU/L Because no information on reproductive cycle or oral anticonception are known, data was only interpreted for males: Male: <1IU/L	<1%(43)

Supplemental Table 1. definitions of organ involvement

			Estimated prevalence in Dutch population*
	Low FSH in males	FSH in blood in IU/L Because no information on reproductive cycle or oral anticonception are known, data was only interpreted for males: Male: <1IU/L	<1%(43)
	Low testosterone	Testosterone in blood in nmol/L Testosterone <0,3 in females or <11 in males	2,1(44)
	Vitamin D deficiency	25-OH-vitamin D <50nmol/L in blood	49% (45)
	IGF>-2SD from normal	IGF>-2SD from normal	2,3% (based on SD)
	Thyroid disease	Thyroid disease, based on TSH and FT4 at any timepoint: Hypothyroidism: (TSH>5mU/L, FT4<10pmol/L) subclinical hypothyroidism (TSH>5mU/L, FT4 10-22pmol/L), Hyperthyroidism (TSH<0,35mU/L, FT4>22pmol/L), subclinical hyperthyroidism	3,2% (46)
	Diabetes (all types)	*Medical history based parameter (diabetes type 1, 2, diabetes gravidarum)*	6,4% (47)
Inflammatory	Increased CRP	CRP>10mg/L measured in blood (48)	1%(49)
Audiovisual	Hearing loss	Any hearing loss 15 or more dB in either ear (50)	3,7% (51)
	Retinopathy	Retinal changes on fundoscopy at any timepoint	0,1% (52)
Other	*Fibromyalgia*	*Medical history based parameter*	1,8%(53)
	Depression	*Medical history based parameter*	3,3% (54)

*Estimated prevalence in Dutch population: prevalence from volksgezondheidenzorg.info, NHG.org and Nivel reflects year prevalence as registered in the National Health Care Registry as provided by the Dutch government. Data is based on all registered cases of named diseases at the general practitioners' office. Prevalence from other references is based on published information in the cited articles If no information on Dutch population was available, comparable healthy population was used.

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SCREENING FOR PULMONARY HYPERTENSION IN RARE HEREDITARY HEMOLYTIC ANEMIA

ABSTRACT

Introduction

Pulmonary hypertension (PH) is an uncommon complication of hereditary hemolytic anemia (HHA) and is associated with early death. The prevalence of PH is claimed to be 6-10% in sickle cell disease (SCD), for other forms of HHA information is scarce. Here we evaluated PH in patients with HHA. Secondly, we evaluated whether results of a 6-minute walk test (6MWT) could be an added diagnostic value in the diagnosis of PH.

Methods

This is a cross-sectional, observational study in 100 patients with HHA. Patients were regarded as having PH based on results of right heart catheterization. For triscupid regurgitant jet velocity (TRV) a threshold of ≥2.5 m/s was used.

Results

None of the patients included had a clinical diagnosis of PH. Of the 46 patients that underwent cardiac ultrasound seven (15%) had TRV≥2.5m/s. There was no difference in 6-minute walk distance between patients with and without TRV≥2.5m/s (p=0.888).

Conclusion

In this study we did not identify any patients diagnosed with PH. We did identify elevated TRV in several non-SCD HHA patients. Given the relation of elevated TRV with early death and PH in SCD, we suggest to follow patients with rare HHA that have elevated TRV with the same care.

INTRODUCTION

Pulmonary hypertension (PH) is an uncommon complication of hereditary hemolytic anemia (HHA) and is strongly associated with early death.(1-5) The prevalence of PH is claimed to be 6-10% in sickle cell disease (SCD), but for other forms of HHA information about prevalence is scarce.(6, 7)

In PH increased pulmonary vascular resistance and elevated pulmonary artery pressure are accompanied by restricted blood flow through the pulmonary arterial circulation. The exact cause of PH in HHA is unclear and expected to be multifactorial, including splenectomy, thromboembolisms, increased cardiac output, left heart disease, hyperviscosity and possibly inactivation of nitric oxide by free plasma hemoglobin due to chronic hemolysis, although the latter is controversial.(8-10) In SCD, PH is not purely pulmonary artery hypertension as some individuals also show signs of pulmonary venous hypertension.(11) As a result, in the official PH classification of the World Health Organisation, PH associated with chronic hemolytic anemia, was moved from group 1 (pulmonary artery hypertension) to group 5 (unclear/multifactorial mechanism).(12)

PH, if untreated, eventually leads to right heart failure and ultimately death. The gold standard for diagnosis of PH is right heart catheterization. However, because of the invasiveness if this test, often other, non-invasive screening tools, like Doppler echocardiography to determine triscuspid regurgitant jet velocity (TRV) are preferred, despite their suboptimal correlation with diagnosis. In SCD an elevated TRV is a well-known indicator of possible PH and is associated with increased mortality risk.(13)

Possibly, the accuracy of the non-invasive tests could be improved by using a multimodality approach. In primary PH and SCD this multimodality approach, with prediction models combining echocardiography, laboratory values and the results of a simple 6-minute walk test (6MWT), was already studied and the American Thoracic Society added the 6-minute walking test as a diagnostic tool to their 2014 guidelines for diagnosis of PH in SCD.(6, 13, 14) As an illustration: in SCD the positive predictive value of TRV ≥2.5m/s was reported to be only 25%, combining TRV≥2.5m/s with either NT-pro-BNP>164pg/ml or 6MWT<333 meters improved positive predictive value to 62%.

In this study we evaluated PH in patients with HHA. Secondly, we aimed to evaluate whether 6MWT correlates with signs of heart disease and whether it has an added diagnostic value in the diagnosis of PH.

METHODS

This is a cross-sectional, observational study. Patients were participants of the ZEbRA-study (NTR5337), in the University Medical Center Utrecht, the Netherlands. Adult patients with HHA, with and without transfusion history, were eligible to enrol. All patients who were willing and able performed a 6MWT. The 6MWT was performed

according to the practical guidelines of the ATS statement.(15) Six-minute walk distance was calculated in meters (6MWT in meters) and was analyzed based on percentage of predicted distance according to the equation Tveter et al (6MWD in %), because of good predictability in a wide age range.(16)

Cardiologic data was collected through chart review. Cardiac ultrasound was not part of the ZEbRA-study but was performed at the discretion of the treating physician. Patients were regarded as having pulmonary hypertension based on results of right heart catheterization (elevated mean pulmonary arterial pressure with pressures above 25 mmHg in rest or 30 mmHg during exercise and a normal capillary wedge pressure (≤15 mmHg)). A threshold of 2.5 m/s was used for TRV.(13) If there was no TRV mentioned, but the ultrasound report mentioned "no tricuspid regurgitation", "no tricuspid insufficiency" or "trace of tricuspid regurgitation" TRV was scored as 1.3m/s. For BNP, patients were categorized as BNP<30pmol/L (cardiac strain unlikely), BNP 30-120pmol/L, and BNP>120 (cardiac strain). All procedures followed were in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Statistical analysis was performed using non-parametric tests because of the abnormal distribution of some of the data.

RESULTS

Baseline characteristics

One hundred patients were included in the ZEbRA-study. Fifteen patients were diagnosed with SCD, 18 with other hemoglobin disorders (12 with β-thalassemia, 5 with unstable hemoglobin variants (4 cases of Hb Adana with heterozygous α-thalassemia and 1 case heterozygous for Hb Savanna) 1 with a HbH disease variant), 32 patients with enzyme deficiencies (23 pyruvate kinase deficiency, 6 glucose-6-phosphate dehydrogenase deficiency, 2 hexokinase deficiency, 1 glutamate cysteine ligase deficiency) and 32 with membrane disorders (22 hereditary spherocytosis, 8 hereditary xerocytosis, 2 hereditary elliptocytosis). Three patients did not yet receive a molecular diagnosis at the moment of inclusion and were labeled as hemolytic anemia *e cause ignota*. Patients had a median age of 40 years and 47% was female. Baseline characteristics are depicted in Table 1.

Cardiac stress and pulmonary hypertension

None of the patients included had a clinical diagnosis of PH. Forty six patients, including all patients with SCD included in this study, underwent a cardiac ultrasound. Patients that underwent cardiac ultrasound had lower hemoglobin, erythrocyte, LDH and BNP levels compared to patients who did not (Table 2). Of the 46 patients that underwent cardiac ultrasound seven (15%) had TRV≥2.5m/s. These patients were diagnosed with

Table 1. Baseline characteristics

Baseline characteristics	SCD	Other hemoglobin disorders	Enzyme disorders	Membrane disorders	All patients
Age in years	30 (27-35, N=15)	30 (27-61, N=18)	45 (32-51, N=32)	47 (36-59, N=32)	40 (28-52, N=100)
Gender: female	9/15 60%	7/18 39%	13/32 41%	17/32 53%	47/100 47%
Hb in mmol/L	6.1 (5.4-7.6, N=15)	5.7 (5.2-6.0, N=17)	7.0 (5.4-8.2, N=31)	8.6 (7.6-9.2, N=32)	7.1 (5.7-8.5, N=983.6
Erytrocytes $x10^{12}$/L	2.8 (2.6-3.7, N=15)	3.5 (3.2-3.8, N=16)	3.3 (2.5-3.9, N=31)	4.1 (3.6-4.7, N=32)	(3.0-4.4, N=97)
Reticulocytes $x10^{9}$/L	178 (107-260, N=15)	143 (74-217, N=14)	366 (156-754, N=24)	303 (150-564, N=29)	236 (139-511, N=85)
BNP in pmol/L	15 (6-20, N=13)	17 (9-21, N=14)	9 (3-13, N=19)	13 (3-23, N=10)	11 (6-18, N=57)
LDH	313 (263-393, N=15)	185 (149-297, N=15)	195 (168-332, N=29)	202 (186-264, N=21)	213 (178-321, N=83)
TRV >2.5m/s	0/15 0%	5/14 36%	2/1315%	0/4 0%	7/46 (15%)
6MWT in m	545 (488-615, N=10)	522 (462-603, N=16)	590 (530-640, N=27)	554 (499-600, N=29)	566 (509-610, N=85)
6MWD in %	87 (74-91, N=10)	82 (76-89, N=16)	87 (82-90, N=27)	84 (80-90, N=29)	85,8 (79,0-90,1, N=85)

Numbers depict median (IQR) or numerator/denominator (percentage)
BNP= brain natriuretic peptide, TRV=tricuspid regurgitant jet velocity, 6MWT= six minute walk distance in meters, 6MWD= six minute walk distance as a percentage of predicted distance

β-thalassemia (4), pyruvate kinase deficiency (2) and unstable Hb variant (1). There was no difference in anemia or hemolysis parameters between patients who did or did not have TRV≥2.5 (Table 3). Six out of seven patients were splenectomized. No patients had TRV≥3m/s. None of the patients with TRV≥2.5 were selected by their clinician to undergo further cardiac evaluation through heart catheterization. NT-pro-BNP levels were not available. BNP was available for 64 patients. Six had BNP levels>30pmol/L of which one patient had a BNP-level>120pmol/L. Cardiac ultrasound of this patient did not show TRV≥2.5m/s.

6MWT

Eighty five patients were willing and able to perform 6MWT. Patients had a median 6MWD of 86% of predicted distance (IQR 79-90). There was no difference in 6MWD between patients with sickle cell disease, other hemoglobin disorders, enzyme disorders or membrane disorders (p=0.445).

Of the 85 patients who performed 6MWT, six had TRV≥2.5m/s and four had BNP>30pmol/L. There was no difference in 6MWD between patients with TRV<2.5m/s and patients with TRV≥2.5m/s (See table 3). There was also no difference in 6MWD

Table 2. difference between patients with and without TRV measurements available

	No cardiac US TRV available	Cardiac US TRV available	P-value
Hb in mmol/L	8.2 (7.1-9.1)	6.0 (5.4-7.2)	<0.001
Erytrocytes $x10^{12}$/L	4.1 (3.5-4.7)	3.2 (2.7-3.8)	<0.001
Reticulocytes $x10^{9}$/L	278 (135-564)	193 (119-369)	0.416
BNP in pmol/	6 (3-14)	14 (8-19)	0.009
LDH in U/L	195 (171-257)	255 (185-350)	0.038
6MWT in m	566 (512-618)	550 (506-628)	0.776
6MWD in %	87 (80-91)	84 (77-89)	0.080

Numbers depict median (IQR), test performed; Mann Whitney U

Table 3. difference between patients with and without TRV≥2,5m/s

	TRV<2.5m/s	TRV≥2.5m/s	P-value
Hb in mmol/L	6.0 (5.4-7.6)	5.4 (4.9-6.0)	0.074
Erytrocytes $x10^{12}$/L	3.2 (2.7-4.1)	3.4 (2.4-3.7)	0.398
Reticulocytes $x10^{9}$/L	193 (127-338)	389 (66-972)	0.821
BNP in pmol/	13 (8-18)	16 (8-21)	0.433
LDH	277 (192-350)	185 (149-350)	0.201
6MWT in m	546 (508-603)	617 (456-661)	0.511
6MWD in %	84 (77-88)	84 (70-92)	0.888

Numbers depict median (IQR), test performed; Mann Whitney U

between patients with BNP<30 or ≥30pmol/L. There was no correlation between 6MWD and TRV (p=0.519) but there was an inverse correlation between 6MWD and BNP (ρ=-0.318, p=0.029).

Of the 85 patients who performed 6MWT, one patient had a 6MWT <333m. This patient had to quit the 6MWT early because of symptoms of dizziness. This patient had normal TRV, and BNP was not available.

DISCUSSION

In this study we did not identify any patients diagnosed with PH. Because there were no patients diagnosed with PH, we could not test the added diagnostic value of 6MWT. However, none of our patients met both of the criteria based on the earlier used cut-off points in SCD (TRV≥2.5m/s ánd 6MWT<333m).

Based on the percentage of PH in SCD described in literature of 6-10%, we did expect to identify at least one patient diagnosed with PH. It is possible that, because patients were not evaluated by heart catheterization and not all patients underwent cardiac ultrasound, the diagnosis of PH was missed. Patients that were not screened with cardiac ultrasound were less anemic and showed lower levels of hemolysis. In SCD the combination of TRV≥2.5m/s and 6MWT<333m is associated with increased risk of PH. (14) None of the patients in our cohort met these criteria.

Even though our study did not contain patients diagnosed with PH, published research reported a prevalence of PH as diagnosed by heart catheterization in β-thalassemia of 2.1% (17) A recently published study in PKD identified eight patients clinically diagnosed with PH out of the 237 patients studied. These numbers imply an increased risk for PH in other forms of HHA besides SCD as well and therefore warrants screening.

Our study contained seven patients with TRV≥2.5m/s. None of these patients was diagnosed with SCD. The cut-off suggestive for PH in non-anemic subjects is TRV>3.0m/s.(18) However, what constitutes increased PH is different in SCD-related PH compared with other forms of PH because patients with SCD have anemia-induced elevation of their cardiac output and reduction in their blood viscosity, which result in a lower baseline pulmonary vascular resistance than observed in non-anemic patients.(19) We reason that the same hemodynamic changes are present in other patients with anemia as well. We therefore suggest that patients with rare HHA and TRV≥2.5m/s should be followed with more attention to heart failure than normal routine requires. In SCD, the American Thoracic Society only advises to perform right heart catheterization in patients with TRV between 2.5 and 2.9m/s if they have symptoms of PH, an elevated NT-pro-BNP or a decreased 6MWT. Although the American thoracic Society does not specify the cut-off for 6MWT, their recommendations regarding 6MWT are based on the published research suggesting a cut-off of 333m. In absence of any evidence in rare HHA we suggested to follow these guidelines for patients with rare HHA as well.

In this study we chose to not only evaluate 6MWT in meters, but to also analyze 6MWD as a percentage of predicted distance. This is different from the approach that was used previously for SCD.(14) For future research we suggest to use 6MWD as a percentage of predicted distance instead of 6MWT in meters because it compensates for age, length, weight and gender of the patient, making it more sensitive and precise.(16)

In conclusion, although our study did not contain any patients diagnosed with PH, we did identify elevated TRV in several non-SCD HHA patients. As more evidence on the connection between HHA and PH is emerging, and because of the possible life threatening consequences of the disease, we suggest to screen for PH in patients with HHA and follow those with increased TRV with more attention.

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II

FROM CLINICAL SYMPTOMS TO PATHOPHYSIOLOGY

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PHOSPHATIDYLSERINE-EXPOSING EXTRACELLULAR VESICLES MIGHT INCREASE THE PRO-COAGULANT POTENTIAL OF BLOOD PLASMA AFTER SPLENECTOMY IN HEREDITARY HEMOLYTIC ANEMIA

ABSTRACT

Background

Thrombosis is a common complication of hereditary hemolytic anemia (HHA). Etiology involves hemolysis, inflammation and splenectomy, although their intertwined contribution to thrombosis is not yet fully determined. In the present study, we hypothesized that increased concentrations of phosphatidylserine (PS) exposing extracellular vesicles (EVs) in splenectomized patients contribute to a hypercoagulable state. Therefore, we analyzed the origin and coagulant activity of EVs in various forms of HHA.

Methods

In this cross-sectional, observational study we studied concentration of PS-exposing EVs and coagulant activity of EVs in splenectomized and non-splenectomized adult patients with HHA (sickle cell disease, other hemoglobin disorders, red cell enzyme disorders, red cell membrane disorders). To determine origin, cluster of differentiation markers were measured with a dedicated flow cytometer for EVs. Coagulant activity of EVs was studied by a fibrin generation test (FGT). Clotting time (CT) V1/2max<1,500s was regarded as fibrin generation.

Results

Ninety-seven patients were included. Splenectomized patients had more PS-exposing EVs compared to non-splenectomized patients (317 versus $173x10^6$/mL, $p<0.001$). Patients who had CT<1,500s had higher concentrations of PS-exposing EVs compared to patients with CT≥1,500 (376 vs $189x10^6$/ml, <0,001). There was a strong inverse correlation between the concentration of PS-exposing EVs and CT (Spearman-ρ=-0.631, $p<0.001$).

Conclusion

Our results suggest that increased concentrations of PS-exposing EVs in splenectomized patients might contribute to the increased thrombotic risk after splenectomy in patients with HHA.

INTRODUCTION

Hereditary hemolytic anemia (HHA) is globally one of the top causes of years lived with disability and due to better survival of patients it is an increasing health problem.(1-3) Apart from stem cell transplantation and gene therapy, HHA does not have a curative treatment option. Therefore, treatment is mainly supportive, including regular red blood cell transfusions, chelation therapy for iron overload and splenectomy.(4, 5) Splenectomy is performed to manage anemia, based on the evidence that defected or damaged red cells that pass through the spleen are removed by the splenic macrophages.(5) However, in HHA, both ongoing hemolysis and splenectomy are associated with an increased risk of early and late venous and arterial thrombosis.(5-8) As an example: a review published in 2008 showed that 11/89 patients with HHA developed splenic or portal vein thrombosis after splenectomy, versus 0/122 trauma patients and 2/118 auto-immune thrombocytopenia patients.(9) However, the intertwined contributions of splenectomy and ongoing hemolysis to thrombosis are not yet fully determined.(8)

Circulating cell derived, membrane enclosed extracellular vesicles (EVs) may contribute to the hypercoagulable state in HHA and other diseases. (10-15) Patients with HHA are reported to have an increased concentration of EVs, compared to healthy controls.(10, 16-19) After splenectomy, even higher concentrations of EVs are present, of which phosphatidylserine (PS) exposing EVs are particularly interesting with regards to the hypercoagulable state.(12, 18) Normally, the negatively charged PS is present only in the inner layer of the plasma membrane. However, both sickle cell disease (SCD) and thalassemia are characterized by abnormal exposure of PS.(20-23) The exposure of PS serves as an apoptotic signal for splenic macrophages, but is also a cofactor for coagulation by serving as a pro-coagulant surface that acts to orient coagulation proteases. (24, 25).

Possibly, in HHA splenectomy reduces the efficacy of clearance of the PS-exposing EVs, resulting in higher concentrations of circulating PS-exposing EVs, which may promote coagulation and contribute to hypercoagulation in HHA. In this study, we measured the concentration of PS-exposing EVs in splenectomized and non-splenectomized patients with HHA, and studied their procoagulant phenotype in an *in vitro* plasma clotting assay.

METHODS

This is a cross-sectional study on patients with HHA. Patients were all participants of the ZEbRA-study (NTR5337), a monocenter study conducted in the University Medical Center Utrecht in Utrecht, the Netherlands, focusing on the clinical sequelae and pathophysiology of HHA. The study required a single visit, one blood donation and consent to a chart review. All adult patients with HHA were eligible to enrol. Both patients with and without transfusion history and with or without splenectomy were

able to participate in this study. Because autosplenectomy is highly prevalent in SCD, SCD patients were all regarded as splenectomized.(26)

Blood collection and sample preparation

Blood samples were collected with a 21-gauge butterfly needle (Vacuette) without use of a tourniquet and collected in 9 mL citrate phosphate dextrose adenine (CPDA) vacutainers (Vacuette). The tubes were mixed gently and centrifuged twice (2,000G, 10 minutes and 4,000g, 10 minutes) at room temperature to obtain platelet depleted plasma and aliquots were frozen in safe lock vials at -80 °C until use. The time between blood collection and centrifugation was maximum one hour.

Fluorescent labeling of EVs

EVs were stained with antibodies against CD14 (monocytes, allophycocyanin (APC) labeled, clone 61D3, eBioscience San Diego, California), CD61 (platelets, APC, clone Y2/51, Dako Denmark A/S, Glostrup, Denmark), CD62e (E-selectin, marker of endothelial activation, phycoerythrin (PE) labelled, clone 68-5H11, BDbiosciences, Franklin Lakes, New Jersey), CD62p (P-selectin, marker of platelet activation, PE, clone CLB-thromb/6, Beckman-Coulter, Woerden, The Netherlands), CD71 (reticulocytes, PE, clone CY1G4 Biolegend, San Diego, California), CD144 (endothelial cells, APC, clone 16B1 eBioscience, San Diego, California), CD235a (erythrocytes, PE, clone JC159, Dako, Santa Clara, California). In addition, EVs were stained with fluorescein isothiocyanate (FITC) labelled lactadherin (Haematologic Technologies Incorporated Essex Junction, Vermont) to detect PS-exposing EVs. Isotyping was performed with IgG1-APC, IgG1-PE and IgG2a-PE (clone MOPC-21, X40 and clone X39 BDbioscience).

Flow cytometry

Flow cytometry was performed with a dedicated flow cytometer for EVs (A60-micro, Apogee Flow sysems Hemel Hempstead, UK), during 120 seconds at a flow rate of 3,01ul/min, gated on Small Angle Light Scatter and the respective gates (638 D-Red, 488-Orange, 488-Green, lower limit of detection 170-180 nm single EVs). To avoid swarm detection, samples were diluted to achieve an event rate of maximum 5000 events/s.

D-dimer

A routine quantitative D-dimer latex immunoassay (STA® -Liatest® D-Di Plus, Diagnostisca Stago, Asnieres-sur-Seine, France) was used for D-dimer measurements. D-dimer was interpreted as a continuous variable. However, as the small population of the data and the abnormal distribution did not allow us to correct for age, we also tested D-dimer as a dichotomous variable, with a discriminant value of 0.5ug/mL for

patients younger than 50, or a reference value of age in years/100 for patients >50 years old.

Coagulant activity measurements

The coagulant properties of EVs were determined in the fibrin generation test (FGT). (11, 27) Briefly, after recalcification of patients' platelet-depleted plasma, time to fibrin formation (clotting time (CT) in seconds (s), 1/2max) is measured based on turbidity during one hour using optical densitometry (λ = 405 nm). A clotting time (cut off of V1/2max) of <1,500 s was used to consider the FGT as "positive". Samples with >25% difference between duplicates or from patients using anticoagulant medication were excluded from analysis.

C1 esterase inhibitor enzyme complex measurements

C1 esterase inhibitor (C1inh) enzyme complex capture ELISA using 5ug/mL 1B12 capture nanobody were performed as described elsewhere.(28)

Statistical analysis

For non-parametric measurements of FGT, samples that did not clot in one hour were scored as 3,600s (the maximum time of the assay). As a sensitivity test, correlation tests between FGT and other parameters was performed both with patients that did not clot scored as 3,600 and with patients that did not clot excluded.

Continuous variables were expressed as medians and interquartile range (IQR, presented here as Q1-Q3). Statistical analysis was carried out with use of non-parametric tests because of the abnormal distribution. Correlation was performed using Spearman's correlation test. Nominal data was compared using Mann Whitney U or Kruskall Wallis tests when appropriate. Statistical significance was considered as $P \leq 0.05$.

RESULTS

Between 2016 and 2017, a total of 100 patients were included. Patients were diagnosed with SCD (N=15), other hemoglobin disorders (β-thalassemia, N=12, unstable hemoglobin variants, N=5 (4 Hb Adana with heterozygous α-thalassemia and 1 heterozygous Hb Savanna), HbH-disease variant, N=1), red cell enzyme disorders (pyruvate kinase deficiency, N=23, glucose-6-phosphate dehydrogynase deficiency, N=6, hexokinase deficiency, N=2, glutamate cysteine ligase deficiency, N=1) and membrane disorders (hereditary spherocytosis, N=22, hereditary xerocytosis, N=8, hereditary elliptocytosis, N=2). Three patients were excluded because molecular diagnosis was pending at the moment of analysis. At baseline, patients had a median age of 40 years (IQR 18-84), 46 patients (47%) were female, 52 (54%) were splenectomized and 34 patients (35%) had never received red cell

transfusions. Patients' median Hb was 7 mmol/L (IQR 5.7-8.4), reticulocytes 232x10^9/L (IQR140-507) and platelets 330x10^9/L (IQR 232-498). Baseline characteristics per disease category are depicted in Table 1.

Concentration and origin of EVs

Median concentrations of EVs in HHA are shown in Table 2. A median of 246x10^6/mL EVs exposed PS (Table 2). There was a difference in PS exposure between disease categories.

In the total patient population, the bulk of EVs originated from reticulocytes (CD71+), followed by those originating from platelets (CD61+) and red blood cells (CD235a+). All EV types showed a positive correlation with PS-exposing EVs ($p<0.001$). EVs originating from platelets showed the highest correlation (Spearman-ρ=0.683).

Concentration of EVs after splenectomy

The concentration of EVs in splenectomized and non-splenectomized patients is shown in Table 3, Figure 1. In the total group, splenectomized patients had more PS-exposing EVs compared to non-splenectomized patients (317 vs 173x10^6/ml, $p<0.001$). As a sensitivity analysis, analysis was repeated excluding patients with SCD: also then, splenectomized patients had more PS-exposing EVs compared to non-splenectomized patients (305 vs 173x10^6/ml, $p<0.001$). When the subgroups were analyzed separately, the difference in PS-exposing EVs was significant for hemoglobin disorders (p=0,001) and enzyme disorders (p=<0.001), but not for membrane disorders (p=0.267).

Splenectomized patients also had increased concentrations of EVs originating from platelets (CD61+, $p<0.001$) and from red cells (CD235a+) and reticulocytes (CD71+, both $p<0.01$, but not from endothelium (CD144+) and monocytes (CD14+, Table 3). When the subgroups were analyzed separately, in samples of patients with enzyme disorders, splenectomized patients had increased concentrations of EVs derived from reticulocytes (CD71+, $p<0.001$) platelets (CD61+, p=0.019) and red cells (CD235a+, p=0.032). In patients with membrane disorders splenectomized patients had increased concentrations of EVs derived from reticulocytes (CD71+, p=0.009). In hemoglobin disorders, there was no difference (Figure 1).

D-dimer

D-dimer was measured as a marker of *in vivo* coagulation activation. D-dimer measurements were available for 92 patients. Twenty patients (22%) had elevated D-dimer levels. Of these patients twelve had SCD, five had another hemoglobin disorder, none had an enzyme disorder and three had a membrane disorder. PS-exposing EVs correlated with D-dimer (Spearman-ρ 0.335, p=0.001). Patients who had abnormal D-dimer levels had increased concentrations of PS-exposing EVs

Table 1. Baseline characteristics

		Sickle cell disease (N=15)		Other hemoglobin disorders (N=18)		Red cell enzyme disorders (N=32)		Red cell membrane disorders (N=32)	
Age (years)		30	(27-35)	30	(27-61)	45	(32-51)	47	(36-59)
Female gender		9/15	(60%)	7/18	(39%)	13/32	(41%)	17/32	(53%)
Splenectomy		NA		10/18	(56%)	16/32	(50%)	11/32	(34%)
Transfusion	never	3/14	(21%)	1/17	(6%)	15/30	(50%)	15/28	(54%)
	sporadically	3/14	(21%)	4/17	(24%)	6/30	(20%)	12/28	(43%)
	dependent	8/14	(57%)	12/17	(71%)	9/30	(30%)	1/28	(4%)
Hemoglobin (mmol/L)		6.1	(5.4-7.6)	5.7	(5.2-6.0)	7.0	(5.4-8.2)	8.6	(7.6-9.2)
Reticulocytes (E+09/L)		178	(107-260)	143	(74-217)	365	(156-754)	303	(150-564)
Platelets (E+09/L)		385	(318-481)	508	(184-767)	334	(253-721)	248	(191-407)
Leukocytes (E+09/L)		7.8	(5.2-13.3)	10.0	(6.7-13.7)	8.3	(6.2-10.0)	6.6	(5.9-9.7)

Numbers represent median (IQR), or numerator/denominator (percentage)

Table 2. Cellular origin and PS exposure of EVs in plasma per disease category

	All patients		Sickle cell disease		Other hemoglobin disorders		Enzyme deficiencies		Membrane deficiencies		P-value
PS exposing EVs#	246.0	(172.0-341.5)	343.0	(279.0-463.0)	284.0	(174.5-360.5)	247.0	(180.8-369.5)	190.5	(139.5-241.5)	0.000
Reticulocyte EVs	147.0	(42.1-454.3)	374.0	(107.0-665.0)	333.0	(186.1-584.5)	212.5	(36.5-856.5)	52.9	(19.3-179.0)	0.000
Platelet EVs	91.0	(67.6-132.5)	91.0	(81.3-134.0)	132.0	(83.4-156.5)	98.7	(69.5-130.0)	79.5	(49.4-98.7)	0.030
Red cell EVs	87.2	(53.3-112.5)	95.3	(75.0-117.0)	101.5	(81.8-181.5)	55.4	(38.4-86.2)	96.4	(60.1-115.0)	0.001
Endothelium EVs	9.8	(4.9-19.1)	7.5	(3.8-13.7)	14.4	(5.2-21.5)	12.4	(6.4-20.8)	6.9	(4.3-15.7)	0.161
Monocyte EVs	8.1[#]	(4.2-16.3)	6.3	(4.2-11.5)	12.5	(4.1-18.3)	9.7	(5.8-19.8)	7.2	(3.9-14.3)	0.494

#Numbers represent median (IQR) in E+06/mL, test performed: Kruskal-Wallis H

Table 3. Differences in vesicle concentration related to splenectomy and fibrin generation

	PS exposing EVs	Reticulocyte EVs	Platelet EVs	Red cell EVs	Endothelium EVs	Monocyte EVs
Splenectomy						
yes	316.5 (247.8-402.5)	326.0 (80.8-665.0)	101.6 (82.4-146.0)	95.0 (65.9-152.0)	11.3 (4.4-17.4)	8.3 (4.9-17.8)
no	173.0 (130.0-220.0)	69.6 (34.6-246.5)	75.0 (49.7-105.0)	72.6 (41.4-101)	7.4 (5.1-20.6)	8.1 (4.0-15.0)
P-value	*0.000*	*0.001*	*0.000*	*0.001*	*0.646*	*0.675*
FGT						
CT<1,500 s	376.0 (310.5-582.0)	561.0 (241.0-1540.0)	126.0 (96.6-297.0)	113.0 (80.4-287.0)	15.1 (4.8-24.1)	10.8 (5.2-21.8)
CT≥1,500 s	189.0 (136.0-266.0)	60.6 (32.8-340.0)	76.1 (50.3-117.0)	77.9 (44.9-101.0)	7.3 (4.8-20.0)	7.6 (4.0-14.9)
P-value	*0.000*	*0.000*	*0.000*	*0.002*	*0.245*	*0.194*

#Numbers represent median (IQR) in E+06/mL, test performed:Mann-whitney U

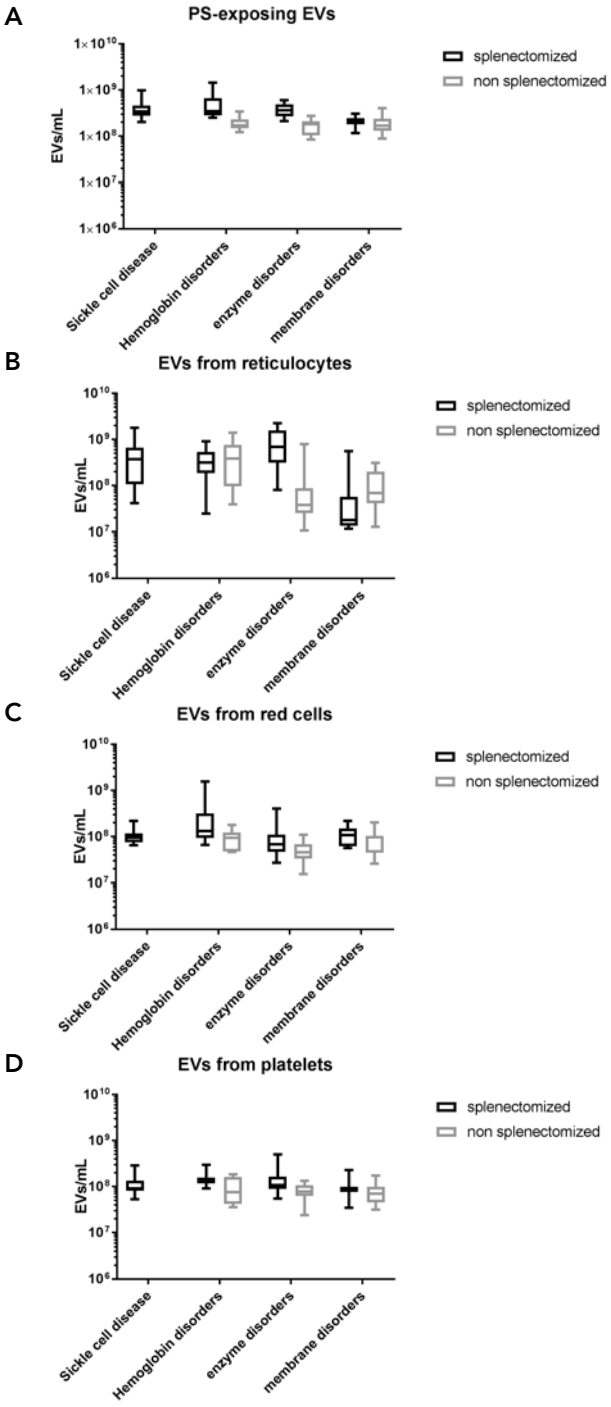

Figure 1. concentration of EVs with and without splenectomy per disease category. Black represents splenectomized and grey non-splenectomized patients. SCD patients were all regarded as splenectomized.

compared to patients who had normal D-dimer levels (264x10^6/mL versus 212 x10^6/mL p=0.021).

Fibrin generation test

FGT results of 60 patients were available for analysis. Results are shown in Table 3 and Figure 2. Thirteen patients had CT<1,500 s. These patients were diagnosed with SCD (2), other hemoglobin disorders (3), enzyme disorders (6) and membrane disorders (2).

There was a strong inverse correlation between PS-exposing EV concentration and CT (Spearman-ρ=-0.631, p<0.001 patients who did not clot were excluded from analysis. As a sensitivity analysis, test was repeated with patients who did not clot scored as 3,600s (Spearman-ρ=-0.579, p<0.001, Figure 3). Patients who had CT<1,500 s had higher concentrations of PS-exposing EVs, than patients with CT≥1,500s (Table 3). When the subgroups were analyzed, the difference was significant for hemoglobin disorders (N=12, p=0.009) and enzyme disorders (N=22, p<0.001), but not for SCD (N=5, p=0.800) or membrane disorders (N=21, p=0.152). In line with the results from splenectomized patients, patients with a CT<1,500s had increased concentrations of EVs originating from reticulocytes (CD71+), platelets (CD61+) and red cells (CD235a+), compared to patients with CT>1,500s (all p<0.05), but not from monocytes (CD14+) and endothelial cells(CD144+).

In the 13 samples with CT<1,500s, the median CT was 865s (IQR 792-1139). Only one sample had CT <600s. This was also the patient with the highest concentration of PS-exposing EVs (1450x10^6/ml, Figure 3). Eleven out of these 13 patients (85%) were splenectomized, versus 14 patients (30%) in the FGT-negative group (p=0.001).

C1-inhibitor complex assays

The relatively long CT could argue for FXII consumption through ex vivo contact activation. Therefore, we measured factor XIIa-C1inhibitor complexes and plasma kallikrein-C1inhibitor as a reflection of recent contact activation *in vivo* in a selected sample of 26 patients (data not shown).(29) Results were interpolated to a reference curve of contact-activated plasma. In none of the patients there were increased complexes measured.

DISCUSSION

In this study, we showed that in HHA, splenectomized patients have increased concentrations of circulating PS-exposing EVs, compared to non-splenectomized HHA patients. The concentration of PS-exposing EVs correlates with coagulation activation *in vivo* (as represented by D-dimer levels) and with clotting time *in vitro*. As the spleen is the main organ removing PS-exposing cells, higher levels of PS-exposing EVs in splenectomized patients may be due to reduced clearance, which in turn may contribute to the increased risk of thrombosis in patients after splenectomy.(30)

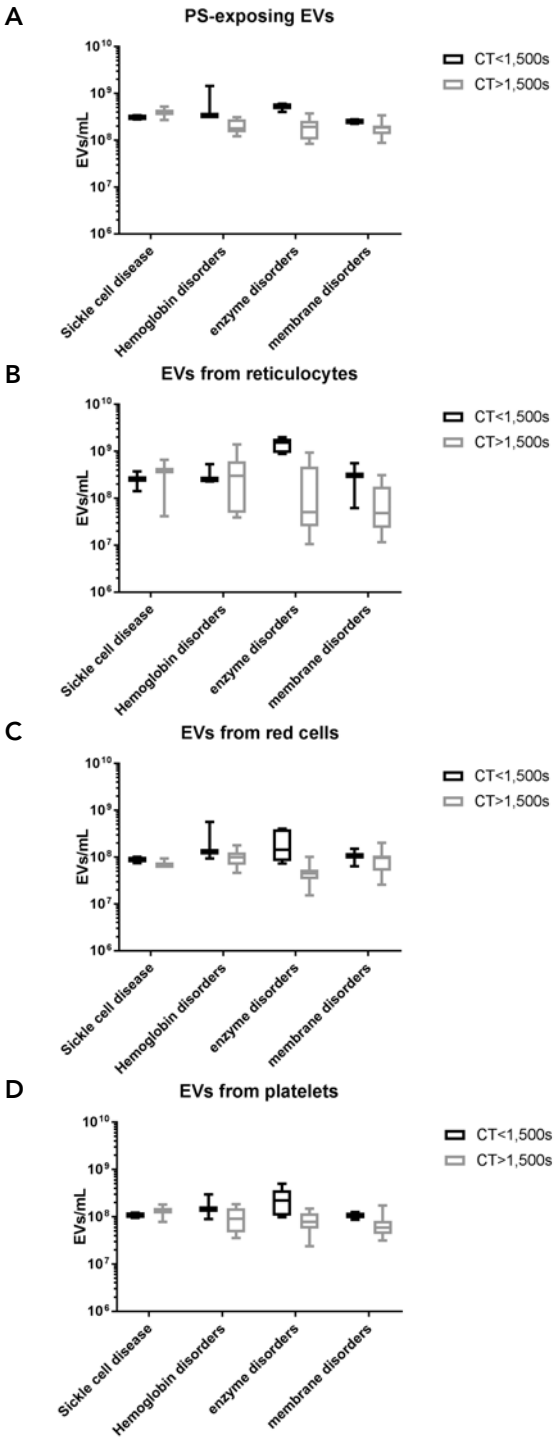

Figure 2. concentration of EVs with CT<1,500 and >1,500 per disease category. Black represents CT<1,500 s and grey CT>1,500 s.

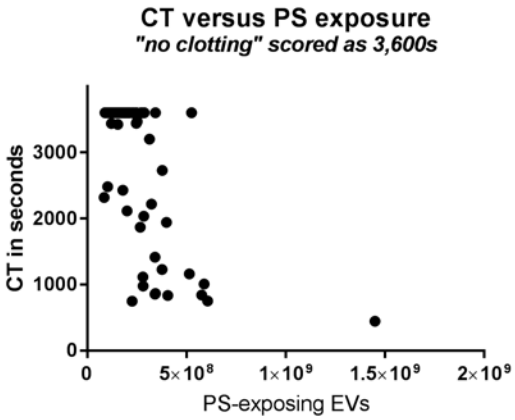

Figure 3. Correlation between PS-exposing EVs and fibrin generation.

Several studies in SCD, cancer and healthy donors suggested EV-mediated coagulation to be caused by tissue factor (TF) expression on EVs, however other studies in SCD and other forms of HHA were unable to identify TF-positive EVs. (10, 11, 31, 32) Some investigators suggested a role for EVs in contact activation, and pointed out low plasma levels of factor XII, pre-kallikrein and high molecular weight kininogen in SCD as a sign of consumption through on-going contact activation.(10, 33-35) The role of the EV-TF mediated hypercoagulability in patients is suggested to be caused by TF exposure on EVs originating form monocytes and endothelial cells.(31, 32) In our study, EVs originating from monocytes and endothelial cells represented the smallest population of EVs in our patients. There was no difference in concentration of EVs originating from monocytes and endothelial cells between patients who did and did not show fibrin generation *in vitro* or between patients that did or did not have a splenectomy. Also, in our C1-esterase inhibitor complex assay, we did not see any signs of on-going contact activation.

To further establish the role of EV-TF mediated coagulation, we performed a fibrin generation test. This in-house assay is especially developed to study pro-coagulant effect of EVs exposing TF, as, unlike a standard thrombin generation assay, the coagulation cascade is not started by adding TF as a trigger. Earlier research with this assay with plasma from healthy controls containing EVs showed a CT of 1274±197s and plasma completely depleted from EVs showed CT>3600s. Therefore, in this assay we used a very conservative cut-off for CT of 1,500 s to be regarded as positive.(27, 36) Based on earlier research and published results of platelet depleted plasma spiked with EVs originating from lipopolysaccharide stimulated monocytes from healthy donors and the amount of EVs identified in our population, we expected EV-TF mediated coagulation in our experiments to occur consistently and within the first 600s.(32)

In our study, fibrin generation was relatively slow and inconsistent, a pattern that is generally accepted not to be consistent with any functional TF present in the assay and likely is caused by contact activation with an *ex vivo* trigger. Interestingly, the one patient with CT<600s was also the patient with the highest concentration of PS-exposing EVs. Taken together, the relatively slow and inconsistent CT, the correlation between CT and PS-exposing EVs suggest that PS-exposing EVs seem to be able to mediate hypercoagulability by facilitating coagulation propagation, rather than initiating coagulation directly in HHA.

Although PS-exposing EVs might play a role in creating a pro-coagulant state by serving as a pro-coagulant surface, PS-exposure on EVs alone is not enough to fully explain hypercoagulability. Especially our membrane disorders patients, of which hereditary xerocytosis patients are known to have very high thrombosis risk after splenectomy, actually did not show an increase in PS-exposing EVs after splenectomy. (7, 37-41) This fits with earlier research showing that in hereditary spherocytosis and elliptocytosis there is no abnormal exposure of PS on red cells, as was seen in SCD and β-thalassemia.(42-44)

In conclusion, our study suggests a possible role for PS-exposing EVs serving as a pro-coagulant surface after splenectomy. This could contribute to the increased thrombotic risk after splenectomy in patients with HHA.

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6

ACQUIRED DECREASED STABILITY OF RED CELL PYRUVATE KINASE IN SICKLE CELL DISEASE

ABSTRACT

Background

Sickle cell disease (SCD) represents the top cause of years lost to disability by anemia in Western Europe and North America. Although the pathophysiology is complex and multifactorial, increased oxidative stress is an important feature of pathophysiology. We postulate that in SCD, increased levels of oxidative stress cause an acquired form of pyruvate kinase (PK) deficiency, characterized by impaired stability of the PK-enzyme, which could explain high levels of 2,3-diphosphoglycerate found in these patients.

Methods

PK thermal stability testing was performed by heating red cell lysates at 53'C. To mimic oxidative stress, two samples were incubated with tert-butylhydroperoxide (tBHP) prior to thermal stability measurements.

Results

Patients with SCD, showed marked decreased thermal stability of PK after heating to 53'C compared to healthy controls. Incubating red cell lysates of a healthy control and SCD-patient with tBHP reduced thermal stability in a dose dependent way. In two patients with unstable hemoglobin variants and in two patients with glutathione cycle defects, there was also decreased thermal stability.

Conclusion

Our pilot study showed that patients with SCD do show reduced *ex vivo* thermal stability. Similar results in unstable hemoglobin variants and glutathione-cycle defects suggest a role for oxidative stress and impaired anti-oxidant defense in the pathophysiology of this phenomenon. This theory is strengthened by the finding that the oxidizing agent tBHP could reduce thermal stability both in a SCD-patient and in a healthy control.

INTRODUCTION

Sickle cell disease (SCD) represents the top cause of years lost to disability by anemia in Western Europe and North America.(1) Apart from stem cell transplantation and experimental gene therapy, currently there are no curative treatments for SCD.

The pathophysiology of SCD involves polymerization of tense (T-state) deoxygenated sickle hemoglobin (HbS), leading to the formation of polymers and rigid sickle shaped red cells.

Therefore, inhibition of polymerization by increasing the proportion of high oxygen affinity relaxed (R-state) HbS or destabilizing low oxygen affinity T-state HbS is an important treatment strategy in the field of SCD.(2-4) HbS has a reduced oxygen affinity compared to normal hemoglobin.(5-7) The low oxygen affinity is caused at least partially by elevated levels of red cell 2,3-diphopshoglycerate (2,3-DPG), a glycolytic intermediate. 2,3-DPG modulates the allosteric equilibrium of hemoglobin by preferentially stabilizing Hb towards a T-state, thereby shifting the oxygen binding curve to the right, producing low-affinity Hb that readily releases oxygen to the tissues.(4-12) However, the pathophysiology behind elevated levels of 2,3-DPG in SCD is not yet fully established. As increased 2,3-DPG levels make HbS more prone to polymerization, lowering of 2,3-DPG levels in SCD has been a hot topic for the last years.(9, 13-16)

Synthesis of 2,3-DPG is controlled in the phosphoglycerate cycle of the Rapoport-Luebering shunt, which is a bypass in the Embden Meyerhof pathway.(17) The Embden Meyerhof pathway is the main route for the red cell to generate energy, as mature red cells lack mitochondria and therefore are dependent on anaerobic glycolysis. A metabolic block in the Embden Meyerhof pathway below the level of the Rapoport-Luebering shunt is shown to lead to increased levels of 2,3DPG by retrograde accumulation through the Embden Meyerhof pathway. (17, 18) This is evident in the disease pyruvate kinase (PK) deficiency. PK is the last and rate limiting step of the Embden Meyerhof pathway and as a result, in PK-deficiency the two major abnormalities are a reduction in ATP and an increase of 2,3 DPG. In PK-deficiency this is believed to be beneficial for the tolerance of anemia, since oxygen is more readily transferred to the tissues.(18)

In red cells, under influence of red cell O_2 content, energy metabolism either proceeds through the Embden Meyerhof pathway, or through the alternate hexose monophosphate shunt, (also called the pentose phosphate pathway).(19-21) The main goal of the hexose monophosphate shunt is to create NADPH, which is required to generate sufficient amounts of reduced glutathione (GSH). GSH is the red cell's major defense mechanism against oxidative stress. Sickle red cells, are shown to have a constrained hexose monophosphate flux. This leads to less NADPH and glutathione recycling, leaving the cell more prone to oxidative stress.(20) In SCD, there is already a baseline state of oxidative stress, due to auto-oxidation of hemoglobin

and enhanced NADPH-oxidase activity.(22-26) Importantly, the PK-enzyme is highly susceptible to oxidative stress, and glutathione plays an essential role in protecting PK-activity. (15, 24, 27).

We postulate that in SCD, oxidative stress can cause an acquired form of PK-deficiency, characterized by impaired stability of the PK-enzyme, leading to an accumulation of 2,3-DPG. In this pilot study, we aim to explore the stability of PK in SCD. Furthermore, we aim to test our hypothesis by performing *in vitro* oxidative stress experiments and by studying PK-stability in patients with hemolytic mutations disrupting GSH homeostasis.

METHODS

This is a pilot study conducted in the University Medical Center Utrecht, in Utrecht, The Netherlands, as part of the TaPIR-study (NTR6462). After informed consent, residual material from diagnostic venipunctures of patients was collected. Results were compared to samples of healthy controls.

Sample collection and enzymatic testing

The blood samples provided were collected in ethylenediaminetetraacetic acid (EDTA) tubes.

PK-stability was determined by measuring *ex vivo* thermal stability according to standard methods.(28-30) In short: purified red cells were isolated from whole blood samples to create packed red cells and hemolysates were created and diluted in a β-mercapto-stabilizing solution. The hemolysates were incubated at 53'C for 0, 5, 10, 20, 40 and 60 minutes. After dilution in a tris-HCl based buffer and the addition of appropriate substrates, the decrease in optical density, created by the oxidation of NADH to NAD+ was measured at 340nm. The decrease in PK-activity is expressed as residual activity as a percentage of activity at time point T=0 minutes.

For the oxidative stress mimicking experiment red cells were incubated with tert-butylhydroperoxide (tBHP) diluted with Ringer buffer for 30 minutes at room temperature. After this, cells were washed, diluted in β-mercapto-stabilizing solution and used for the thermal stability measurements.

RESULTS

We tested twelve samples of SCD patients (ten patients homozygous HbSS (one of which had a heterozygous alpha(+)thalassemia), one heterozygous HbS-beta(+), one heterozygous HbS-beta(+) combined with a heterozygous alpha(+)thalassemia) and seven control samples. Baseline characteristics of the SCD patients are depicted in Table 1.

Thermal stability testing was performed according to protocol. Previous research in healthy controls showed that PK can be expected to be very stable under these

Table 1. baseline characteristics

	Sickle cell disease (N=12)
Age (years)	26 (14-31)
Gender: female	6/12 (50%)
Percentage HbF	15.5 (4.6-24.6)
Percentage HbS	59.8 (54.2-77.6)
Transfused (last 12 months)	7/11 (64%)
Crises (last 12 months)	4/11 (36%)
Hydrea use	6/12 (50%)
Hb (mmol/L)	6.1 (4.9-6.9)
Erytrocytes ($x10^{12}$/L)	2.7 (2.6-3.7)
Leuko ($x10^{9}$/L)	11.2 (7.7-14.4)
Platelets ($x10^{9}$/L)	350 (277-533)
Retics ($x10^{9}$/L)	255 (147-316)
MCV (fL)	93 (78-113)

Numbers represent median (IQR) or numerator/denominator (percentage)

conditions, with a median residual activity of 70-75%. Meanwhile patients with PK-deficiency can show markedly decreased stability of PK in these conditions, with a large range of residual activity, from as low as 5% residual activity, to 95% after 60 minutes.(31).

Control samples showed a slight decrease in residual activity in the thermal stability test, with a median residual activity after 60 minutes of 73%, range 66-78% (Figure 1a).

In the samples of our SCD patients, several patients showed a marked decreased stability of PK, with a median residual activity after 60 minutes of 49%. There was a large inter sample variation, with a range 17-71% (Figure 1b).

In vitro effects of oxidative stress on PK thermal stability

In order to explore whether oxidative stress has a causal relation to decreased PK-stability we performed *in vitro* oxidative stress experiments. Purified red cells of one SCD patient and one healthy control were incubated with two concentrations of tBHP to mimic oxidative stress (Figure 2). In both samples, incubation with 1mM tBHP resulted in a marked decrease of residual activity at T=60 minutes compared to the residual activity without tBHP. Incubation with 1.5mM tBHP resulted in an almost complete loss of residual activity (control: T=60min, tBHP 0mM: 78%, tBHP 1mM: 35% and tBHP 1.5mM: 1%, SCD: 43%, 17% and 3% respectively).

Disorders of Hb auto-oxidation and impaired oxidative stress defense

In order to further explore the role of intracellular oxidative stress we analyzed PK thermal stability in two patients with unstable Hb variants (Hb Volga, Hb

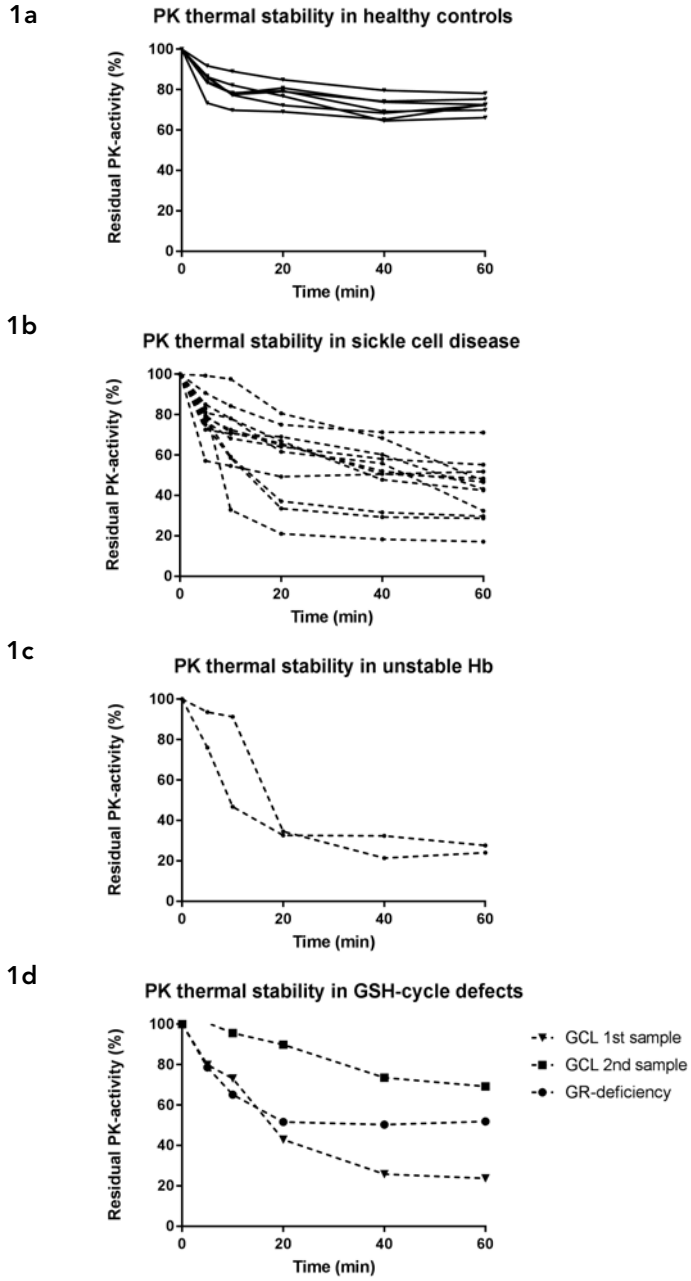

Figure 1. PK thermal stability

Figure 1a. PK thermal stability in healthy controls. Lines depict individual samples

Figure 1b. PK thermal stability in sickle cell disease. Lines depict individual samples

Figure 1c. PK thermal stability in unstable Hb. Lines depict individual samples

Figure 1d. PK thermal stability in GSH-cycle defects. Dashed line with triangular marks and dashed line with square marks represent the same GCL-deficient patient, measured at two different timepoints. Dashed line with circular marks represent a GR-deficient patient

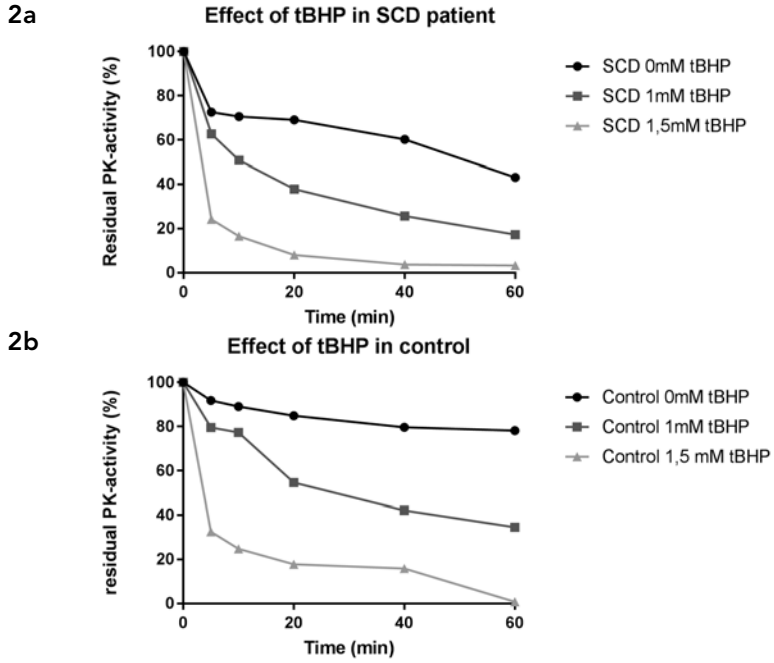

Figure 2. PK thermal stability after incubation with tert-butylhydroperoxide
Figure 2a. effect of tBHP in one SCD patient. Lines depict samples of one SCD patient incubated with several concentrations of tBHP
Figure 2b. effect of tBHP in one healthy control. Lines depict samples of one healthy control incubated with several concentrations of tBHP

Hammersmith). Both variants are characterized by low oxygen affinity and high rate of auto-oxidation.(32, 33) Both patients were anemic (Hb Volga patient: Hb 6.7 mmol/L, Hb Hammersmith patient: Hb 4.2 mmol/L). The two samples of patients with unstable Hb, showed an even more profound pattern of lost residual activity after heating, as is depicted in Figure 1c. After 60 minutes, the residual activity was 27% for the Hb Volga patient and 24% for the Hb Hammersmith patient.

In order to study whether impaired oxidative stress defense due to impaired functioning of the GSH-cycle is related to PK thermal stability we analyzed PK thermal stability in two patients with a glutathione cycle defect. One patient had a glutamate cysteine ligase deficiency (GCL-deficiency) and one patient had a glutathione reductase deficiency (GR-deficiency). The GCL-deficient patient had a fully compensated anemia (Hb 8.6 mmol/L, reticulocytes 328x10^9/L, The GR-deficient patient did not have anemia (Hb 7.74, reticulocytes 65 x10^9/L).

Both patients also showed a profound pattern of lost residual activity after heating (T=60min: 24% in GCL-deficiency and 52% in GR deficiency, Figure 1d). Notably, PK stability of the GCL-deficient patient as measured on a second sample, obtained one month after the first sample, was normal (69%).

DISCUSSION

This study showed a marked reduced *ex vivo* thermal stability of PK in patients with SCD, suggesting that a secondary or acquired form of PK-deficiency is present in SCD red cells. This could explain the increased levels of 2,3-DPG in SCD red cells, similar to PKD red cells.

Although we cannot exclude the possibility of an inherited PK-gene mutation in the included patients, the decrease in PK-stability is likely caused by increased levels of oxidative stress. We showed that oxidative stress, created by incubation of red cells with tBHP *ex vivo*, could reduce thermal stability in a dose dependent manner in both a SCD patient and a healthy control.

To strengthen our theory we performed experiments in patients with GSH-cycle defects. The fact that these patients too showed decreased PK thermal stability suggests that acquired PK-deficiency could indeed be related to inadequate oxidative stress defense through the GSH-cycle. These results are supported by published research describing the effect of oxidized glutathione on pyruvate kinase thermal stability of healthy volunteers.(34) In this publication, incubating isolated red blood cells with oxidized glutathione, markedly decreased PK thermal stability. Adding reduced glutathione partially restored thermal stability and incubation with 1mM β-mercapto-ethanol restored stability almost completely.

In this light, also earlier published findings of acquired PK-deficiency in alcoholics are very interesting. Patients with a longstanding history of alcohol abuse are thought to be at risk for the very rare Zieve's syndrome, which is defined as a triad of hemolytic anemia, hypertriglyceridemia and jaundice. These patients temporarily show decreased PK thermal stability.(35, 36) It is proposed that decreased PK-stability in these patients would be a result of alcohol induced vitamin E deficiency causing oxidation of reduced erythrocyte glutathione in combination with altered erythrocyte membrane lipid composition.(36) Vitamin E is found to also be decreased in patients with SCD.(37) Paradoxically, in a reduced milieu, vitamin E and GSH work together as antioxidants and in a situation of more reactive oxygen species production a supplementation of either GSH or vitamin E can protect the cell.(38) However, during severe oxidative stress, both vitamin E and GSH can display pro-oxidative action and accelerate oxidative damage by reacting with traces of transition metal ions, eventually leading to apoptosis or necrosis of the cell. This might explain why supplementation of vitamin E in sickle cell anemia was actually found to increase instead of decrease markers of anemia. (37)

The fact that not all patients show the same level of decreased PK thermal stability, and the difference in results between the first and second measurement of the GCL-deficient patient, suggest that acquired decreased PK-stability might be an intermittent or dynamic phenomenon. This is in line with findings in the abovementioned Zieve's syndrome, as most patients recover after a few weeks of alcohol withdrawal.(35, 36)

Interestingly, during the first measurement, due to a presumably unrelated medical condition, the GCL-deficient patient tested was in a state of prolonged starvation with consequently large weight loss. During the second measurement, patients health condition had significantly improved and the patient was gaining weight. In patients with anorexia nervosa, high levels of oxidative stress are reported, that improve after oral re-alimentation, even without full weight normalization.(39, 40)

The results of this pilot study are encouraging, especially because recently the first drug to improve PK-functioning was developed. First *ex vivo* results in PKD patients are promising.(41, 42) Interestingly, the phase one study showed decreased 2,3-DPG and increased ATP levels in healthy volunteers(43). This is likely due to increased glycolytic flux caused by enhanced activity of PK. This would be especially relevant for SCD patients, because decreasing 2,3-DPG is an important treatment goal. However, as SCD patients already appear to have a decreased hexose monophosphate flux, necessary for anti-oxidant defense, it is also conceivable that interfering with PK, and thereby possibly shifting metabolism through glycolysis would be unfavorable.

At the same time, preliminary experiments with the drug in murine β-thalassemia showed an increase in ATP and decrease in 2,3-DPG, together with an increase in red cell lifespan and subsequent amelioration of anemia.(44) This is the first evidence suggesting that the drug could have the potential to improve anemia in non-PKD patients.

Further investigations of this phenomenon are necessary, and will be performed in the TApIR-study (NTR6462). In this study we aim to investigate PK-activity and PK thermal stability in hemoglobin disorders, and in other forms of non-PK hereditary hemolytic anemia. In this study we will also measure other intermediates of the Embden Meyerhof pathway, hexose monophosphate shunt and GSH-cycle and ATP levels. We will further investigate the role of oxidative stress as a cause of decreased PK thermal stability by use of oxidative stress mimicking experiments. Lastly we aim to investigate the possibility of stimulation of PK-activity and thermal stability by use of allosteric activators.

In conclusion, our pilot study showed that patients with SCD show reduced *ex vivo* thermal stability. Similar results in unstable hemoglobin variants and GSH-cycle defects suggest a role for oxidative stress and impaired anti-oxidant defense in the pathophysiology of this phenomenon. This theory is strengthened by the finding that the oxidizing agent tBHP could reduce thermal stability both in SCD-patients and in healthy controls. Altogether, these findings not only support the possibility of acquired PK-deficiency in SCD, but also suggest a role for oxidative stress and antioxidant defense in the genesis of acquired PK-deficiency.

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III

FROM PATHOPHYSIOLOGY TO TREATMENT STRATEGIES

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IRON OVERLOAD IN PATIENTS WITH RARE HEREDITARY HEMOLYTIC ANEMIA: EVIDENCE BASED SUGGESTION ON WHOM AND HOW TO SCREEN

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ABSTRACT

Introduction

Iron overload is an important complication in rare hereditary hemolytic anemia. Research on iron overload to date has mainly focused on β-thalassemia. Evidence on the prevalence of iron overload in rare forms of hereditary hemolytic anemia such as red cell enzyme, membrane, and hemoglobin disorders other than β-thalassemia, is lacking.

Methods

We performed a cross-sectional analysis of iron overload in a cohort of 44 patients with rare hereditary hemolytic anemia which included: pyruvate kinase deficiency, G6PD deficiency, hereditary spherocytosis, hereditary xerocytosis, HbH-disease and unstable hemoglobins. As reference, findings were compared to a group of 86 patients with sickle cell disease and β-thalassemia.

Results

Iron overload (liver iron concentration (LIC)≥3 mg/g dry weight liver on MRI) was present in 28/44 patients, moderate to severe liver iron overload (LIC≥7 mg/g) was present in 16/44 patients. Importantly, LIC≥3 mg/g was found in 12/17 of patients who never received red cell transfusions. The sensitivity of ferritin>1000 ng/ml for LIC≥7 mg/g was only 47%. At a cut-off of 500 ng/ml this increased to 100% in both transfused and never-transfused patients. In the reference group, sensitivity of ferritin>500 ng/ml ór transferrin saturation (TSAT) of 45% for LIC≥7 was also 100%.

Conclusion

Iron overload occurs in all forms of rare hereditary hemolytic anemia studied, even without transfusion history. The traditionally used ferritin cut-off of 1000 ng/ml has a poor sensitivity for iron overload in rare hereditary hemolytic anemia and in patients with β-thalassemia and sickle cell disease. We suggest that all, possibly except those with ferritin below 500 ng/ml ánd TSAT<45%, should be evaluated for iron overload with MRI.

INTRODUCTION

Hereditary hemolytic anemia encompasses a heterogeneous group of diseases characterized by premature destruction of the red cell. (1, 2) It can roughly be divided into three disease categories: hemoglobin disorders (e.g. β-thalassemia, sickle cell disease (SCD)), red blood cell enzyme disorders (e.g. pyruvate kinase deficiency (PKD), glucose-6-phosphate dehydrogenase (G6PD) deficiency), and red blood cell membrane disorders (e.g. hereditary spherocytosis, hereditary xerocytosis).

Patients with hereditary hemolytic anemia are at risk for iron overload either due to episodic or chronic blood transfusion or due to inappropriately high dietary iron absorption as a result of ineffective and increased erythropoiesis. (3) The notable exception is SCD, in which intravascular hemolysis provides a potential mechanism for iron elimination through increased urinary or biliary excretion. (4, 5) Excess iron can be stored in the liver, endocrine organs, and heart. Exposure of these organs to the toxic effects of iron causes organ damage and subsequent morbidity and mortality.

Iron overload has been extensively studied in β-thalassemia.(6) In particular, cardiac iron overload has demonstrated to be responsible for mortality in patients with transfusion dependent β-thalassemia (TDT) while severe hepatic iron overload is frequently found in non-transfusion dependent β-thalassemia (NTDT).(7, 8) However, data on the prevalence of iron overload in more rare forms of hereditary hemolytic anemia is limited to descriptive reports and case reports on PKD, hereditary spherocytosis and G6PD-deficiency. (9-25) No data are available on iron overload in hexokinase deficiency.(24, 25) Therefore, most guidelines available on the diagnosis of iron overload in hereditary hemolytic anemia are based on experience in TDT and NTDT. (26-29)

In this study, we aim to evaluate the occurrence of iron overload in patients with rare hereditary hemolytic anemia, and to determine the predictive value of ferritin and transferrin saturation (TSAT) levels to diagnose liver iron overload.

METHODS

Patient selection and study design

This cross-sectional study included patients who were treated at three Dutch expert centers for rare anemias (Haga Hospital, The Hague, Amsterdam University Medical Center, and University Medical Center, Utrecht). All adult patients (18 years and older) with rare hereditary hemolytic anemia from who MRI (T2* or R2) results of the liver were available were eligible for enrollment. Patients with SCD and β-thalassemia in whom MRI-results of the liver were available were enrolled as a reference group.

Most patients in Utrecht were enrolled as part of the ZEBRA-study (NTR5337). In the other centers data was collected from chart review only. Data included information on most recent radiographic and laboratory iron overload parameters, laboratory inflammation parameters, and transfusion and chelation regimen.

Definitions and statistical analysis

We compared the predictive value of ferritin levels for liver iron overload measured by MRI (T2* or R2 MRI (Ferriscan)). The MRI threshold for liver iron overload was defined as LIC ≥3 milligram ferritin per gram dry weight (mg/g) and for moderate to severe iron overload of LIC ≥7 mg/g.(30, 31) If patients had LIC levels exceeding the maximum measurable level of 19.6 mg/g they were considered as having LIC 19.6 mg/g.

The T2*MRI threshold for cardiac iron overload was defined as a cardiac T2* result of <20ms.(32)

To compare ferritin with LIC, we used the ferritin level closest to MRI date with a maximum time interval of 120 days. For this comparison we used the standard ferritin cut-off of 1000 ng/ml as suggested by many guidelines and several lower cut-off values.(31)

To analyze the differences between transfused and not transfused patients, very strict transfusion thresholds were used. Only patients whose complete medical history was known, who never had a single red cell transfusion were categorized as never transfused. Patients who did receive transfusions in the past but did not receive any red cell transfusions in the 12 months prior to MRI, or patients whose complete transfusion history was not available, but did not receive any red cell transfusions in the 12 months prior to MRI were categorized as sporadically transfused. Patients who received one or more transfusions in the last 12 months were regarded as transfusion dependent. One rare hereditary hemolytic anemia patient and four reference group patients whose recent transfusion history were not known were excluded from the transfusion analyses._

Continuous variables were expressed as medians and interquartile range (IQR, presented as Q1-Q3). Statistical analysis was carried out with use of non-parametric tests because of the abnormal distribution. Correlation was performed using Spearman's correlation test. Nominal data was compared using Mann Whitney U or Kruskall Wallis tests when appropriate. Categorical data was compared using Fisher's exact for binomial tables. Statistical significance was considered as P≤0.05.

RESULTS

Baseline characteristics

From February 2016 to June 2017 we included 44 patients with rare hereditary hemolytic anemia. Median age at enrollment was 43 years (IQR 32-51) and 20 patients (46%) were female. Seventeen patients (40%) had never received red cell transfusions. Median Hb was 7.0 mmol/L (IQR 5.5-8.6) and median reticulocyte number was $309x10^9$/L (IQR 159-645). As a reference group, we included 86 patients with SCD and β-thalassemia. Baseline characteristics per disease category are depicted in Table 1 and Supplemental table 1.

Table 1. Baseline characteristics per subgroup of rare hereditary hemolytic anemia

	Rare enzyme disorders				Rare membrane disorders				Rare hemoglobin disorders	
	Pyruvate kinase deficiency (N=17)		Other enzyme disorders[1](N=7)		Hereditary spherocytosis (N=12)		Hereditary xerocytosis (N=2)		HbH disease/ unstable Hb (N=6)	
Female	9/17	(53%)	1/7	(14%)	5/12	(42%)	1/2	(50%)	4/6	(67%)
Age in years	42	(27-47)	49	(44-54)	40	(35-56)	59	(na)	32	(26-49)
Hb in mmol/L	5.8	(5.1-7.5)	8.2	(6.0-9.1)	8.8	(7.2-9.7)	8.5	(na)	5.0	(4.6-5.8)
Retic x10^9/L	601	(256-930)	234	(109-676)	238	(115-431)	597	(na)	238	(124-541)
Direct bilirubin in umol/L	10	(9-14)	9	(8-12)	7	(5-11)	6	(na)	7	(6-12)
Total bilirubin in umol/L	64	(48-88)	85	(31-134)	25	(14-59)	28	(na)	45	(23-246)
Plasma ferritin in ng/ml	553	(305-977)	834	(185-1371)	437	(146-494)	539	(na)	391	(123-1564)
Transferrin saturation in %	50	(33-78)	27	(26-68)	32	(22-53)	49	(na)	37	(na)
LIC in mg/g	5.3	(2.5-10.6)	9.0	(3.1-14.6)	4.3	(2.6-6.4)	7.8	(na)	5.9	(1.3-14.6)
Percentage never transfused	7/16	44%	3/7	43%	5/12	42%	2/2	100%	0/6	0%

Data expressed as counts (within group percentage) or medians (IQR)
1: Group of "other enzyme disorders" contains patients with: G6PD deficiency (4), hexokinase deficiency (2), glutamate cysteine ligase deficiency (1).
na: not applicable

Iron overload in rare hereditary hemolytic anemia based on MRI LIC

LIC ≥3 mg/g on MRI was present in 71% (31/44) of patients with rare hereditary hemolytic anemia. LIC ≥7 mg/g was present in 36% (16/44) of patients and occurred in all forms of rare hereditary hemolytic anemia included (Table 2). Three patients had LIC values ≥15 mg/g of which two had LIC values exceeding the maximum measurable value (19.6 mg/g).

Seventy one percent (12/17) of patients who had never received a red cell transfusion did have LIC ≥3 mg/g. Eighteen percent (3/17) had LIC ≥7 mg/g. Of the patients who were sporadically transfused 65% (11/17) had LIC ≥3 mg/g and 41% (7/17) had LIC ≥7 mg/g. Of the transfusion dependent patients this was 89% (8/9) and 67% (6/9).

Patients with LIC ≥7 mg/g differed significantly from patients with LIC<7 mg/g. They were significantly more anemic and levels of plasma iron, plasma ferritin, and TSAT were higher(data not shown).

Correlation between ferritin and LIC in rare hereditary hemolytic anemia

Of the 44 patients with rare hereditary hemolytic anemia that underwent MRI, 40 had ferritin levels measured in the same period of time. Ferritin levels correlated significantly to LIC in patients with rare hereditary hemolytic anemia (ρ=0.832, p=<0.001, Figure 1a) Correlation was also seen within the subgroup of patients that were never transfused (ρ=0.700, p=0.004), were sporadically transfused (ρ=0.945, p=<0.001), and transfusion dependent (ρ=0.882, p=0.002, Figure 1b). Despite this strong correlation, there was a poor sensitivity of ferritin levels>1000 ng/ml for LIC ≥7 mg/g (47%, Table 3). Twenty four percent (8/33) of patients with ferritin<1000 ng/ml did have LIC ≥7 mg/g. Sensitivity for LIC ≥7 mg/g in never-transfused, sporadically transfused and transfusion dependent was respectively 0%, 43%, and 67%.

In order to rule out a possible confounding effect of chelation therapy, sensitivity analysis was repeated in patients who did not receive any chelation therapy or phlebotomy for iron removal in the 12 months prior to MRI and in never transfused patients who did not receive any chelation therapy or phlebotomy in the 12 months prior to MRI. Again sensitivity was poor (sensitivity of 0% in both groups). Decreasing the ferritin cut-off to 800 ng/ml showed little improvement, but decreasing it to 500 ng/ml increased the sensitivity of ferritin for LIC ≥7 mg/g to 100% in all groups (table 3).

Correlation between ferritin and LIC in sickle cell disease and β-thalassemia

To validate the results, analysis was repeated in the reference group (Supplemental table 2). Of the 86 patients that underwent MRI, 72 had ferritin levels measured in the same period of time. A correlation between ferritin and LIC was seen in the whole group (ρ =0.856, p=<0.001), sporadically transfused (ρ=0.629, p=0.009), and

Table 2. Iron overload per patient group of rare hereditary hemolytic anemia

		Rare enzyme disorders				Rare membrane disorders				Rare hemoglobin disorders	
		Pyruvate kinase deficiency		Other enzyme disorders		Hereditary spherocytosis		Hereditary xerocytosis		HbH disease/ unstable Hb	
Never transfused	Ferritin ≥1000 ng/ml	0/6	(0%)	0/3	(0%)	0/5	(0%)	0/1	(0%)	-	
	LIC ≥3 mg/g	4/7	(57%)	2/3	(67%)	4/5	(80%)	2/2	(100%)	-	
	LIC ≥7 mg/g	2/7	(29%)	0/3	(0%)	0/5	(0%)	1/2	(50%)	-	
Sporadically tranfused	Ferritin ≥1000 ng/ml	0/4	(0%)	2/3	(67%)	0/4	(0%)	-		1/4	(25%)
	LIC ≥3 mg/g	2/4	(50%)	3/3	(100%)	4/6	(67%)	-		2/4	(50%)
	LIC ≥7 mg/g	1/4	(25%)	3/3	(100%)	1/6	(17%)	-		2/4	(50%)
Transfusion dependent	Ferritin ≥1000 ng/ml	3/5	(60%)	0/1	(0%)	0/1	(0%)	-		1/2	(50%)
	LIC ≥3 mg/g	5/5	(100%)	1/1	(100%)	1/1	(100%)	-		1/2	(50%)
	LIC ≥7 mg/g	4/5	(80%)	1/1	(100%)	0/1	(0%)	-		1/2	(50%)

Numbers are counts (within group percentage);
-: not applicable

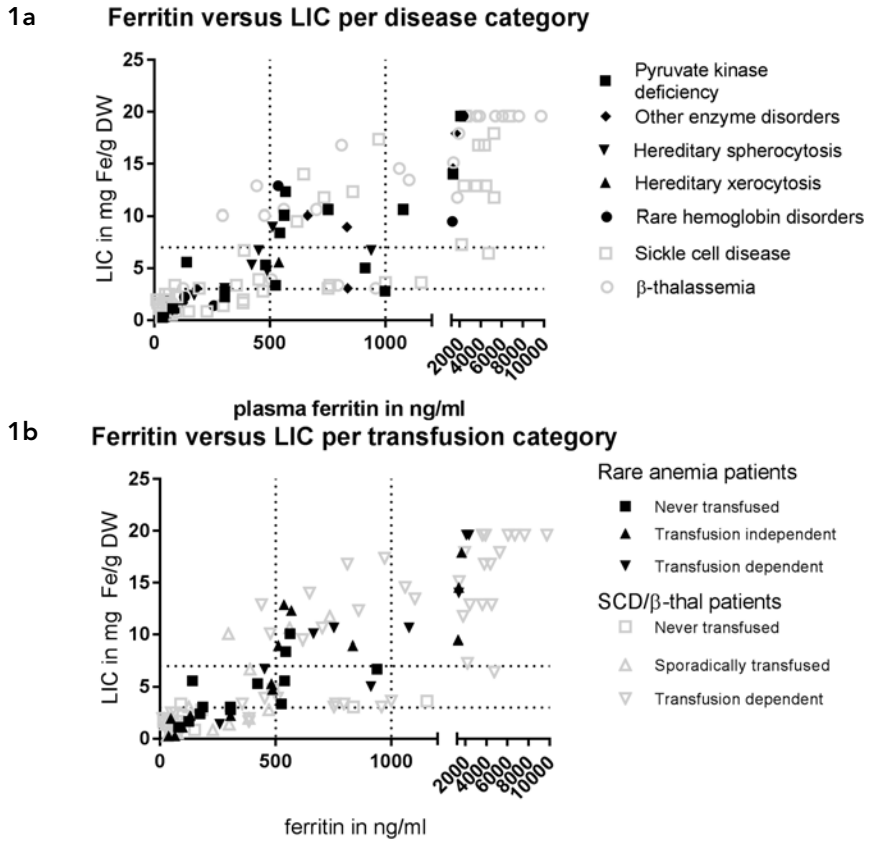

Figure 1. ferritin versus LIC
Figure 1a. ferritin versus LIC per disease category, 1b: ferritin versus LIC per transfusion category

transfusion dependent (ρ=0.807, p=<0.001) subgroups, but not within the never transfused group (ρ=0.487, p=0.268), and again sensitivity of ferritin levels>1000 ng/ml to identify patients with LIC ≥7 mg/g (64%) was poor. Twenty five percent (20/81) of patients with ferritin<1000 ng/ml did have LIC ≥7 mg/g. Sensitivity for LIC ≥7 mg/g in never transfused, sporadically transfused, and transfusion dependent subgroups was respectively 0%, 30%, and 67%. In the not-chelated, and never-transfused/not-chelated group it was respectively 11% and 0%. Decreasing the cut off to 500 ng/ml again improved the sensitivity of ferritin for LIC albeit not to 100% in all groups.

Correlation between transferrin saturation, ferritin, and LIC

Of the 44 patients with rare hereditary hemolytic anemia that underwent MRI, 28 had transferrin saturation (TSAT) levels measured in the same period of time. There was no correlation between TSAT and LIC (ρ=0.326, p=<0.090). In the reference group TSAT was available for 23 patients. In this group there was a significant correlation between

Table 3. predictive value of ferritin, TSAT and LIC in rare hereditary hemolytic anemia

		ferritin ≥1000		ferritin ≥800		ferritin ≥500		ferritin ≥500 or TSAT≥45		
		LIC≥3	LIC≥7	LIC≥3	LIC≥7	LIC≥3	LIC≥7		LIC≥3	LIC≥7
Total	Sensitivity	25%	47%	39%	53%	71%	100%	*N=28*	74%	100%
N=40	Specificity	100%	100%	92%	84%	92%	76%		56%	53%
Never transfused	Sensitivity	0%	0%	18%	0%	55%	100%	*N=14*	60%	100%
N=15	Specificity	100%	100%	100%	85%	100%	69%		75%	58%
Sporadically transfused	Sensitivity	33%	43%	44%	57%	78%	100%	*N=8*	100%	100%
N= 15	Specificity	100%	100%	100%	100%	100%	100%		50%	40%
Transfusion dependent	Sensitivity	50%	67%	63%	67%	88%	100%	*N=6*	80%	100%
N=9	Specificity	100%	100%	100%	67%	100%	67%		0%	50%
No chelation at time of	Sensitivity	0%	0%	14%	0%	50%	100%	*N=19*	67%	100%
MRI *N=22*	Specificity	100%	100%	100%	89%	100%	83%		71%	60%
Never transfused, no	Sensitivity	0%	0%	22%	0%	56%	100%	*N=12*	63%	100%
chelation at MRI *N=13*	Specificity	100%	100%	100%	82%	100%	73%		75%	60%

TSAT and LIC (ρ=0.483, p=<0.020). Of note, the three patients of the reference group with ferritin <500 ng/ml and LIC ≥7 mg/g that had TSAT levels available, all had very high TSAT levels (85%, 88%, and 95% respectively). The sensitivity of plasma ferritin>500 ng/ml ór TSAT>45% for LIC ≥7 mg/g was 100% in all groups.

Cardiac iron overload

Cardiac MRI data was available for 33 patients with rare hereditary hemolytic anemia. None of the patients had cardiac iron overload.

In the reference group, seven patients had cardiac iron overload, all of which were transfusion dependent at time of inclusion. One β-thalassemia patient with cardiac iron overload had ferritin levels of <1000 ng/ml.

Hereditary hemochromatosis

For 38 out of 44 patients test results for hereditary hemochromatosis were available. Five patients were heterozygous for the H63D mutation in HFE and six for the C282Y mutation. One patient was homozygous for C282Y. There were no significant differences in LIC between patients heterozygous for either one of the HFE mutations, or between patients who carried a mutation versus patients that did not.

DISCUSSION

Our study shows that iron overload occurs in all forms of rare hereditary hemolytic anemia included in this study. Importantly, it shows that iron overload is not limited to patients with ferritin levels >1000 ng/ml, >800 ng/ml or to patients with a history of red cell transfusions. The results in rare forms of hereditary hemolytic anemias were very similar to the results found in the reference group consisting of patients with β-thalassemia and SCD.

Our study shows a correlation between ferritin and LIC, which is in line with earlier studies in several forms of hemolytic anemia.(33, 34) However, to accurately diagnose iron overload in the individual patient, it is important to also evaluate sensitivity of the screening tests used. Our data clearly show that ferritin levels at a cut-off of 1000 ng/ml have a low sensitivity for iron overload. Iron overload detected by MRI in patients with ferritin levels lower than 1000 ng/ml occurred in all types of hereditary hemolytic anemia studied, including patients that were never transfused. Therefore, a ferritin cut-off of 1000 ng/ml cannot be safely applied to patients with hereditary hemolytic anemia. We recommend that MRI LIC measurement should be performed in all patients.

Recently, in a large study in patients with NTDT a ferritin cut off of 800 ng/ml was demonstrated to have the best predictive value for iron overload (defined as LIC ≥5 mg/g) instead of 1000 ng/ml when MRI is unavailable.(35) Both in our rare hereditary hemolytic anemia sample as well as in the reference group, the sensitivity of a cut-off

of 800 ng/ml for LIC ≥7 mg/g remained poor. Notably, our reference group did not include never-transfused β-thalassemia patients. At a ferritin threshold of 500 ng/ml, sensitivity for LIC ≥7 mg/g increased to 100% in rare hereditary hemolytic anemias and increased to ≥67% in the reference group.

We did not find a correlation between TSAT and LIC in patients with rare hereditary hemolytic anemia. Ideally, to interpret TSAT measurements, patients should withhold iron chelation for at least one day before measurement, as the presence of iron chelation in the bloodstream may influence the results.(36) Notably, patients with LIC ≥7 mg/g despite low ferritin levels, did have very high TSAT levels suggesting that TSAT may be helpful in recognizing patients with iron overload despite a low ferritin. Therefore, it could be interesting to repeat these measurements in a prospective study with controlled settings to evaluate diagnostic potential of TSAT as a marker for iron overload in hereditary hemolytic anemia. Meanwhile, we suggest that, especially in situations where MRI is not available, patients who have ferritin levels <500 ng/ml ánd transferrin saturation <45% are unlikely to have iron overload.

We used a reference group of patients with SCD and β-thalassemia. Percentage iron overload in our transfused SCD patients was comparable to published data. (37) However, we did also find two patients with SCD (one Hemoglobin SS, one Hemoglobin SE) who were never transfused but did have LIC ≥3 mg/g. This is interesting, as in general it is perceived that patients with SCD do not suffer from non-transfusion related iron overload. (38)

For this study, we included only patients who had MRI LIC data available. This creates a selection bias, but since we aimed to study the occurrence of iron overload and not its prevalence, the data can be considered representative. HFE test results were not available for all patients. For the patients that had data available, the study did not show an association between HFE-mutations and iron overload.

We did not correct for chelation therapy. Therefore, it is possible that the real prevalence of iron overload in hereditary hemolytic anemia, is even higher than described here, as effective chelation therapy reduces or even normalizes LIC levels.

When studying the difference between transfusion related iron overload and non-transfusion related iron overload, patients are often categorized as either transfusion-dependent or non-transfusion dependent. However, the definition of (non-)transfusion dependent patients differs in various studies. In this study, in order to completely eliminate the confounding effect of red cell transfusions, we added a third, very strictly defined group of never-transfused patients. Patients who received as little as one transfusion during their lifetime, or patients who had no complete medical follow-up from birth available were excluded from this group. Even with this very strict definition there were patients in the never-transfused group with moderate to severe iron overload. This indicates that non-transfusion related iron overload, due to inappropriately increased dietary uptake, does occur in rare forms of hereditary hemolytic anemia similar to patients with β-thalassemia.

In conclusion, this study demonstrates that iron overload is present in all forms of rare hereditary hemolytic anemia, even in patients who were never transfused, confirming an inappropriate dietary iron uptake. The traditionally used cut-off of plasma ferritin >1000 ng/ml and even >800 ng/ml appears to be a poor predictor for liver iron overload. We suggest therefore that all patients with rare hereditary hemolytic anemia, possibly except those with ferritin levels below 500 ng/ml ánd transferrin saturation below 45%, should be evaluated for iron overload with MRI.

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SUPPLEMENTAL DATA

Supplemental table 1. baseline characteristics reference group

	Reference group			
	Sickle cell disease (N=60)		B-thalassemia (N=26)	
Female	36/60	(60%)	15/26	(58%)
Age in years	33	(26-49)	31	(27-42)
Hb in mmol/L	6.3	(5.4-7.0)	5.4	(4.9-5.6)
Retic x10^9/L	200	(96-293)	227	(110-411)
Direct bilirubin in umol/L	9	(6-12)	9	(7-11)
Total bilirubin in umol/L	32	(18-46)	37	(27-67)
Plasma ferritin in ng/ml	384	(65-1078)	958	(477-3610)
Transferrin saturation in %	56	(36-83)	87	(76-95)
LIC in mg/g	5.3	(2.5-10.6)	12.8	(8.9-19.6)
Percentage never transfused	11/57	(20%)	0/25	0%

Data expressed as counts (within group percentage) or medians (IQR)

Supplemental table 2. predictive value of ferritin, TSAT and LIC in sickle cell disease and β-thalassemia

		ferritin ≥1000		ferritin ≥800		ferritin ≥500		ferritin ≥500 or TSAT≥45		
		LIC≥3	LIC≥7	LIC≥3	LIC≥7	LIC≥3	LIC≥7		LIC≥3	LIC≥7
Total	Sensitivity	51%	64%	60%	73%	79%	88%	*N=23*	100%	100%
N=72	Specificity	100%	92%	100%	90%	100%	79%		100%	43%
Never transfused	Sensitivity	50%	-	50%	-	50%	-	*N=0*	-	-
N=7	Specificity	100%	86%	100%	86%	100%	86%		-	-
Sporadically transfused	Sensitivity	0%	0%	0%	0%	40%	67%	*N=4*	100%	100%
N= 16	Specificity	100%	100%	100%	100%	100%	100%		100%	100%
Transfusion dependent	Sensitivity	56%	67%	67%	78%	86%	89%	*N=20*	100%	100%
N=45	Specificity	100%	89%	100%	83%	100%	61%		-	43%
No chelation at time of MRI	Sensitivity	27%	20%	36%	40%	55%	80%	*N=6*	100%	100%
N=22	Specificity	100%	93%	100%	93%	100%	93%		100%	75%
Never transfused, no chelation at MRI	Sensitivity	50%	-	50%	-	50%	-	*N=0*	-	-
N=7	Specificity	100%	86%	100%	86%	100%	86%		-	-

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PREVALENCE AND MANAGEMENT OF IRON OVERLOAD IN PYRUVATE KINASE DEFICIENCY: REPORT FROM THE PYRUVATE KINASE DEFICIENCY NATURAL HISTORY STUDY

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ABSTRACT

Pyruvate kinase deficiency is the most common red cell glycolytic enzyme defect causing non-spherocytic hemolytic anemia. The objective of this study was to determine the prevalence of iron overload in patients with pyruvate kinase deficiency with a focus on those who are not regularly transfused. Of 242 patients, 175 (72%) had ferritin levels and 65 (27%) had magnetic resonance imaging (MRI) for liver iron concentration (LIC) within the prior 12 months. The median ferritin was 583 ng/ml (range: 17-5630); the median LIC was 5.4 mg/g dry weight (range 1-33.4). The overall prevalence of iron overload in those tested was 45% by ferritin and 85% by MRI. At enrollment, 82% (198/242) were not regularly transfused; 38% of these patients had iron overload by ferritin and 82% by MRI. The patients with iron overload were older, more anemic, more often splenectomized, and had higher bilirubin levels. Despite these correlations, iron overload by ferritin was seen at all ages (range: 1.4-60.4 years) and hemoglobin levels (range: 4.0-7.4 mmol/L), and in those never transfused (4/22,18%). Five patients, ages 3-34 years, had cardiac iron overload (5/75). LIC and ferritin significantly correlated in those not receiving regular transfusions ($p<0.0001$), but ferritin >1000 ng/ml had a sensitivity for LIC >3 mg/g of only 53%. Transfusion independent iron loading is common in pyruvate kinase deficiency. Given the high rate of iron loading and poor predictive value of ferritin in this population, regardless of transfusions, we recommend routine MRI iron screening starting in childhood with continued regular monitoring.

INTRODUCTION

Pyruvate kinase (PK) deficiency is the most common red cell glycolytic enzyme defect causing hereditary non-spherocytic hemolytic anemia, with a prevalence estimated between 1:20,000 and 1:300,000 in the Caucasian population.(1-3) The prevalence is higher in certain subpopulations (e.g., Pennsylvania Amish) due to a founder effect and is also likely higher in malaria endemic regions, where PK deficiency may confer a protective advantage.(4-10)

Current treatments are mainly supportive and include red cell transfusions and splenectomy.(11) Chronic red cell transfusions cause iron overload; however, the prevalence and spectrum of transfusion-independent iron overload in PK deficiency has not been established. Red cell disorders that are associated with ineffective erythropoiesis, such as thalassemia intermedia, have been associated with transfusion-independent iron overload due to relatively low hepcidin expression.(12-14) Small cohort studies of patients with PK deficiency have reported transfusion-independent iron loading.(10, 15)

Iron overload can cause end-organ damage and is associated with significant morbidity and mortality.(16, 17) Given that iron loading is clinically silent until quite severe and can be treated with iron chelation, identifying the appropriate patient population for screening and the most sensitive screening measure is critical.

The PK Deficiency Natural History Study (NHS) was developed as a retrospective and prospective cross-sectional cohort study to characterize the clinical spectrum and complications of patients with PK deficiency.(18) The objective of this analysis was to determine the prevalence and clinical characteristics of iron overload in this population with a focus on those patients who are not regularly transfused.

METHODS

Study design

The PK deficiency NHS protocol was approved by each site's Institutional Review Board and/or Ethics Committee, and study procedures were in accordance with the Helsinki declaration. All patients gave informed consent. Eligible patients had molecularly diagnosed PK deficiency. This NHS enrolled 278 patients with PK deficiency from June 2014 through April 2017 at 31 centers in 6 countries. Twenty-one patients, despite manifesting a PK deficiency phenotype, were excluded due to the inability to confirm two pathogenic *PKLR* mutations, and 3 patients were excluded because genetic results were pending at the time of the analysis. Patients less than one year old at enrollment were also excluded (n=12) from this analysis, because ferritin is less reliably related to iron overload in this youngest age group, leaving 242 participants reported herein.

Data collection

Baseline and retrospective enrollment data were used, including detailed information regarding clinical, radiographic, and laboratory data. Data also included information on transfusion regimen, lifetime transfusion burden, iron measurements, chelation regimen, and therapy adherence. In Lancaster, Pennsylvania, in which all enrolled patients are Amish, additional laboratory and radiologic data were collected under a site-specific IRB-approved protocol.

End points and definitions

Patients were considered to have iron overload if (i) their highest ferritin was over 1000 ng/ml, or (ii) they received chelation therapy in the 12 months prior to enrollment, or (iii) their highest liver iron concentration (LIC) was >3 mg/g dry weight liver (DW) on T2* MRI, or (iv) they had cardiac iron overload as defined by a cardiac T2* ≤20ms at any time in their history. The definition of iron overload as an LIC >3 mg/g DW was based on guidelines for β-thalassemia.(19, 20)

Statistical analysis

Tests of association were performed using Fisher's exact test (categorical) and Wilcoxon rank sum test (continuous). General linear regression models were used to identify the association of LIC with ferritin. Sensitivity cut-off values of ferritin (500 ng/ml and 1000 ng/ml) for iron overload by MRI were determined prior to analysis. P-values were two-sided, and p-values <0.05 were considered statistically significant.

RESULTS

Patients

The median age at enrollment was 19.8 years (range: 1.3-69.9 years). The cohort was 51% (123/242) female, and 22% (53/242) of patients were from the Pennsylvania Amish community. Splenectomy had been performed in 62% (149/242) at the time of enrollment.

Iron overload as defined by ferritin

Of the 242 patients, 175 (72%) had ferritin levels measured within 12 months prior to enrollment. The median ferritin level was 583 ng/ml (range: 17-5630 ng/ml). The overall prevalence of iron overload as defined by ferritin (ferritin >1000 ng/ml or chelation treatment) was 45% (82/181). Differences between patients with and without ferritin data are presented in Table 1. Patients without ferritin monitoring had fewer transfusions (1% vs 25% regularly transfused, p<0.0001) and a higher hemoglobin level (median Hb 6.0 vs. 5.5 mmol/L, p=0.01).

At enrollment, 82% (198/242) of patients were not receiving regular transfusions; 38% (53/138) of these patients had iron overload as defined by ferritin. Characteristics

Table 1. Patient characteristics, comparing those with iron monitoring to those without monitoring, in the 12 months prior to enrollment.

	Ferritin monitoring (n=242)			MRI monitoring for liver iron assessment, in the non-Amish patient cohort (n=189)		
	Obtained in the 12 months prior to enrollment (n=175)*	**Not obtained in the 12 months prior to enrollment (n=67)***	**P-value****	**Obtained in the last 12 months prior to enrollment (n=18)***	**Not obtained in the last 12 months prior to enrollment (n=171)***	**P-value****
Median*** Age at enrollment	21.8 (1.3-69.9)	16.3 (1.4-60.4)	0.3	21.1 (2.2-46.0)	19.1 (1.3-69.9)	0.5
Transfused in the 12 months prior to enrollment (%)	72/175 (41%)	9/67 (13%)	<0.0001	11/18 (61%)	67/171 (39%)	0.08
Regularly Transfused (%)	43/175 (25%)	1/67 (1%)	<0.0001	7/18 (39%)	36/171 (21%)	0.1
Median*** Hemoglobin value (mmol/L)	5.5 (3.2-8.8) n=174	6.0 (4.0-8.1) n=65	0.01	5.3 (3.8-6.9)	5.4 (3.2-8.8) n=168	0.3
Splenectomized (%)	112/175 (64%)	37/67 (55%)	0.2	15/18 (83%)	83/171 (49%)	0.006

*Number of patients with known data (n) are presented in the columns headers unless otherwise indicated in the table. **Using Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables. ***Median presented with ranges.

of non-regularly transfused patients with iron overload are presented in Table 2. In this cohort, 40 patients had never been transfused; seven of these (18%) had iron overload as defined by ferritin. The patients with iron overload were older, more anemic, and more often splenectomized, and had a higher median total bilirubin than patients who did not have iron overload as defined by ferritin (Table 2).

At enrollment, 18% (44/242) of patients were receiving regular transfusions. Of these patients, 67% (29/43) had iron overload as defined by ferritin.

Iron overload as defined by LIC

An MRI for liver iron assessment was conducted in 65 (27%) patients in the 12 months prior to enrollment; 47 (72%) were from the Amish cohort and obtained per protocol. The median LIC was 5.4 mg/g DW (range: 1.0-33.4 mg/g DW).

Of the patients who were not receiving regular transfusions that had MRI or chelation data available, 82% (67/82) had iron overload as defined by MRI or chelation (Table 2). The median LIC in patients who were not regularly transfused was 5.4 mg/g DW (range: 1-33 mg/g DW). Of those patients who had never been transfused and had MRI or chelation data available, 6 of 7 patients met criteria for iron overload (6/7 splenectomized). These six patients, who had iron loading defined by MRI but had never been transfused, had a median age of 32.3 years (range: 5.9-58) with a median Hb of 6.1 mmol/L (range: 5.6-7.4 mmol/L).

Of the patients receiving regular transfusions at enrollment, 96% (22/23) had iron overload as defined by MRI or chelation. In this group, the median LIC was 5.2 mg/g DW (range: 1.7-20 mg/g DW).

MRI for cardiac iron assessment was available for 75 (31%) patients. Five patients (7%) had cardiac iron overload (median T2*=17.8 ms, range: 5-19.7 ms); of these five patients, only one patient had LIC measured (5 mg Fe/g DW). These patients' ages ranged from 3-34 years at the time of the MRI. Four of the five patients were male. The median hemoglobin was 5.4 mmol/L (range 4.3-5.8 mmol/L), the median number of lifetime transfusions was 39 (range 10-90 transfusions), and the median ferritin was 1343 ng/ml (range 346-3890 ng/ml).

Correlation between MRI and ferritin

Forty-five patients had paired ferritin and LIC measurements available (Figure 1). There was a significant correlation between LIC and ferritin in patients receiving regular transfusions (R^2=0.83, p=0.01, n=6) and in patients who were not receiving regular transfusions (R^2=0.51, p<0.0001, n=39, Figure 1). Using a ferritin cut-off of 1000 ng/ml, the sensitivity to predict LIC >3 mg/g DW was 53% and the specificity was 100%. At a ferritin cut-off of 500 ng/ml, the sensitivity for LIC >3 mg/g DW was 90% and the specificity was 67%.

Table 2. Characteristics of non-regularly transfused patients with PK Deficiency and iron overload

	Ferritin >1000 ng/ml or chelation*			LIC >3 mg/g DW or chelation*		
	Absent (85/138, 62%)	**Present (53/138, 38%)**	**p****	**Absent (15/82, 18%)**	**Present (67/82, 82%)**	**p****
CHARACTERISTICS						
Female sex	49/85 (58%)	24/53 (45%)	0.2	12/15 (80%)	36/67 (54%)	0.08
Amish	27/85 (32%)	13/53 (25%)	0.4	14/15 (93%)	33/67 (49%)	0.001
Age at enrollment (y)	22.6 (1.6-69.9)	38.9 (2.2-60.4)	0.005	23.4 (7.4-53.6)	34.7 (3.1- 60.4)	0.3
Hemoglobin (mmol/L)	5.8 (3.8-8.8)	5.4 (4.0-7.4) n=52	0.003	6.1 (4.7-6.8)	5.6 (3.8-7.4) n=66	0.2
Absolute reticulocyte count (10^6/µL)	0.2 (0.1-5.3) n=41	0.5 (0.1-1.2) n=15	0.2	0.9 (0.9-1.0) n=2	0.6 (0.1-0.9) n=13	0.1
Total Bilirubin (mg/dl)	3.6 (0.9-9.0) n=79	4.3 (1.3-17.6) n=48	0.01	3.5 (1.0-7.2)	3.6 (1.1-17.6) n=62	0.3
Ferritin (ng/ml)	388.0 (31.0-971.5)	1335.0 (171.5-5630.0) n=47	ND	362.5 (126.0-1065.0) n=12	969.0 (171.5-5630.0) n=53	<0.001
LIC (mg/g DW)	4.0 (1.0-8.4) n=26	8.0 (2.2-33.4) n=21	<0.001	2.0 (1.0-3.0)	6.4 (2.2-33.4) n=43	ND
Transferrin saturation (%)	43.3 (8.8-100.0) n=51	62.0 (18.4-100.0) n=29	0.02	42.4 (23.2-96.6)	50.1 (12.1-100.0) n=43	0.3
Splenectomized	51/85 (60%)	47/53 (89%)	0.0002	14/15 (93%)	63/67 (94%)	1

Table 2. (continued)

	Ferritin >1000 ng/ml or chelation*			LIC >3 mg/g DW or chelation*		
	Absent (85/138, 62%)	Present (53/138, 38%)	p**	Absent (15/82, 18%)	Present (67/82, 82%)	p**
Chelation in prior 12 months	0/85 (0%)	31/53 (58%)	ND	0/15 (0%)	31/67 (46%)	ND
Never Transfused	18/85 (21%)	4/51 (8%)	0.054	1/15 (7%)	6/65 (9%)	1
<10 lifetime transfusions	22/61 (36%)	7/39 (18%)	0.07	2/9 (22%)	9/51 (18%)	0.7
≥10 lifetime transfusions	39/61 (64%)	32/39 (82%)	0.07	7/9 (78%)	42/51 (82%)	0.7

Numbers are medians (range) or absolute numbers/group total (corresponding percentage). n is shown only for those groups that do not contain the total.
* Iron overload by ferritin was defined as the number of patients with ferritin>1000 ng/ml or who were treated with chelation therapy in the 12 months prior to enrollment. If a patient had >1 ferritin measurement in the 12 months prior to enrollment, the maximum ferritin value was used. Iron overload based on liver iron concentration (LIC) was defined as an LIC >3 mg Fe/g dry weight liver (DW) on T2* MRI in the 12 months prior to enrollment or who were treated with chelation therapy in the 12 months prior to enrollment. **p values of Fisher's Exact test or Wilcoxon Rank Sum test. ND: Not Done, testing is not appropriate or necessary within the same factor

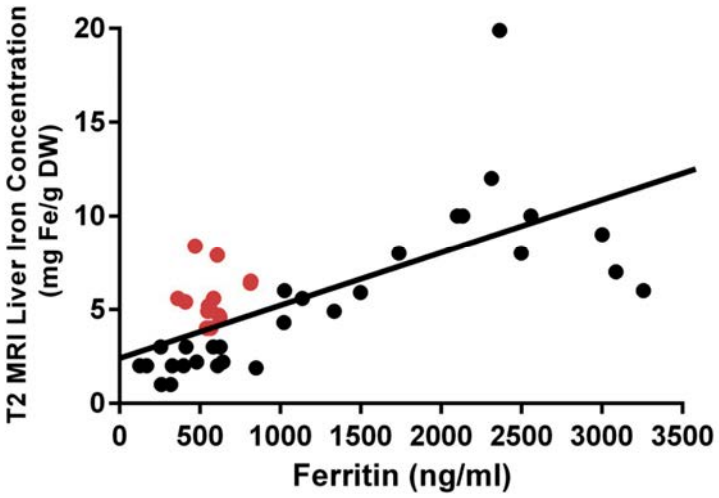

Figure 1. Correlation between ferritin and liver iron concentration as measured by MRI
Correlation between ferritin and LIC (r=0.45, p<0.0001, n=45). Red circles indicate the individuals with a median ferritin <1000 ng/mL but a LIC >3 mg/g DW.

Iron overload in children less than 10 years of age

The study enrolled 68 patients ages 1-<10 years old. Within this age cohort, of those who were not regularly transfused at enrollment, 28% (7/24) had iron overload as defined by ferritin. These patients had a median of 12 lifetime transfusion episodes (range: 1-112) and a median hemoglobin of 5.1 mmol/L (range: 4.2-7.3).

Of those who were not regularly transfused at enrollment but had received occasional transfusions, 82% (9/11) had iron overload as defined by MRI. The median number of lifetime transfusions in this group was 22.5 (range: 11-85). Correlation between LIC and age is depicted in Figure 2. Of those <10 years old who had never been transfused (n=9), data regarding iron overload by ferritin were known for only two patients, neither of whom had iron overload by ferritin. MRI was available for one patient which confirmed iron overload.

Of those children who were regularly transfused at enrollment, 52% (14/27) had iron overload as defined by ferritin and 90% (9/10) by MRI.

Treatment of Iron Overload

Of the 242 patients, 82 (34%) had been prescribed chelation therapy prior to enrollment. The median age at the time chelation therapy was first initiated was 10.4 years (range: 0.7-47.9 years). Of those who had never been transfused, 10% had received chelation therapy. Of those patients ages 1-<10 years at enrollment, 19% had been on chelation therapy starting at a median age of 2.4 years (range: 2-5 years). Patients spent a median of 5.3 years (range: 0-26.6) on chelation therapy.

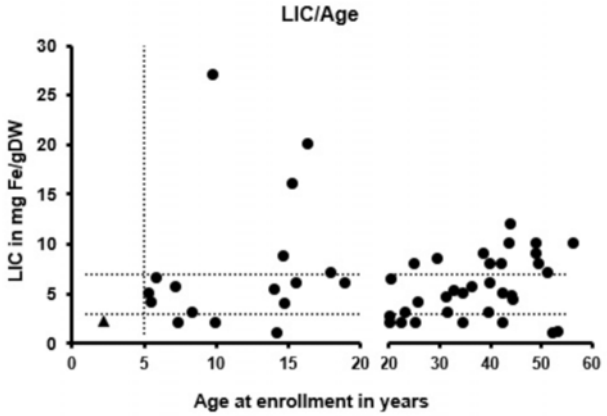

Figure 2. Relationship between age and liver iron concentration as measured by MRI
N=52, excluding patients on chelation in the 12 months prior to enrollment. Only one patient (represented as a triangle in the figure) was receiving regular transfusions at the time of enrollment. Moderately increased LIC (lower horizontal dotted line) is defined as 3 mg Fe/g DW and significantly increased LIC (upper horizontal dotted line) is defined as 7 mg Fe/g DW.

Twenty-eight patients were on chelation and then had a median of 3.4 years (range: 5 days-21.4 years) in which they were off chelation before restarting. Five patients were stopped and restarted on chelation more than twice over many years.

Phlebotomy was used for iron removal in five patients. In these patients, the median Hb was 5.4 mmol/L (range: 3.8-7.0 mmol/L) and a median of 500 mL per phlebotomy (range: 125-550 mL) was removed at a median interval of every 3 weeks (range: 2-12 weeks).

Iron overload in the Amish Cohort

Disease treatment of Amish patients differed significantly from the other patients, as these patients are typically uninsured and the cost of standard supportive care can be prohibitive. All but two Amish patients were splenectomized; none of the splenectomized patients received regular transfusions. Only one Amish patient had ever received chelation therapy. Instead of chelation therapy, Amish patients were managed with an iron restricted diet and a combination of proton pump inhibitors and calcium citrate to reduce dietary iron uptake. The Amish cohort was not significantly different from the non-Amish cohort with regard to the prevalence of iron overload as defined by ferritin.

DISCUSSION

Several small studies in PK deficiency have suggested a high prevalence of iron overload.(15, 21, 22) This NHS is the first international cohort study describing iron overload in PK deficiency. We found that the prevalence of iron overload defined

by LIC (LIC >3 mg/g DW or chelation therapy) was 82% in non-regularly transfused patients and 96% in regularly transfused patients. Although ferritin correlated with LIC, ferritin levels of >1000 ng/ml had a sensitivity for LIC >3 mg/g DW of only 53%. This finding indicates that a conservative ferritin based definition of iron overload will underestimate the true prevalence of iron overload in PK deficiency and highlights the need to screen patients with PK deficiency for iron overload using MRI. Given that the ferritin >500 ng/ml had sensitivity of 90%, this is a better cut-off if ferritin is used as a screening test for selecting patients for an MRI.

We used LIC >3 mg/g DW as our definition of iron overload based on guidelines for β-thalassemia.(19, 20) The upper limit of normal LIC in healthy volunteers is 1.8-2 mg/g DW.(23, 24) Therefore, a LIC 3 mg/g DW is an appropriate cut-off to define patients with abnormal iron loading. The threshold for treatment with chelation will be greater than 3 mg/g DW; however, we suggest this LIC for identifying patients with PK deficiency who have iron loading and are at risk for developing complications associated with iron overload.

Despite the conservative ferritin threshold of >1000 mg/dl, 38% of all patients who were not regularly transfused and 18% of those who were never transfused met this definition of iron overload. Furthermore, 82% of the patients who were not regularly transfused and had an MRI or chelation data had a LIC >3 g/mg DW. These data clearly show that iron overload is not limited to regularly transfused patients but is also common in patients who are not regularly transfused and even in those who have never been transfused. This is consistent with the findings in thalassemia intermedia in which iron loading occurs both in transfused and non-transfused patients.(25) Given the high rate of iron loading even in non-regularly transfused patients with PK deficiency, we recommend that all patients with PK deficiency have regular screening for iron overload.

There is no consensus definition of when patients with PK deficiency are regarded as regularly transfused. Factors influencing transfusion frequency are often transient and patient or physician dependent. Transfusion frequency is most often determined by the treating physician. Transfusion triggers vary from hospital to hospital and the degree of transfusion dependence of the patient might also differ based on characteristics, such as age, growth, and daily activities. Patients with PK deficiency may tolerate a lower hemoglobin due to increased 2,3-diphosphoglycerate levels leading to increased oxygen off-loading. For the definition of regularly transfused patients, we used a conservative cut-off of at least 6 transfusions per year to distinguish between regularly and not-regularly transfused patients. By choosing a conservatively high cut-off, we minimized the chance of confounding transfusion-related iron loading with transfusion independent iron overload.(26-31) Moreover, as a sensitivity analysis, we also analyzed the cohort using a definition of regularly transfused of 4 transfusions over a 12 month period, and the conclusions remained the same.

These data show that iron overload is not limited to patients with a ferritin level >1000 ng/ml. In order to improve the sensitivity of ferritin for LIC, we analyzed several cut-off values of ferritin. At a cut-off of 500 ng/ml, the sensitivity of ferritin for LIC >3 mg/g DW improved to 90%. In addition to its poor sensitivity, ferritin can be influenced by non-iron related factors, such as inflammation and hepatic disease. (32, 33) Therefore, we recommend that all patients with PK deficiency have screening for iron overload using T2*MRI or ferriscan, regardless of ferritin level. If access to T2*MRI or ferriscan is limited, a cut-off value of 500 ng/ml is a reasonable threshold to indicate the need for an MRI.

In this study, in non-regularly transfused patients, iron overload had also already occurred in many patients at a very young age. Moreover, the youngest patient with cardiac iron overload in this cohort was three years old. Similar results are found in thalassemia major, in which cardiac iron overload can be present in children.(34-36) In non-regularly transfused patients, we recommend the first MRI screening at least at the earliest age when the procedure can be done without sedation, particularly in patients with ferritin levels >500 ng/mL. In regularly transfused patients, MRI or ferriscan should be considered annually after one year of transfusions.

Cardiac iron overload resulting in heart failure remains the major cause of death in patients with β-thalassemia major in most parts of the world.(37, 38) In this cohort, only five patients (7%) had cardiac iron overload by MRI. Cardiac iron overload in PK deficiency appears to be slightly more common than in sickle cell disease, in which cardiac iron overload is seen rarely and only in heavily transfused patients. (39) One patient in the cohort developed cardiac iron overload after only 10 lifetime transfusions. This indicates that the number of prior transfusions might not predict which patients are most in need of screening for cardiac iron overload.

This cohort included a large subgroup of patients from the Amish community. The Amish patients all underwent MRI LIC measurement as part of a site-specific protocol. Thus, the Amish population is over-represented in the MRI results. The Amish do not participate in insurance-based or public-payer based health care systems; thus, expensive medications, such as iron chelators, are unaffordable and, therefore, underutilized compared to other patients in high-resource countries. Amish patients were splenectomized at a very young age with the goal of increasing Hb and decreasing the need for transfusions and consequent iron overload. In addition, these patients are also treated with iron-restricted diet and a combination of proton pump inhibitors and calcium citrate to reduce dietary iron uptake. Because of this variation in care, it is difficult to distinguish which factors drive the differences in the prevalence of iron overload between the Amish and the remainder of the cohort. However, despite all these management differences, the high rate of iron overload by MRI in non-regularly transfused Amish patients is striking.

In the non-Amish cohort, splenectomy was associated with iron loading.(40-45) However, this association is not clearly causal. Since splenectomy typically occurs in the more severely affected patients with PK deficiency, other factors associated with splenectomy, such as a lower hemoglobin or increased transfusion burden, may be contributing to this relationship. Further study is needed to understand whether splenectomy is independently associated with iron loading in PK deficiency.

The study is biased by its retrospective nature and challenges related to rare disease registries, including variability between diagnostic and treatment standards. However, the results clearly show that in PK deficiency, there is a high prevalence of iron overload both in regularly transfused and not regularly transfused patients. Ferritin levels <1000 ng/ml, hemoglobin levels 5.6 mmol/L, young age, and/or an absence of regular transfusions do not exclude the possibility of iron overload in patients with PK deficiency. Therefore, iron screening is important in all patients with PK deficiency and should be regularly monitored starting in childhood. Although the optimal criteria and timing for MRI monitoring in young children who are not regularly transfused remains unclear, routine MRI testing should be considered when sedation is no longer needed. Regular monitoring for iron overload and treatment, when needed, is imperative to the management of this patient population.

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9

WORLDWIDE STUDY OF HEMATOPOIETIC ALLOGENEIC STEM CELL TRANSPLANTATION IN PYRUVATE KINASE DEFICIENCY

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ABSTRACT

Currently, allogeneic stem cell transplantation is the only curative treatment for pyruvate kinase deficiency. Outcome data are very scarce with case reports of only four patients published. The total number of cases transplanted worldwide is unknown. We performed a worldwide inventory and report the indication, transplant procedures, complications and success rate of 16 cases transplanted between 1996 and 2015. Three year survival rate was 65%. Surviving patients were significantly younger. There was a 90% survival rate in cases <10 years of age, versus a 33% survival rate in older cases. Compared to published survival rates after transplantation in other hereditary anemias, overall survival in PK deficiency is relatively low. The observed trend in better survival in younger patients could be of guidance in future decision making in stem cell transplantations.

INTRODUCTION

Pyruvate kinase deficiency (PKD) is the most frequent glycolytic enzyme defect causing hereditary non-spherocytic hemolytic anemia.[1] PKD leads to energy deprivation of the red cell, ultimately resulting in premature red cell death. Premature red cell death causes clinical symptoms of hemolytic anemia. The degree of hemolysis can vary widely, from very mild and fully compensated forms, to life-threatening anemia with transfusion dependency.[2] The treatment for PKD is mainly supportive, and consists of regular red blood cell transfusions, splenectomy and chelation therapy for iron overload.[3] Hematopoietic allogeneic stem cell transplantation (HSCT) has the potential to cure PKD. However, there is little experience of applying HSCT in PKD. The current knowledge of HSCT in PKD is predominantly based on animal studies and guidelines are not available. [4,5] To date, only four human cases of HSCT have been published in literature. [6-8] The total number of cases transplanted worldwide is unknown.

The aim of this study is to make a worldwide inventory of PKD cases that have been treated by HSCT, and to evaluate indication, procedures employed and outcome as a first step towards the establishment of guidelines for HSCT in PKD.

METHODS

In order to achieve this goal queries were sent to national and international databanks, including the European Society for Blood and Marrow Transplantation (EBMT), the Center for International Blood and Marrow Transplant Research (CIBMTR), and the National Institute of Health (NIH), as well as to physicians known to be involved in HSCT in PKD patients. For each case found, a specifically designed questionnaire was sent to the physician involved. The questionnaire contained questions on disease characteristics, pre-transplant condition, transplant regimen and post-transplant outcome.[9] All data were evaluated by an experienced physician and institutions were contacted in case of inconsistencies. An adapted EBMT score (i.e. age, donor type and donor-recipient sex combination) was calculated based on the answers provided.[10] In addition, data from two additional cases, published recently, were extracted from the literature and included.[8] To the best of our knowledge, we have included all cases worldwide.

RESULTS

In total, sixteen cases were found to be treated by HSCT between 1996 and 2015. Patient characteristics are summarized in Table 1. Patients had all been treated in either European or Asian centers. No cases resulted as being transplanted in the USA. Patient's median age at transplantation was 6.5 years. All patients were transfusion dependent before transplantation, with median transfusion needs of thirteen units of packed red blood cells per year (range: 6 to 34 units). Conditioning and prophylaxis characteristics are summarized in Table 1. All patients

Table 1. patient characteristics

Sex	center	PKLR genotype	Splenectomy	№. Transf. 12mo	aEBMT	Age at HSCT	Max Ferritin	Pre. Trans. Ferritin
	Pt 1: Severe chronic anemia and progressive splenomegaly							
M	Asia	unknown	no	14	Good (0)	5y	-	950
	Pt 2: concerns regarding progressive liver and heart hemosiderosis							
F	EU	c.[721G>T;1594C>T] p.[(Glu241*);(Arg532Trp)]	yes	8	Intermediate (2)	15y	-	596
	Pt 3: Transfusion dependency							
F	Asia	c.[1044G>T;1076 G>A] p.[(Lys348Asn);(Arg359His])]	no	14	Intermediate (2)	1y, 7mo	3357	206
	Pt 4: high transfusion dependency and secondary hemochromatosis							
F	EU	c.[721G>T;1463G>A] p.[(Glu241*);(Arg488Gln)]	no	10	Intermediate (2)	3y	2444	1161
	Pt 5: Transfusion dependency							
M	Asia	c.[119G>A;1015G>A] p.[(Arg40Gln);Asp339Asn)]	no	13	Intermediate (1)	2y, 6mo	-	-
	Pt 6: Transfusion dependency							
F	EU	c.[1123_1133dup11; 1123_1133dup11] p.[(Met377fs;Met377fs)]	Yes	20	Intermediate (1)	17y	1888	1888
	Pt 7: Progressive transfusion dependency, decreasing quality of life							
F	EU	c.[494G>T;1529G>A] p.[(Gly165Val);(Arg510Gln)]	Yes	12	Intermediate (2)	39y	1311	650

Trans. year	Donor	Regimen	Matching	Stem cell source	conditioning regimen	GvHD	Infection	Outcome	Follow up time (Mo)
1996	MSD	Myelo ablation	8/8	Bone marrow	Cycph 200mg/kg Bu 16mg/kg p.o.	No	Febrile neutropenia unknown origin	alive[%]	235
2002	MFD	Myelo ablation	10/10	Bone marrow	ATG 20mg/kg Cycph 90mg/kg Flu 100mg/m2 Bu 16mg/kg p.o.	grade 4 (S/G/L)	Primary CMV infection, asperg. pneum	deceased	15
2009	Cord	Myelo ablation	7/8	Cord blood	ATG 7,5mg/kg Cycph 200mg/kg Bu 19,2mg/kg	grade 1 (S)	Bacterial infection	alive	72
2009	MUD	non-myelo ablation/ RIST		Bone marrow	ATG 30mg/kg Flu 160mg/m2 Thio 8mg/kg Treo 42mg/kg	No	No	alive	65
2009	MUD	non-myelo ablation/ RIST	8/10	peripheral blood	ATG 15mg/kg Cycph 200mg/kg Flu120-160mg/m2 Bu3,2-4,8mg/kg	grade 2 (S)	Fever unknown origin	alive	88
2010	MFD	Myelo ablation	8/8	Peripheral blood	Cycph 120mg/kg Bu 12,8mg/kg	grade 4 (S, G)	e. Faecum sepsis, susp. fungal pneumonia	deceased	5
2011	MUD	non-myelo ablation/ RIST	8/8	Bone marrow	ATG 600mg/m2 Flu120mg/m2 Bu 10,8mg/m2	grade 4 (S,G,L)	No	deceased	25

Table 1. (continued)

Sex	center	PKLR genotype	Splenectomy	№. Transf. 12mo	aEBMT	Age at HSCT	Max Ferritin	Pre. Trans. Ferritin
	Pt 8: Transfusion dependency							
F	EU	c.[1532G>A;1612G>T] p.[(Gly511Glu);(Glu538*)]	yes	8	Intermediate (2)	7y	771	771
	Pt 9: Transfusion dependency							
M	EU	c.[1481T>C;1675C>T] p.[(Ile494Thr);(Arg559*)]	no	12	Intermediate (1)	6 y	675	675
	Pt 10: Transfusion dependency							
M	Asia	c.[848T>C;941T>C] p.[(Val283Ala);(Ile314Thr)]	no	13	Intermediate (1)	1y, 6mo	-	593,5
	Pt 11: Transfusion dependency, problems with iron overload treatment because of compromised renal function							
M	EU	c.[1618+37_2064del;1618+37_2064del] p.[(Lys541fs);(Lys541fs)]	yes	6	Intermediate (1)	10y	4149	7026
	Pt 12: Transfusion dependency							
M	Asia	c.[661G>T;941T>C] p.[(Asp221Tyr);(Ile314Thr)]	no	9	Intermediate (1)	9mo	-	-
	Pt 13: Transfusion dependency							
M	Asia	c.[848T>C;848T>C] p.[(Val283Ala);(Val283Ala)]	no	13	Intermediate (1)	1y, 2mo	-	297,3
	Pt 14: Transfusion dependency, secondary hemochromatosis and hepatocarcinoma							
M	EU	c.[993C>A;1015G>C] p.[(Asp331Glu);(Asp339His)]	yes	34	Intermediate (2)	41y	-	1650

Trans. year	Donor	Regimen	Matching	Stem cell source	conditioning regimen	GvHD	Infection	Outcome	Follow up time (Mo)
2013	MFD	non-myelo ablation/ RIST	10/10	Bone marrow	ATG 4mg/kg Flu 160mg/m2 Thio 8mg/kg Treo 42mg/m2	No	CMV reactivation	alive	29
2013	MUD	non-myelo ablation/ RIST	9/10	Bone marrow	ATG 6mg/kg Flu 160mg/m2 Thio 8mg/kg Treo 42mg/m2	grade 4 (S, G,L)	CMV and EBV reactivation	deceased	2
2013	MUD	Myelo ablation	9/10	peripheral blood	ATG 15mg/kg Cycph200mg/kg Flu120-160mg/m2 Bu3,2-4,8mg/kg	grade 4 (G)	No	alive	34
2014	MUD	Myelo ablation	9/10	Bone marrow	ATG 8mg/kg Flu 160mg/m2 Bu TD, target AUC 90	grade 3 (S,G)	Asperg. Pneum.	deceased	13
2014	Cord	Myelo ablation	7/10	Cord blood	ATG 15mg/kg Cycph200mg/kg Flu120-160mg/m2 Bu3,2-4,8mg/kg	grade 4 (unknown)	Pneumonia	alive	24
2015	MUD	non-myelo ablation/ RIST	10/10	peripheral blood	ATG 15mg/kg Cycph200mg/kg Flu120-160mg/m2 Bu3,2-4,8mg/kg	no	No	alive	12
2015	MSD	Myelo ablation		Bone marrow	ATG 30mg/kg Flu 160mg/m2 Thio 8mg/kg Treo 42mg/kg	no	Susp. asperg. Pneum.	alive	12

Table 1. (continued)

Sex	center	PKLR genotype	Splenectomy	№. Transf. 12mo	aEBMT	Age at HSCT	Max Ferritin	Pre. Trans. Ferritin
	Pt 15: Transfusion dependency secondary hemochromatosis, spinal compression fracture due to osteoporosis							
M#	Asia	c.[1270-3C>A;1618G>T] p.[(?);(Gly540*)]	yes	-	-	11y	-	2000
	Pt 16: Transfusion dependency							
F#	Asia	c.[1270-3C>A;1618G>T] p.[(?);(Gly540*)]	no	-	-	8y	-	-

#data retrieved from Kim, 2016 [8]
[%]at last follow up
M=Male, F=Female, Y=Years, Mo=Months, №.Transf.12mo= estimated number of red blood cell transfusions in 12 months prior to HSCT, $=no biopsy data of liver available, Max. Ferritin=maximum ferritin reported in ng/ml, Pre. Trans. Ferritin=Pre-transplant ferritin level in ng/ml, Underlined ferritin levels: under chelation regimen MUD=Matched unrelated donor, MSD=Matched sibling donor, MFD=Matched family donor, Cord=Cord blood, ATG=Anti-thymocyte globuline, FLU=Fludarabine, Bu=Busilvan, Thio=Thiothepa, Treo=Treosulvan, Cycph=Cyclophosphamide- =unknown, RIST=Reduced intensity hematopoietic stem cell transplantation, S=Skin, G=GI tract, L=Liver, CMV= Cytomegalovirus, EBV=Epsteinn-Barr virus, susp=suspected, asperg=aspergillus, pneum=pneumonia

received graft-*versus*-host disease (GvHD) prophylaxis. *Ex vivo* T-cell depletion was performed in one transplant. In another, red cell depletion was performed. Five transplants were sex-matched, four were female receiver-male donor and four male receiver-female donor; his information was not available for three cases.

Median follow up time after transplantation was 2.3 years (range: 2 months to 19 years). Fifteen patients showed engraftment. The sixteenth patient initially showed pancytopenia and mixed chimerism. Following splenectomy six months post-transplantation, this patient's cell count spontaneously transitioned to normal with full donor chimerism. Two patients suffered from secondary graft loss; in one there was recovery to 91% donor chimerism after donor lymphocyte infusion. The outcome in the second patient was unknown.

Infectious complications and occurrence of GvHD are summarized in Table 1. The most significant infectious complications were aspergillus pneumonia (two patients), suspected aspergillus pneumonia (one patient), suspected fungal pneumonia (one patient), pneumonia (one patient), sepsis (one patient) and bacterial infection *e causa ignota* (one patient). GvHD grade 4 was reported in 6/16 cases (38%). Seven out of sixteen cases (44%) did not show symptoms of GvHD. There was no correlation between GvHD prophylaxis or any other clinical factors and the occurrence of GvHD grade 2-4 in the patients.

Five out of sixteen patients (31%) did not survive. All died of transplant-related causes. They had a median survival time of thirteen months (range: 2-25 months).

	Trans. year	Donor	Regimen	Matching	Stem cell source	conditioning regimen	GvHD	Infection	Outcome	Follow up time (Mo)
-		MUD	Myelo ablation	9/10	peripheral blood	ATG 7,5mg/kg Cycph200mg/kg Flu 120mg/m2	no	-	alive	36
-		MUD	Myelo ablation		peripheral blood	ATG 7,5mg/kg Cycph200mg/kg Flu 200mg/m2	no	-	alive	30

The two-year cumulative survival was 74%. Two patients had not yet reached the two-year milestone at the time of the questionnaire. Three-year cumulative survival was 65% (Figure 1); seven patients had not yet reached the three-year milestone.

Patients who did not survive differed significantly from surviving patients (Figure 1, Table 2). They were significantly older (p=0.036). Nine out of ten patients (90%) <10 years of age survived transplantation, whereas 2/6 (33%) ≥10 survived. Patients <10 years were less often splenectomized (p=0.001) and had lower pre-transfusion hemoglobin levels prior to HSCT (p=0.04). Patients who did not survive had all been treated in European centers. All patients treated in Asian centers survived transplantation (8/8). Patients treated in Asian centers were younger (p=0.001), less often splenectomized (p=0.041) and had lower ferritin levels prior to HSCT (p=0.048). In addition, they were more often transplanted using peripheral blood stem cells as a source (p=0.014) and had been conditioned more often on a cyclophosphamide regimen (p=0.007). Furthermore, patients who did not survive had frequently suffered GvHD grade 2-4 (p=0.031). Notably, four out of five deceased patients had suffered from both GvHD grade 3-4 and infection or viral reactivation. There were no significant differences in sex, plasma ferritin level, use of pre-transplant chelation therapy, transfusion burden in 12 months prior to HSCT, adapted EBMT-score, conditioning regimen, relation to donor, graft type, donor-recipient sex combination or transplant source.

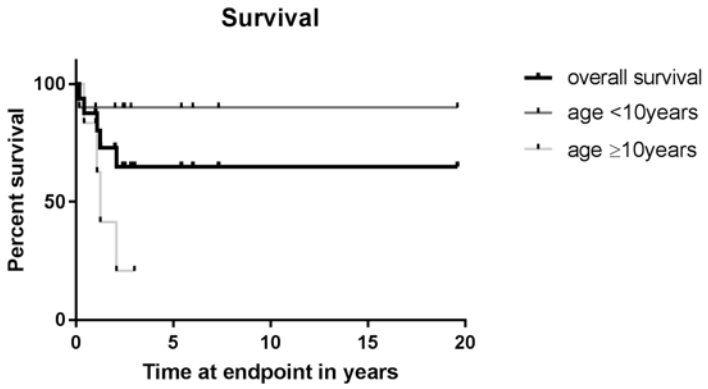

Figure 1. Overall survival, according to age

Table 2. Statistical differences between surviving and non-surviving patients

	Survivor	Non-survivor	P value
Age in years	7.5 – 3.0 (0.8-41)	17.4 – 15.2 (6-39)	0.036*
Asian hospital	8/11 (73%)	0/5	0.026*
Splenectomy performed	3/11 (27%)	4/5 (80%)	0.106
mean Hb (mmol/L) (N=13)	3.7-3.4 (2.8-4.9)	4.4-4.3 (3.7-5.0)	0.112
Pre-transplant ferritin (ng/ml) (n=12)	804 – 771 (206-1650)	2167 – 675 (596-7026)	0.432
Myeloablation	6/11 (55%)	4/5 (80%)	0.588
Graft type			0.507
MSD	2/11 (18%)	0/5	
MUD	6/11 (55%)	3/5 (60%)	
CORD	2/11 (18%)	0/5	
MFD	1/11 (9%)	2/5 (40%)	
Transplant source			0.333
Bone marrow	4/11 (36%)	4/5 (80%)	
Peripheral blood	5/11 (45%)	1/5 (20%)	
Cord blood	2/11 (18%)	0/5	
GvHD			0.015*
None	7/11 (64%)	0/5	
Grade 1	1/11 (9%)	0/5	
Grade 2	1/11 (9%)	0/5	
Grade 3	0/11	1/5 (20%)	
Grade 4	2/11 (18%)	4/5(80%)	

(descriptive statistics: mean – median (range) (N), frequencies number/total (percentage)
*P<0.05
Continuous variables were expressed as mean, median and range and subgroups were compared using Mann Whitney U tests. Categorical data was compared using Fisher's exact for binomial and Fisher-Freeman-Halton for contingency tables larger than 2x2. Statistical significance was considered as P≤0,05. All tests were 2-sided. No *post-hoc* multiple comparison correction. Graft-*versus*-host disease (GvHD) was defined and graded according to international criteria.[15] Pre-transplant laboratory results from splenectomized patients were from the period after splenectomy.

DISCUSSION

In conclusion, herein we discuss the first global study on the outcome of all patients known to have undergone HSCT in PKD. Since guidelines for HSCT in PKD are lacking, this report may be a helpful first step toward future protocols. Compared to published survival rates in other forms of hereditary anemias, cohorts that are otherwise comparable in age, time period and transplant hospital, the overall survival rate after HSCT in PKD is relatively low.[11-13]. The present analysis of all sixteen PKD patients known to be transplanted to date showed a three-year overall survival of 65%. Significantly better survival was observed for patients transplanted before the age of ten. A negative effect of age on survival is also reported for other forms of hereditary anemia.[11,12] Concurrently, we noticed a striking difference in survival between patients treated in Asian and European centers, which could possibly also be explained by the difference in age at which patients were transplanted. In addition, Asian patients were non-splenectomized in many instances, and had lower pre-transplantation ferritin levels, which could also be related to the young age at which HSCT was performed.

Asian patients were more frequently transplanted with peripheral blood stem cells as opposed to bone marrow derived stem cells. Peripheral blood stem cells are easier to collect from the donor, but reportedly increase the risk of chronic GvHD.[14] Our cohort, however, was too small to analyze the specific effect of stem cell source on the occurrence of chronic GvHD.

An important limitation of this study is its retrospective character, and the fact that the small sample size did not allow us to perform post-hoc correction for multiple testing. Therefore, the quantitative analysis of this data should be interpreted with care. Other limitations include the heterogeneity of conditioning regimens, and heterogeneity in pre-transplant risk classification systems used. However, we did observe a better survival for patients transplanted prior to the age of ten. This effect of age might also play a role in the observed differences in survival between patients treated in European centers and those treated in Asian centers.

Although HSCT should be considered an investigational treatment, the strong decline in survival of patients treated after the age of ten suggests the need to evaluate HSCT as a treatment option for early in life. However, since the rate of grade 3-4 GvHD is relatively high (7/16 = 44%), and death resulting from GvHD was likewise high (5/16 = 31%), transfusion dependency alone should not be an indication for performing HSCT in PKD.

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10

SUMMARY AND GENERAL DISCUSSION

This thesis was designed to contribute to unraveling various aspects of hereditary hemolytic anemia (HHA), and to outline the first steps towards creating an evidence based framework for future guidelines regarding diagnosis and treatment of HHA. As outlined in chapter 1, the aims of this thesis were:

1. to improve understanding of the disease burden of HHA, by studying both shared and distinct features of the diseases involved

2. to study pathophysiology of HHA

3. to evaluate effectiveness of current treatment strategies

The thesis was built up in three parts to answer the abovementioned three questions. Every part contained several research questions, which will be addressed in the summary:

Part 1: From diagnosis to clinical symptoms:

- What is the quality of life of patients with HHA?

- Do patients with rare forms of HHA suffer from organ involvement to the same extend as patients with sickle cell disease (SCD) and thalassemia, and do we need guidelines for screening?

- Is the 6-minute walk test a useful tool to diagnose pulmonary hypertension in HHA?

Part 2: From clinical symptoms to pathophysiology

- Do cell derived extracellular vesicles play a role in creating a hypercoagulable state after splenectomy in patients with HHA?

- Is acquired decreased stability of red cell pyruvate kinase a common pathophysiological feature of SCD and other hemoglobin disorders, and does this affect clinical symptoms and disease severity?

Part 3: From pathophysiology to treatment strategies

- Do all patients with HHA suffer from iron overload, even patients who never received red cell transfusions? Can the currently used guidelines for screening

for iron overload, based on experience in β-thalassemia, safely be applied in rare HHAs?

- Is stem cell transplantation a successful treatment option in pyruvate kinase deficiency?

SUMMARY

Part 1: From diagnosis to clinical picture:

In the first part of the thesis we focused on the clinical burden of HHA. After a brief general introduction on HHA and a short outline of the thesis in **chapter 1**, we started with the most important subject: the patient. Therefore, **chapter 2** provided insight in the patient-perceived health related quality of life (HR-QoL) of patients with HHA. Eighty three patients filled out two validated questionnaires; EQ5D5L, a generic, five dimensional questionnaire focusing on general HR-QoL, and FACT-An, a multi domain questionnaire with both general and anemia-specific domains.

One of the most encouraging findings in this chapter was that generally, HR-QoL from a patient's perspective was similar to that of the US-citizen reference population. However, patients with hemoglobin disorders and patients who were transfusion dependent experienced lower HR-QoL than their peers with other forms of HHA and their non-transfusion dependent peers. We did not find a correlation between patient-perceived quality of life and any laboratory parameter reflecting the severity of the anemia, like hemoglobin levels or reticulocyte count. In our study, HR-QoL was correlated to 6-minute walking distance and disease-related organ involvement. This finding highlights a very important feature of HHA: the severity of the disease is not solely represented by the degree of hemolysis. Our study also highlighted the differences in social quality of life between patients that experienced relatively high and relatively low quality of life. Therefore, we suggested that treating organ involvement and improving social support could be an interesting future goal to increase HR-QoL, especially for transfusion dependent patients or patients with hemoglobin disorders.

Conclusion: In general, patients with HHA reported a HR-QoL similar to healthy peers. However, especially patients with hemoglobin disorders, and transfusion dependent patients had a lower HR-QoL compared to other patients with HHA. HR-QoL correlated to hemolysis-associated organ involvement. Improving social quality of life and adequately treating organ involvement could be an interesting goal to increase HR-QoL, especially for transfusion dependent patients or patients with hemoglobin disorders.

As a consequence of improved life expectancy of HHA, more and more patients will be confronted with chronic organ involvement. In SCD and β-thalassemia the occurrence of disease-related organ involvement is an important determinant of morbidity and prognosis. (1-3). However, for the other forms of HHA, not much was known about disease related organ involvement and there were no clear guidelines determining if and how to screen for it. **Chapter 3** focused on this issue. We reviewed the medical charts of a total of 90 patients with HHA and scored organ involvement

based on information from medical history and (bio)markers. We showed that organ involvement, including thrombosis and organ iron overload, is not limited to patients with SCD and β-thalassemia , but also occurs in other forms of HHA. An important finding of this study was that organ involvement was not limited to (heavily) transfused patients, but also occurred in patients who never received any blood transfusion in their life.

The observational, retrospective nature of this study rendered two important limitations: our study was hampered by information bias, and it was impossible to draw conclusions on causality. It is conceivable that, because some patients had less screening results available in their medical charts, organ involvement was underrepresented. Even though both our research as well as earlier publications on this topic could not define a high risk profile for organ involvement, it is possible that clinicians were somehow able to only select high risk patients for screening.

It is interesting that in chapter 2 we found a correlation between organ involvement and HR-QoL. As stated, possibly treating organ involvement could also improve patients' HR-QoL.

Although in this chapter we could not extrapolate our data to calculate incidence, our study did show that patients do suffer from various forms of organ involvement. More research would be necessary to evaluate the exact incidence of organ involvement in our population, but we suggested that in the meantime patients are screened for at least those forms of organ involvement that are expected to have high prevalence and good treatment options. Based on our findings we suggested this could include screening for iron overload, osteoporosis, vitamin D deficiency, sex hormone deficiencies and microalbuminuria.

Conclusion: Organ involvement was not limited to patients with SCD or β-thalassemia, but occurred in all forms of HHA included in our study. Importantly, it also occurred in never-transfused patients. Although more research is necessary, we suggested to at least screen for highly prevalent forms of organ involvement that have treatment options.

One of the most dangerous, but rare forms of organ involvement in HHA, is pulmonary hypertension.(4-8) The prevalence of pulmonary hypertension is claimed to be 6-10% in sickle cell disease, but for other forms of HHA information about prevalence is scarce.(9, 10)

In pulmonary hypertension increased pulmonary vascular resistance and elevated pulmonary artery pressure are accompanied by restricted blood flow through the pulmonary arterial circulation. Pulmonary hypertension, if untreated, can lead to right-sided heart failure and eventually death. It is important to have proper screening tools to be able to identify the patients at risk. The gold standard for diagnosis of

pulmonary hypertension is right heart catheterization, however this is an invasive method. Therefore often other non-invasive screening tools are used, despite their suboptimal correlation with diagnosis, for instance Doppler echocardiography to determine triscuspid regurgitant jet flow (TRV). Research in SCD and in primary pulmonary hypertension showed improved diagnostic accuracy using a multimodality approach combining TRV with the results of the 6-minute walk test.(9, 11) Based on this research, the American Thoracic Society added the 6-minute walk test as diagnostic tool to their 2014 guidelines for diagnosis of pulmonary hypertension in SCD.(12) In SCD, elevated TRV is not only a well-known indicator of possible pulmonary hypertension, but is also associated with increased mortality risk.(12)

In **chapter 4** we evaluated pulmonary hypertension based on right heart catheterization and TRV. In our study we did not identify any patients with pulmonary hypertension as diagnosed by right heart catheterization. Therefore, we were not able to test the added diagnostic value of the 6-minute walk test. We did identify TRV>2,5m/s on cardiac ultrasound of several non-SCD HHA patients. The TRV cut-off suggestive for pulmonary hypertension in non-anemic subjects is TRV>3,0m/s.(13) However, in SCD a lower cut-off of TRV>2,5m/s is used, because patients with SCD have anemia-induced elevation of their cardiac output and reduction in their blood viscosity, which result in a lower baseline pulmonary vascular resistance than observed in non-anemic patients.(14) We reasoned that the same hemodynamic changes are present in other patients with HHA as well. We therefore suggested that patients with HHA should be screened for pulmonary hypertension and that patients with TRV≥2,5m/s should be followed with more attention to heart failure than normal routine requires.

Conclusion: Our study identified elevated TRV in several non-SCD HHA patients. We suggested to screen for pulmonary hypertension in patients with HHA and follow those with increased TRV with more attention.

Part 2: From clinical symptoms to pathophysiology

The second part of the thesis was dedicated to the association between clinical symptoms and pathophysiology. As we discussed in chapter 3, thrombosis is a relatively common complication of HHA. The pathophysiology is not yet fully determined. In **Chapter 5** we hypothesized that increased concentrations of phosphatidylserine (PS) exposing circulating extracellular vesicles after splenectomy could contribute to a hypercoagulable state in patients with HHA. In a cross-sectional study of 97 patients we studied membrane labeled extracellular vesicles (EVs) in platelet depleted plasma by means of a dedicated flow cytometer. This study was performed together with the Vesicle Observation Centre, from Amsterdam UMC in Amsterdam. We tested the ability of EVs to induce clotting in patients with and

without splenectomy with a special interest in PS-exposing EVs. PS is normally found on the inner layer of the cell membrane, and exposure of PS can promote coagulation, phagocytosis and vesiculation. Splenic macrophages normally play an important role in removing PS-exposing cells from the circulation.(15) In our study we did indeed see that splenectomized patients had higher concentrations of PS-exposing vesicles than non-splenectomized patients. The concentration of PS-exposing EVs correlated with coagulation activation *in vivo* (as represented by D-dimer levels) and with clotting time *in vitro*. Based on our results, we suggested a possible role for PS-exposing EVs serving as a pro-coagulant surface after splenectomy, contributing to the increased thrombotic risk after splenectomy in patients with HHA.

Conclusion: Increased concentrations of PS-exposing EVs might increase the pro-coagulant potential of blood plasma after splenectomy in patients with HHA by serving as a pro-coagulant surface. This could contribute to the increased thrombotic risk after splenectomy in patients with HHA.

Second, we studied the role of the currently not fully understood elevated levels of red cell 2,3-diphopshoglycerate (2,3-DPG) in SCD and their relation to oxidative stress. The glycolytic intermediate 2,3-DPG can modulate the allosteric equilibrium of oxygen binding to hemoglobin by shifting the oxygen binding curve to the right. As a consequence, hemoglobin will more readily release oxygen. However, in SCD, deoxygenated hemoglobin tends to polymerize and form rigid sickle shaped red cells that are easily destroyed. Red blood cell sickling is the most important cause of intravascular hemolysis in SCD and increases the risk of end organ damage .(16, 17) In **chapter 6** we reported on a pilot study focusing on the role of the enzyme pyruvate kinase (PK) in red cells of patients with hemoglobin disorders. We postulated that in SCD, oxidative stress causes an acquired form of PK-deficiency (PKD), characterized by impaired stability of the PK-enzyme. This leads to an accumulation of 2,3-DPG through retrograde accumulation of intermediates of the Embden Meyerhof pathway. Therefore, we tested red cell PK thermal stability. Compared to healthy controls, patients with SCD showed markedly decreased thermal stability of PK. In order to further explore the role of oxidative stress in causing PKD, we treated samples from both a healthy volunteer and a sickle cell patient with tert-butylhydroperoxide (tBHP) to mimic oxidative stress. Similar effects on thermal stability in these samples were seen in samples from patients with unstable hemoglobin disorders and patients with glutathione cycle defects. This suggests a role for oxidative stress and/or impaired anti-oxidant defense in the pathophysiology of these diseases. However, our study was carried out only as a pilot study and more research is necessary. Therefore, to further investigate PK thermal stability in hemoglobin disorders, and in other forms of non-PK HHA, we launched a new study, called the TApIR-study, that will run in 2018-2019.

Conclusion: Red cell PK of patients with SCD showed reduced *ex vivo* thermal stability. Similar results in unstable hemoglobin variants and glutathione-cycle defects suggest a common role for oxidative stress and/or impaired anti-oxidant defense in the pathophysiology of this phenomenon. This theory was strengthened by the finding that the oxidizing agent tBHP could reduce thermal stability both in SCD-patients and in healthy controls.

Part 3: From pathophysiology to treatment strategies

One of the main complications of HHA is iron overload, either due to blood transfusions, or due to inappropriately high dietary iron absorption as a result of ineffective and increased erythropoiesis. (18) Currently, iron overload has been extensively studied in β-thalassemia, but not much information is available on iron overload in the other, more rare forms of HHA that we focused on in earlier chapters of this thesis. Therefore, **chapter 7** described a cross-sectional analysis of iron overload in 44 patients with rare HHA, performed in collaboration with the Amsterdam UMC in Amsterdam and Haga Hospital in The Hague. Eighty six patients with β-thalassemia and SCD were included as a reference group. We concluded that iron overload occurs in all forms of HHA included in our study and that also patients who never received a red cell transfusion are at risk for iron overload. Equally as important from a clinical perspective, we discovered that the traditionally used ferritin cut-off of 1000ng/ml had a poor sensitivity for iron overload as diagnosed by T2* MRI of the liver, not only in patients with rare HHA, but also in SCD and β-thalassemia. This has important consequences for current clinical practice, as it implies that all patients, possibly except those with ferritin levels below 500ng/ml ánd transferrin saturation levels <45% should be evaluated for iron overload with MRI.

Conclusion: Iron overload occurred in all forms of rare HHA studied, even without transfusion history. The traditionally used ferritin cut-off of 1000ng/ml had a poor sensitivity for iron overload in rare HHA and in patients with β-thalassemia and SCD. We suggested that all patients with HHA, possibly except those with ferritin below 500ng/ml ánd TSAT<45%, should be evaluated for iron overload with MRI.

So far, in the thesis we focused on all forms of HHA and only studied adults. For the last two chapters however, we focused on one disease in particular, namely PKD. In **chapter 8**, similar to chapter 7 we analyzed iron overload, however this time in a large cohort of PKD patients of all ages. This study was performed in collaboration with the Children's Hospital Boston, as part of the Pyruvate Kinase Deficiency Natural History Study(NCT02053480).(19, 20) We performed a retrospective analysis of iron overload in 242 patients diagnosed with PKD and found that iron overload occurred also in patients who were never transfused. In addition, we found that a ferritin cut

off of 1000ng/ml had a poor predictive value for iron overload. Moreover we found that iron overload was actually highly prevalent in PKD and at all ages. Even cardiac iron overload occurred in children as young as three years old. We concluded that regular monitoring for iron overload and treatment, when needed, is imperative to the management of patients with PKD.

Conclusion: Iron overload was common in both transfused and non-transfused patients with PKD. Given the high rate of iron loading and poor predictive value of ferritin in this population, regardless of transfusions, we recommended routine MRI iron screening starting in childhood with continued regular monitoring.

As stated before, most treatment options for HHA are solely supportive. But, as demonstrated in chapter 7 and 8, these supportive treatment options can have severe side effects. Although there are increasing possibilities for gene therapy, especially in β-thalassemia and SCD, for other forms of HHA the only curative option to date is hematopoietic allogeneic stem cell therapy (HSCT). There is little experience in applying HSCT in PKD. The current knowledge of HSCT in PKD is predominantly based on animal studies and guidelines are not available. In **chapter 9**, we described a worldwide inventory of PKD cases that were treated by HSCT. In an international collaboration with PKD and SCT experts, we evaluated indications, procedures employed and outcome, as a first step towards establishments of guidelines for HSCT in PKD. We found 16 cases treated by HSCT between 1996 and 2015 and concluded that, compared to published survival rates for several other hereditary anemias, the overall survival rate after HSCT in PKD was relatively low, with a three-year overall survival of 65%. However we did see a significantly better survival for patients who were transplanted before the age of ten. Also, we found a relatively high rate of grade 3-4 Graft-*versus*-Host Disease (GvHD) and death resulting from GvHD was likewise high. We concluded that, although HSCT should be considered an investigational treatment for patients with PKD, the strong decline in survival of patients treated after the age of ten suggests the need to evaluate HSCT as a treatment option early in life.

Conclusion: Although HSCT should be considered an investigational treatment for patients with PKD, the strong decline in survival of patients treated after the age of ten suggests the need to evaluate HSCT as a treatment option early in life.

DISCUSSION

The discussion of this thesis will be built around several propositions. The discussion is split into three parts: 1: understanding the burden of disease of HHA, 2: future research and 3: clinical perspectives.

Understanding the burden of disease of HHA

No cancer, no problem, is HHA a "benign disease"?

HHA is classified as a benign disease. According to the Concise Medical Dictionary of Oxford University Press "benign" is described as "any disorder or condition that does not produce harmful effects".(21) The online medical dictionary of Merriam Webster defines "benign" as "of a mild type or character that does not threaten health or life", or "having a good prognosis: responding favorably to treatment".(22) However, as can be appreciated from **chapter 3 and 4,** apart from anemia, HHA can produce numerous complications. These can range from relatively mild complications, like vitamin D deficiency, to life threatening complications like cardiac iron overload. And, as discussed in **chapter 9**, for most patients curative treatment strategies are currently lacking and patients are depending on supportive treatment strategies. Unfortunately, as described in **chapter 7 and 8**, these treatment strategies might ameliorate the anemia, but at the same time leave the patient susceptible to serious side effects. Therefore, the term "benign" seems inaccurate do describe the forms of HHA described in this thesis.

Even if "benign" would be interpreted solely as the opponent of malignant, defined as "tending to produce death or deterioration", the comparison is flawed. As an example: although survival estimates have continued to improve, the life expectancy of for instance SCD is still shortened by at least two decades compared to the general population.(23-26) Actually, SCD is one of the most common genetic causes of sickness and death.(27)

Yet, both doctors and patients are "stuck" with the label of "benign" hematology. Or, as a professor of medicine in the Division of Hematology and Oncology at the University of North Carolina School of Medicine wrote in a column for American Society of Hematology Clinical News in 2015: "for better or worse, benign hematology just doesn't seem as exciting as malignant hematology".(28) As much as this "stigma" could be a problem when attracting future research candidates, the real problem of course is for the patients. There are numerous examples of patients who, upon coming to the UMC Utrecht for the studies described in this thesis, had simply heard that living with HHA would not cause any problem for them, as their bodies were adjusted to their anemia level. In that light, our findings in **chapter 2** are very relevant. At first glance, the fact that we found that patients with HHA report similar quality of life to healthy pears seems to confirm the assumption that living with HHA does not cause any problems for our patients, because their bodies are adjusted to their

level of anemia. However, exactly the latter, the fact of not only patients' bodies, but their complete lives being adjusted to HHA, is what makes patient reported research on quality of life difficult to interpret for patients with chronic diseases. After all, it is conceivable that living with a chronic disease has led to normalization of the condition and to a lifestyle that fits not only the patient, but also the disease.

Interpretation tools like the composite Time Trade-off scale used in our study are useful in making some first steps into objectifying quality of life results. With use of these tools, we were able to show "the other side" of quality of life in HHA: two patients included in the study had a quality of life of life score, that, on the composite Time Trade-off scale ranked below zero. This indicates that the Dutch reference population would consider their quality of life status as bad, or even worse than death.

Probably, we will not be able to truly measure quality of life in HHA, until we are able to cure HHA. Only then, when patients are able to judge their lives with and without the disease, will we get a true understanding of what living with HHA means.

The field of medicine is constantly evolving and so should our medical language. Nowadays, some of our originally malignant, incurable diseases have become treatable or even curable. At the same time, this thesis is one of the many examples of benign diseases actually being quite malignant. In society, currently, more and more attention is paid to correct and inclusive language, aiming for communication that avoids using words, expressions or assumptions that would unnecessarily exclude people, conveying respect to all people. Therefore, now might be an excellent time to retire the strict division between the two fields and simply go with the term "hematologic" disease.

When you hear hoof beats…is HHA a "rare disease"?

In first year of medicine, students are being taught a simple phrase: When you hear hoof beats, think horses, not zebra's. In other words: when a patient has a symptom, it is likely that it is related to something very common, instead of something rare and exotic.

According to the European Commission, all the diseases included within the term HHA are classified as "rare".(29) Affecting less than 5 in 10,000 people, rare diseases face specific challenges due to scarcity of both resources and expertise. It is not surprising that these diseases, affecting only a few people per country, in the past failed to get much attention.

As an example: in PKD, reported cases are fewer than predicted. This is thought to be related to underdiagnosis, delayed diagnosis and/or misdiagnosis.(20) For some patients, it takes months or even years before a final diagnosis is made.(30)

At the same time, scientific break-throughs are limited, as from a commercial point of view, the development of for instance new medication that can only be used for a handful of patients, is not very attractive.

However, since the appearance of the definition of rare disease in European Union legislation in 1999, several initiatives were started to bring the spotlight on rare diseases. In 2006, the E-Rare consortium was built to link responsible funding organizations and ministries to combine the scarce resources for rare disease research. (31) They already successfully enabled several transnational research projects via Joint Transnational Calls. In 2011, the European Commission and the US National Institutes of Health initiated the International Rare Diseases Research Consortium (IRDiRC) and currently, there is a special call for funding for rare diseases through the European Research and Innovation program Horizon 2020.

In this light, it is hopeful that in 2018, of the 2404 abstracts submitted to the 23th European Hematology Association Congress, about 6% was related to HHA.(32) In Haematologica, currently one of the most important journals related to hematology, 4% of the articles published between august 2017 and august 2018 was related to HHA.(33)

When there is research, there is hope for progress. This is very necessary, because, although HHA might affect a small number of lives, HHA has the dubious honor to be one of the top causes of years lived with anemia. (34) Moreover, in young children HHA is one of the top causes of years lived with disability.(35, 36)

Much still needs to be done to get healthcare providers, insurance companies and patients adequately educated. It is important to remember that once every now and then, the hoof beats actually will be a zebra coming our way, and when that happens, we should be ready.

No transfusions, no problems?

Transfusion independent patients with HHA are usually considered very mildly affected. This is reflected by clinical practice, as these patients are often only monitored sporadically. Several patients included in our studies were referred back to a general practitioner when transitioning from pediatric to adult care. One of the main focuses of **chapters 2, 3 , 7 and 8** was the question whether the assumption that transfusion independent patients are mildly affected is justified.

In order to properly study this question, correct categorization of patients is essential. Normally, patients are categorized as either transfusion dependent or transfusion independent. However, the cut-off to defining the difference is variable. (37-41) Moreover, often patients that are transfusion independent as an adult, were regularly transfused as a child, before splenectomy was performed.

Therefore, in the studies described in this thesis, we did not split the patients into two, but into three transfusion categories: transfusion dependent, sporadically transfused and never-transfused. Although unusual, adding a "never-transfused" category creates a very solid, non-debatable cut-off, completely eliminating the confounding effects of transfusions.

As can be appreciated from the aforementioned chapters, even patients who never received a transfusion in their life are at risk for organ involvement and iron overload. Therefore, we would suggest that áll patients with HHA, also patients that are seemingly mildly affected, are evaluated by a specialist care team at least once. From the age patients are able to undergo MRI without the need for sedation, a T2*MRI should be performed at least once, and should be repeated at minimum when plasma ferritin becomes >500ng/mL, transferrin saturation level becomes >45%, one of both shows a rising trend, or after a change in treatment such as the start of chronic transfusion therapy.

Research regarding rare diseases: With the patient, for the patient?

This thesis could not have been completed without the use of patient documentation. We performed a chart review of all the patients included in the ZEbRA-study, the PKD natural history study, the Iron in the Netherlands study and of the first patients of the TaPIR study. These charts are a visible reminder of how much is accomplished in the last few years regarding patient registration. Especially for rare diseases like HHA, proper registration and documentation, exchanges of knowledge, documentation and (international) collaboration with experts and patients are key.

The other key factor indispensable for studying rare diseases is of course the patient. One should only study rare diseases wíth the patient and fór the patient. The latter implies that study results are not just communicated back to the research field, but also to the patients involved. By communicating research developments to patients, especially patients with rare diseases, patients get the opportunity to function as their own advocates, working together with their medical care team to analyze what new developments in the field might have implications for them.

Officially, a researcher does not have a legal obligation to share the results of conducted research at all. Although the Medical Research (Human Subjects) Act (WMO: Wet medisch-wetenschappelijk onderzoek met mensen) states that conducted research should be made centrally accessible by the Central Committee on Research Involving Human Subjects (CCMO) (article three, first paragraph, first indent), the researcher has the possibility to object to this.(42) The World Medical Association Declaration of Helsinki points out a moral obligation to publish research (paragraph 36).(43) However, there are no legal or ethical obligations to provide feedback to the participating human subjects. The template Subject Information Sheet as provided by the Medical Ethical Committee of the UMC Utrecht does contain a passage stating that after processing of the data, the investigator will inform the patient about the most important results. However, often communicating information back to the patient is crowded out by all the other duties of a researcher.

At the same time, currently there is a lot of mistrust towards research. Although the public trust in science, with a 7.1 out of 10, is higher than the public trust in for instance the government (5.5 out of 10), 23% of Dutch people think that researchers

change the research to get the answers that they want to get. Around 60% of Dutch people thinks that the government and industry will try to stop publications of results that do not fit in with their vision.(44)

There is much to gain for researchers, patients and public opinion in regards to clear and open communication. Personally, I think that communicating results back to the patients should be an ethical obligation for everyone conducting research involving human subjects. This does not necessarily imply increased workload or the development of new communication tools: a first step could be to publish the Dutch laymen summary that is included in every thesis on an easily accessible website.

Future perspectives

Extracellular vesicles in disease, future or fairytale?

In **chapter 5**, we discussed the possible role of PS-exposing extracellular vesicles (EVs) in HHA. The field of EV-research is relatively young, and exciting studies on the role of EVs as disease markers, intracellular communicators, therapeutic targets and even possibly therapeutic vehicles are published every day.

However, the important challenge in EV-research is the establishment of robust methods to visualize and quantify EVs. In 2010, the International Society for Thrombosis and Hemostasis initiated a project aimed at standardizing enumeration of extracellular vesicles by flow cytometry.(45) In 2013, the ISEV (International Society for Extracellular Vesicles) published a series of position papers on analysis methods for EV-research.(46, 47)

Unfortunately, five years later this has not lead to a generally accepted gold standard method for sample collection, isolation or analysis of EVs. (48, 49) As a matter of fact, the term "extracellular vesicles" was introduced by ISEV, because there are no straightforward criteria to isolate and identify and distinguish cell derived vesicles.(50)

Although no field can show any progress without pioneers, standardization is a prerequisite to validate results. Awaiting gold standards, I personally think that an extensive description of (pre)analytical methods used is indispensable for every article related to EVs.

Pyruvate kinase to the rescue?

In **chapter 6** we described a pilot experiment in which we studied the red cell PK enzyme in SCD. Although our results are preliminary, they implicate that PK might not be only relevant for the small population of patients with PKD, but might be an interesting target in SCD too.

In this light, the development of a new drug AG-348 that is currently being tested in a clinical trial in patients with PKD (51), is very exciting as it might not only mean a treatment option just for patients with PKD, but possibly also for SCD patients.

The phase 1 study showed that in healthy volunteers AG-348 was able to cause dose dependent changes in blood glycolytic intermediates consistent with Embden Meyerhof pathway activation. (51) This implies that, regardless of the assumed underlying secondary PKD in SCD, AG-348 has the potential to restore PK enzymatic function and to improve energy generated in the red cells of not only SCD-patients but also other anemias.

Although not in the scope of this thesis: the potential relevance of unraveling a phenomenon of secondary PKD and consequent retrograde accumulation of 2,3-DPG might be even bigger. Imagine the possibilities if we would be able to mimic secondary PKD. In acute situations, for instance cardiac arrest, systemic shock, or less dramatic: altitude sickness, when there is insufficient time available for 2,3 DPG to adapt to the new circumstances, it might be very interesting to be able to temporarily create high 2,3-DPG levels.

Clinical practice

Recommendations and considerations for future clinical practice.

One of the goals of this thesis was to contribute to taking first steps towards future guidelines for diagnosis and treatment of clinical consequences of HHA. Therefore, we made several recommendations regarding minimal requirements of patient care and considerations related to the care for patients with HHA:
Recommendations and consideration regarding clinical practice:

Recommendations:

- Ferritin levels below 1000ng/ml, young age or absence of a transfusion history do not exclude the possibility of iron overload in HHA. We recommend therefore that all patients with rare HHA, possibly except those with ferritin levels below 500ng/ml and transferrin saturation below 45%, should be evaluated for iron overload with MRI.

- Organ involvement is a point of concern for all patients with HHA. We recommend regular screening for at least iron overload, osteoporosis, vitamin D and sex hormone deficiency and microalbuminuria.

- Pulmonary hypertension is a rare, but dangerous complication of HHA. We recommend to screen for pulmonary hypertension in patients with HHA and follow those with increased tricuspid regurgitant velocity with more attention.

Considerations:

- Although HSCT should be considered an investigational treatment for patients with PKD, the strong decline in survival of patients treated after the age of ten suggests the need to evaluate HSCT as a treatment option early in life.

- Especially transfusion dependent patients and patients with hemoglobin disorders seem to be at risk of experiencing relatively low HR-QoL. Improving social quality of life and treating organ involvement could be an interesting future focus to increase HR-QoL.

Closing remarks

In conclusion, this thesis underscores the malignant side of benign HHA. We described several important features of HHA and discussed recommendations and considerations for future clinical practice. We performed experimental research regarding EVs and the enzyme PK, that could potentially lead to increased understanding of HHA and possibly even new treatment options.

None of this work could have been performed without the help of the patients described in this thesis. We hope that, with the work described, we have made a small contribution towards bringing them and their disease in the spotlight. To cite the Association of American Medical Colleges: research means hope. Every step we take is another one on our way to finally finding a treatment for HHA.

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& ADDENDUM

DUTCH SUMMARY/ NEDERLANDSE SAMENVATTING

Dit proefschrift is het resultaat van het promotieonderzoek van drs. H.A.S. van Straaten naar zeldzame vormen van bloedarmoede, met de naar het Nederlands vertaalde titel: Erfelijke hemolytische anemie: Klinische gevolgen en pathofysiologie.

De hiernavolgende samenvatting bestaat uit een korte inleiding, gebaseerd op hoofdstuk 1 van het proefschrift, gevolgd door een vertaling van de Engelse samenvatting zoals weergegeven in hoofdstuk 10 van het proefschrift. Voor de begrijpelijkheid worden de medische begrippen gevolgd door een korte uitleg tussen haakjes.

Dit proefschrift is geschreven met als doel een bijdrage te leveren aan de kennis op het gebied van zeldzame vormen van erfelijke hemolytische *(versnelde bloedafbraak)* bloedarmoede, als eerste stap in de richting van toekomstige richtlijnen met betrekking tot de diagnose en behandeling van erfelijke hemolytische bloedarmoede.

Het proefschrift had drie concrete doelen:

1. Het verbeteren van de kennis van de ziektelast van erfelijke hemolytische bloedarmoede

2. Het onderzoeken van de pathofysiologie *(het ziektemechanisme)* van erfelijke hemolytische bloedarmoede

3. Het evalueren van behandelingen die op het moment worden toegepast voor erfelijke hemolytische bloedarmoede.

Het proefschrift is opgebouwd in drie delen, om de bovenstaande drie vragen te beantwoorden. Elk deel bestaat weer uit meerdere onderzoeksvragen, die in de samenvatting worden beantwoord.

Deel 1: van diagnose naar klinische symptomen

- Wat is de kwaliteit van leven van patiënten met erfelijke hemolytische bloedarmoede?

- Hebben patiënten met zeldzame vormen van erfelijke hemolytische bloedarmoede, net als patiënten met sikkelcelziekte en thalassemie last van orgaanschade, en moeten we hier in de dagelijkse praktijk alert op zijn en onderzoek naar doen?

- Is de 6-minuten wandeltest een handige manier om pulmonale hypertensie *(verhoogde druk in de longslagaders)* vast te kunnen stellen?

Deel 2: van klinische symptomen naar pathofysiologie *(ziektemechanisme)*

- Spelen extracellulaire microblaasjes *(zeer kleine –enkele nanometers grote-deeltjes van cellen die los in de bloedbaan voorkomen)* een rol bij het ontstaan van hypercoagulabiliteit *(een verhoogd risico op bloedstolsels)* na het verwijderen van de milt in patiënten met erfelijke hemolytische bloedarmoede?

- Hebben rode bloedcellen van patiënten met sikkelcelziekte en andere vormen van hemoglobine afwijkingen een verworven verminderde stabiliteit van het enzym *(eiwit)* pyruvaatkinase in rode bloedcellen? En zo ja, heeft dit invloed op klinische symptomen en de ernst van de ziekte?

Deel 3: van pathofysiologie naar behandeling

- Hebben alle patiënten met erfelijke hemolytische bloedarmoede een verhoogd risico op ijzerstapeling? Kunnen de richtlijnen over ijzerstapeling, gebaseerd op ervaring met β-thalassemie, ook toegepast worden bij andere zeldzame vormen van erfelijke hemolytische bloedarmoede?

- Is stamceltransplantatie een succesvolle behandeloptie voor patiënten met pyruvaatkinasedeficiëntie?

SAMENVATTING

Deel 1: van diagnose naar klinische symptomen

Hoofdstuk 1 is een korte introductie over erfelijke hemolytische bloedarmoede. Erfelijke hemolytische bloedarmoede is een verzamelnaam voor alle aangeboren vormen van bloedarmoede die veroorzaakt worden door een versnelde afbraak van de rode bloedcellen.

Erfelijke hemolytische bloedarmoede kan worden ingedeeld in drie categorieën, gebaseerd op de oorzaak van de verhoogde bloedafbraak. De eerste categorie omvat de hemoglobineafwijkingen: hemoglobine is een gespecialiseerd eiwit in de rode bloedcel dat ervoor zorgt dat de rode bloedcel zuurstof kan opnemen uit de longen en kan vervoeren naar het lichaam, en koolstofdioxide vanuit het lichaam terug kan voeren naar de longen. De belangrijkste vormen van hemoglobineafwijkingen zijn sikkelcelziekte en β-thalassemie. De tweede categorie omvat de enzymafwijkingen. Enzymen zijn gespecialiseerde eiwitten die een rol spelen in de stofwisseling. De enzymen in de rode bloedcel spelen een belangrijke rol in de energievoorziening en bescherming van de rode bloedcel, en een afwijking van een van deze enzymen kan leiden tot versnelde afbraak van de rode bloedcel. De belangrijkste vormen

van enzymafwijkingen zijn glucose-6-fosfaat dehydrogenase (G6PD) deficiëntie en pyruvaatkinasedeficiëntie (PKD).

De laatste categorie zijn de membraanafwijkingen. Een membraan is het buitenste laagje, oftewel de barrière van een cel. De membraan van rode bloedcellen is stevig, maar ook heel buigzaam, waardoor de cel goed van vorm kan veranderen. Dit is belangrijk, omdat de cel door hele nauwe vaten moet kunnen bewegen. Als er iets mis is met de membraan van een rode bloedcel, kan dit de vorm en flexibiliteit van de cel beïnvloeden en ook dit kan leiden tot verhoogde bloedafbraak. De belangrijkste vormen van membraanafwijkingen zijn erfelijke sferocytose en erfelijke xerocytose.

Veel vormen van erfelijke hemolytische bloedarmoede zijn zeldzaam. Daarom is nog niet alles over deze ziektes bekend.

Hoofdstuk 2 verschaft inzicht in de gezondheidsgerelateerde kwaliteit van leven van patiënten met erfelijke hemolytische bloedarmoede. Hiervoor vulden 83 patiënten twee vragenlijsten in over kwaliteit van leven.

Het is hoopvol dat patiënten zeggen gemiddeld genomen een kwaliteit van leven te hebben die vergelijkbaar is met de kwaliteit van leven van een gezonde referentiegroep. Hierbij is het wel van belang dat patiënten met hemoglobineafwijkingen en patiënten die afhankelijk zijn van bloedtransfusies gemiddeld lager scoorden dan patiënten met andere vormen van hemolytische bloedarmoede en patiënten die niet transfusieafhankelijk zijn. In ons onderzoek hing kwaliteit van leven niet samen met de mate van bloedarmoede, maar wel met de afstand die patiënten konden afleggen in de 6-minuten wandeltest en met de mate van orgaanbetrokkenheid en orgaanschade van deze patiënten. Ook viel op dat er met name een verschil was op het gebied van sociale kwaliteit van leven tussen patiënten met hoge en lage kwaliteit van leven scores.

Op basis van deze bevindingen concludeerden wij dat het behandelen van orgaanbetrokkenheid en orgaanschade en het verbeteren van sociale steun mogelijk een interessant doel zou kunnen zijn om in de toekomst kwaliteit van leven van patiënten met erfelijke hemolytische bloedarmoede te verbeteren, met name van transfusieafhankelijke patiënten en patiënten met hemoglobine afwijkingen.

Doordat de levensverwachting van patiënten met erfelijke hemolytische bloedarmoede is toegenomen, krijgen steeds meer patiënten te maken met chronische orgaanbetrokkenheid en orgaanschade. Het is bekend dat in sikkelcelziekte en β-thalassemie orgaanbetrokkenheid een belangrijke voorspeller is van morbiditeit *(mate van ziekzijn)* en prognose *(vooruitzicht)*. Voor de andere vormen van bloedarmoede is dit echter niet bekend en er zijn geen duidelijke richtlijnen met betrekking tot het screenen en behandelen van orgaanbetrokkenheid en orgaanschade.

Voor hoofdstuk 3 hebben we de medische dossiers van 90 patiënten met erfelijke hemolytische bloedarmoede bekeken en beoordeeld op het voorkomen van orgaanbetrokkenheid en orgaanschade. Wij hebben laten zien dat orgaanbetrokkenheid, zoals trombose *(stolselvorming)* en ijzerstapeling in de organen, niet alleen voorkomt bij patiënten met sikkelcelziekte en β-thalassemie, maar ook bij patiënten met andere vormen van erfelijke hemolytische bloedarmoede.

Een belangrijke bevinding was daarnaast dat orgaanbetrokkenheid niet alleen voorkomt bij patiënten die (vaak) bloedtransfusies krijgen, maar ook bij patiënten die nog nooit een bloedtransfusie hebben ontvangen.

Hoewel meer onderzoek nodig is om uit te zoeken hoe vaak orgaanbetrokkenheid precies voorkomt bij patiënten met erfelijke bloedarmoede, stellen wij voor om in de tussentijd patiënten op zijn minst te screenen voor die vormen van orgaanbetrokkenheid die naar verwachting vaak voorkomen en goed behandelbaar zijn. Gebaseerd op dit onderzoek zijn dat bijvoorbeeld ijzerstapeling, osteoporose *(botontkalking)* vitamine D tekort, geslachtshormoontekorten en microalbuminurie *(het voorkomen van kleine hoeveelheden eiwit in de urine)*.

Een van de meest gevaarlijke, maar zeldzame vormen van orgaanbetrokkenheid in erfelijke hemolytische bloedarmoede is pulmonale hypertensie *(verhoogde druk van de longslagaders)*. Pulmonale hypertensie komt voor bij ongeveer 6 tot 10% van de patiënten met sikkelcelziekte, maar voor andere vormen van bloedarmoede is er weinig bekend.

Het is belangrijk om te kunnen vaststellen welke patiënten (een verhoogd risico op) pulmonale hypertensie hebben. De test die hier het meest voor wordt gebruikt is rechterhartcatheterisatie *(een drukmeting in het hart zelf)*. Dit is echter een ingrijpende procedure. Daarom wordt er vaak gekozen voor een alternatieve test, bijvoorbeeld door middel van onderzoek met echografie, waarbij gekeken kan worden naar de tricuspide regurgitant jet flow (TRV); de bloedstroom rondom een van de hartkleppen, maar deze test geeft minder zekerheid. Een verhoogde TRV is niet alleen geassocieerd met pulmonale hypertensie, maar ook met verhoogde kans op overlijden.

Uit onderzoek bij patiënten met sikkelcelziekte en primaire pulmonale hypertensie is gebleken dat een 6-minuten wandeltest, samen met het echografie-onderzoek meer zekerheid kan geven. Patiënten die een verhoogde TRV en een verlaagde afstand op de 6-minuten wandeltest hebben, hebben een grotere kans op pulmonale hypertensie. Daarom is de 6-minuten wandeltest in 2014 opgenomen in de richtlijnen van de American Thoracic Society voor de diagnose van pulmonale hypertensie in sikkelcelziekte. In sikkelcelziekte is een verhoogde TRV niet alleen geassocieerd met pulmonale hypertensie, maar ook met een verhoogd risico op overlijden.

In hoofdstuk 4 hebben we onderzoek gedaan naar de diagnose pulmonale hypertensie. In onze studiegroep bevonden zich geen patiënten die de op basis van

rechterhartcatheterisatie onderzoek de diagnose pulmonale hypertensie hadden gekregen. Daardoor konden we niet onderzoeken of het toevoegen van de resultaten van de 6-minuten wandeltest aan de resultaten (TRV) van het echo-onderzoek bijdroegen aan het stellen van de diagnose pulmonale hypertensie.

Wel waren er meerdere patiënten met andere diagnoses dan sikkelcelziekte die bij echografieonderzoek een TRV hadden van meer dan 2,5 meter per seconde. Voor patiënten zonder bloedarmoede zou dit worden geïnterpreteerd als een hoog-normale uitslag, maar bij patiënten met sikkelcelziekte wordt een TRV van meer dan 2,5 meter per seconde al gezien als verhoogd, omdat de bloedarmoede zelf ook al zorgt voor een andere dynamiek van het bloed dat door het hart gepompt wordt. Omdat ditzelfde opgaat voor patiënten met andere vormen van bloedarmoede, stelden wij voor dat ook patiënten met een andere vorm van bloedarmoede dan sikkelcelziekte onderzocht moeten worden op tekenen van pulmonale hypertensie en dat er bij patiënten met een TRV van meer dan 2,5 meter per seconden meer aandacht moet worden gegeven aan symptomen van hartfalen dan de standaard routine vereist.

Deel 2: van klinische symptomen naar pathofysiologie

Het tweede deel van dit proefschrift is gewijd aan de associatie tussen klinische symptomen en pathofysiologie. Zoals besproken in hoofdstuk 3 is trombose *(stolselvorming)* een relatief veel voorkomende complicatie van erfelijke hemolytische bloedarmoede. De oorzaak hiervan is nog niet helemaal duidelijk. In hoofdstuk 5 onderzochten wij de theorie dat een toegenomen aantal van fosfatidylserine positieve microblaasjes *(zeer kleine – nanometers grote- deeltjes van cellen die los in de bloedbaan voorkomen en die fosfatidylserine, een fosfolipide die normaal alleen aan de binnenkant van de celmembraan aanwezig is, op de buitenkant van het membraan tot expressie brengen)* na een operatieve verwijdering van de milt zouden kunnen bijdragen aan hypercoagulabiliteit (*verhoogde stollingsneiging*) van patiënten met erfelijke hemolytische bloedarmoede. In een onderzoek met 97 patiënten, in samenwerking met het Vesicle Observation Center van het Amsterdam UMC in Amsterdam hebben we onderzoek gedaan naar microblaasjes met een speciaal apparaat om deze te meten: een speciale flowcytometer.

We hebben in het laboratorium getest of microblaasjes in bloedplasma waaruit de bloedplaatjes waren verwijderd van patiënten met en zonder milt in staat waren om stolling te veroorzaken. Daarbij hebben we met name gekeken naar fosfatidylserine-positieve microblaasjes, omdat bekend is dat fosfatidylserine, als het zich aan de buitenkant van de celmembraan bevindt, stolling kan stimuleren. Normaal speelt de milt een belangrijke rol bij het verwijderen van cellen in de bloedbaan die fosfatidylserine aan de buitenkant van de cel hebben.

In ons onderzoek zagen we inderdaad dat bloedplasma van patiënten bij wie de milt verwijderd was meer fosfatidylserine-positieve microblaasjes bevatte dan bloedplasma van patiënten met milt. De hoeveelheid fosfatidylserine-positieve microblaasjes hing samen met de mate van stollingsactivatie in het lichaam, welke was getest door middel van het meten van D-dimeerlevels in het bloed. Ook hing het samen met de mate waarin de microblaasjes in het laboratorium in staat waren om stolling te activeren. Op basis van deze resultaten suggereren wij dat er mogelijk een rol is voor fosfatidylserine-positieve microblaasjes in het veroorzaken van een verhoogde stollingsneiging na het verwijderen van de milt in patiënten met erfelijke hemolytische bloedarmoede.

In dit deel van het proefschrift hebben we ook onderzoek gedaan naar verhoogde hoeveelheden 2,3-difosfoglyceraat (2,3-DPG) in rode bloedcellen van patiënten met sikkelcelziekte en de relatie van deze stof met oxidatieve stress *(een schadelijke disbalans tussen schadelijke vrije zuurstofradicalen enerzijds en beschermende -anti-oxidante- mechanismen anderzijds).* 2,3-DPG is een tussenproduct van de glycolyse *(omzetting van glucose in de cel om energie te produceren)* in de rode bloedcel. 2,3-DPG speelt een belangrijke rol in het evenwicht van zuurstofbinding aan hemoglobine. Onder invloed van 2,3-DPG laat het hemoglobine de zuurstof gemakkelijker los, waardoor de zuurstofafgifte aan weefsels wordt vergemakkelijkt. Echter, de rode bloedcellen van patiënten met sikkelcelziekte hebben juist als ze geen zuurstof gebonden hebben de neiging om te vervormen naar de stijve sikkelvorm die zo karakteristiek is voor de ziekte. Sikkelvorming van rode bloedcellen is de meest belangrijke oorzaak van hemolyse in sikkelcelziekte. Daarnaast vergroot sikkelvorming de kans op orgaanschade van patiënten met sikkelcelziekte.

In hoofdstuk 6 beschreven we een pilot onderzoek naar de rol van het enzym pyruvaatkinase in rode bloedcellen van patiënten met hemoglobine afwijkingen zoals sikkelcelziekte. We onderzochten de theorie dat in sikkelcelziekte, oxidatieve stress kan leiden tot een verworven vorm van PKD, wat zich zou uiten in verminderde stabiliteit van het PK-enzym. Het PK-enzym is betrokken bij de laatste stap van de glycolyse en een verminderde functie zou daardoor kunnen leiden tot retrograde *(achterwaartse)* stapeling van alle andere tussenproducten van de glycolyse, zoals 2,3-DPG. In ons onderzoek zagen we dat patiënten met sikkelcelziekte, in vergelijking met gezonde personen, een verminderde (hitte)stabiliteit van het PK-enzym in de rode cel hadden. Om dit fenomeen verder te onderzoeken, behandelden we een bloedmonster van zowel een gezond persoon als van een patient met sikkelcelziekte in het laboratorium met de stof tert-butylhydroperoxide (tBHP), om oxidatieve stress na te bootsen, waarbij we opnieuw afname van de stabiliteit van het PK-enzym zagen. PK-stabiliteitstesten in bloedmonsters van patiënten met vormen van erfelijke hemolytische bloedarmoede die bekend staan gepaard te gaan met veel oxidatieve stress, lieten opnieuw PK-instabiliteit zien. Dit suggereert dat oxidatieve stress, danwel

een verminderde werking van beschermingsmechanismen tegen oxidatieve stress in de rode cel, mogelijk inderdaad in staat is om PK-instabiliteit te veroorzaken. Echter, onze onderzoek was vooral een vooronderzoek en uitgebreider onderzoek is nodig om de precieze rol van PK stabiliteit in hemoglobine afwijkingen, en andere vormen van hemolytische bloedarmoede, te bepalen. Daarom zijn wij inmiddels gestart met een nieuwe studie, genaamd de TApIR-study, die in 2018-2019 plaats zal vinden.

Deel 3: van pathofysiologie naar behandelstrategie.

Een van de belangrijkste complicaties van erfelijke hemolytische bloedarmoede is ijzerstapeling. Dit kan onder andere optreden door bloedtransfusies, doordat de toegediende rode bloedcellen veel ijzer bevatten. Ijzerstapeling kan ook worden veroorzaakt doordat de darmen van sommige patiënten met hemolytische anemie automatisch te veel ijzer opnemen uit het voedsel, om te gebruiken voor het maken van nieuwe rode bloedcellen.

Ijzerstapeling is uitgebreid onderzocht in β-thalassemie, maar over ijzerstapeling in andere vormen van erfelijke hemolytische bloedarmoede is veel minder informatie beschikbaar.

In hoofdstuk 7 beschreven we daarom een onderzoek naar ijzerstapeling in 44 patiënten met zeldzame vormen van erfelijke hemolytische bloedarmoede. Als vergelijkingsmateriaal gebruikten we de resultaten van 68 patiënten met sikkelcelziekte en β-thalassemie. We concludeerden dat ijzerstapeling voorkomt in alle vormen van erfelijke hemolytische bloedarmoede die onderdeel uitmaakten van de studie. Ook zagen we dat ijzerstapeling kan voorkomen in patiënten die nog nooit een rode bloedceltransfusie hebben ontvangen. Daarnaast ontdekten we dat de afkapwaarde van de meest gebruikte methode om ijzerstapeling vast te stellen, een bepaling van de hoeveelheid van het eiwit ferritine in het bloed, weinig voorspellend was voor de mate van ijzerstapeling in de lever, zoals vastgesteld door middel van een speciale MRI. Dit is een bevinding met belangrijke gevolgen voor de huidige praktijk, omdat het suggereert dat alle patiënten, mogelijk met uitzondering van patiënten met zeer lage waarden van ferritine en transferrine saturatie in het bloed, onderzocht moeten worden op ijzerstapeling door middel van een MRI.

De vorige hoofdstukken van dit proefschrift waren gericht op alle vormen van erfelijke hemolytische bloedarmoede en alleen op volwassenen. In de laatste twee hoofdstukken echter, focusten wij ons specifiek op de ziekte PKD. Net als in hoofdstuk 7 beschreven wij in hoofdstuk 8 een onderzoek naar ijzerstapeling. Echter, dit keer onderzochten wij een groot cohort van PKD-patiënten van alle leeftijden. In een onderzoek met 242 PKD patiënten vonden wij dat ijzerstapeling ook voorkwam in patiënten die nog nooit een bloedtransfusie hadden ontvangen. Ook vonden we opnieuw dat de afkapwaarde voor ferritine in het bloed een matige voorspellende waarde had voor ijzerstapeling in de lever. Daarnaast vonden we dat ijzerstapeling

vaak voorkomt in patiënten met PKD en dat het op alle leeftijden voor kan komen. De jongste patient met ijzerstapeling in het hart was nog maar drie jaar oud. Wij concludeerden dat regelmatig onderzoek naar ijzerstapeling, en het starten van eventuele behandeling van ijzerstapeling, erg belangrijk is in de behandeling van patiënten met PKD.

Bij erfelijk hemolytische bloedarmoede zijn de meeste behandelopties vooral ondersteunend en niet genezend. Voorbeelden hiervan zijn het verwijderen van de milt en het geven van bloedtransfusies. Echter, zoals beschreven in onder andere hoofdstuk 7 en 8, kunnen deze ondersteunende behandelopties gepaard gaan met belangrijke complicaties en bijwerkingen. Met name voor sikkelcelziekte en β-thalassemie zijn er gelukkig steeds meer mogelijkheden om door middel van gentherapie de ziekte te genezen, maar voor andere vormen van hemolytische bloedarmoede is de enige optie voor het genezen van de ziekte een stamceltransplantatie. Hierbij worden de stamcellen van de patient vernietigd, waarna stamcellen van een gezond persoon toegediend worden aan de zieke persoon. Er is nog maar weinig ervaring met het toepassen van stamceltransplantatie in PKD. De meeste informatie die nu beschikbaar is, is gebaseerd op onderzoek in proefdieren, en er zijn nog geen richtlijnen voor stamceltransplantatie in PKD. In hoofdstuk 9 beschreven wij een wereldwijde inventarisatie van alle patiënten met PKD die behandeld waren met een stamceltransplantatie. We onderzochten de indicatie, de gebruikte methoden en de uitkomst van de transplantatie, als eerste stap voor het ontwikkelen van toekomstige behandelrichtlijnen.

We vonden 16 patiënten die tussen 1996 en 2015 een stamceltransplantatie hadden ondergaan. We concludeerden dat, in vergelijking met reeds gepubliceerde overlevingscijfers in andere vormen van erfelijke hemolytische bloedarmoede, de overleving na stamceltransplantatie in PKD relatief laag was. De driejaarsoverleving *(het percentage patiënten dat drie jaar na de transplantatie nog in leven is)* was slechts 65%. Wel zagen we een duidelijk betere overleving van patiënten die op jonge leeftijd, voor het tiende levensjaar, een transplantatie hadden ondergaan.

Daarnaast zagen we dat relatief veel patiënten een ernstige vorm van de belangrijke bijwerking Graft-*versus*-Host Disease kregen en dat de sterfte onder deze patiënten ook hoog was.

Op dit moment wordt stamceltransplantatie niet gezien als een standaard behandeloptie in PKD. Echter, wij concludeerden dat in, het licht van de verslechterende overleving op oudere leeftijd, een stamceltransplantatie met name voor jonge patiënten als behandeloptie kan worden overwogen.

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

2018 **Stephanie van Straaten**, Jean-Louis Kerkhoffs, Jerney Gitz-Francois, Bart J. Biemond, Richard van Wijk and Eduard J. van Beers
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2018 EHA Travel grant, 23rd Congress of the European Hematology Association, Stockholm, Sweden

2017 EHA Travel grant, 22nd Congress of the European Hematology Association, Madrid, Spain

Presentations and Posters

2018 Oral presentation, 60th ASH Annual Meeting and Exposition in San Diego, California, USA:
"Phosphatidylserine-exposing extracellular vesicles after splenectomy are associated with increased D-dimers and fibrin generation in hereditary hemolytic anemia"

2018 Poster presentation, 23th Congress of European Hematology Association, Stockholm, Sweden:
"Iron overload in patients with rare hereditary haemolytic anaemia: evidence based suggestion on whom and how to screen"

2018 Poster presentation, 23th Congress of European Hematology Association, Stockholm, Sweden:
"Quality of life in patients with rare hereditary haemolytic anemia, the importance of social support"

2018 Poster presentation, 23th Congress of European Hematology Association, Stockholm, Sweden:
"Organ involvement occurs in all forms of hereditary hemolytic anemia"

2018 Oral presentation: Dutch Hematology congress, Papendal, The Nederlands: "Iron overload in patients with rare hereditary haemolytic anaemia: evidence based suggestion on whom and how to screen"

2017 Oral presentation, 22nd Congress of European Hematology Congress, Madrid, Spain: *"Stem cell Transplantation in pyruvate kinase deficiency"*

2017 Oral presentation, 21. European Red Cell Society Meeting, Heidelberg, Germany: *"Stem cell transplantation in pyruvate kinase deficiency"*

2017 Oral presentation, Sanquin Spring Seminar, Amsterdam, The Netherlands: "Iron overload in hereditary anemia"

CURRICULUM VITAE

Hélène Antonie Stéphanie van Straaten was born in March 1987 in Nijmegen, The Netherlands. After graduating *cum laude* from Merletcollege in Cuijk, she moved to Amsterdam in 2005 to attend medical school at the University of Amsterdam. Stéphanie combined her studies with several other activities. In 2007-2008 she was chairwoman of the Medical Student Association of Amsterdam (MFAS). In 2008-2009 she was elected as a board member of the central student advisory board of the University of Amsterdam, of which she spent the last months as chairwoman Research and Education. In 2009-2010 Stéphanie spent six months in Boston for a research internship at the Boston Children's Hospital, studying vitamin D deficiency in inflammatory bowel disease at the department of gastro-enterology. In 2011-2012 she spent 3 months in Tanzania for a clinical rotation in Tropical Medicine.

After graduating *cum laude*, Stéphanie worked as a junior doctor not in training at the pediatric department of Reinier de Graaf Gasthuis in Delft, the pediatric ICU of the Leiden University Medical Center in Leiden, the Wilhelmina Children's Hospital in Utrecht and at De Bascule in Amsterdam.

Stéphanie started her PhD-project in April 2015, under supervision of dr. Eduard van Beers, dr. ing. Richard van Wijk, prof. dr. Roger Schutgens and prof. dr. Wouter van Solinge. During her work as a PhD-student she volunteered twice as a volunteer-doctor for the Boat Refugee Foundation in camp Moria on Lesvos.

During her PhD-project, Stéphanie was involved in several (inter)national collaborations. For her scientific work she received several grants and prizes.

Since January 2019, Stéphanie has been working at the pediatric department of the ETZ, Elisabeth-TweeSteden Ziekenhuis in Tilburg, as part of the pediatric residency program at Erasmus MC-Sophia in Rotterdam.

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Talk about funny people**: Anil**! You too always stay positive, except of course when Demian scared you with your first Marktplaats sale. Good luck with your defense soon, but I know you will do great!

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