HEREDITARY ANEMIAS

MORE COMPLEX THAN EVER

Annelies J. van Vuren

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Hereditary anemias: more complex than ever

Erfelijke anemieën: meer complex dan ooit

(met een samenvatting in het Nederlands)

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Annelies Johanna van Vuren

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te Moordrecht

Promotoren:

Prof. dr. R.E.G. Schutgens

Prof. dr. W.W. van Solinge

Copromotoren:

Dr. E.J. van Beers

Dr. H.A. van Wijk

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General introduction

1

GENERAL INTRODUCTION

Despite significant decreases in global anemia prevalence, the burden of anemia is still high, affecting 27% of the world's population in 2013. And, thereby, anemia is responsible for 61.5 million years lived with disability world-wide.[1,2] Iron-deficiency remains the dominant cause of anemia.[1,3] Regional differences in prevalence and the contribution of cases from specific causes vary widely. In high-income regions hereditary anemias (including hemoglobinopathies) are the number-one cause of anemia these days.[1]

Age-standardized numbers suggest that the slow regression of anemia in high-income countries is overshadowed by improved survival of patients with hereditary anemias. Over the last decades, life expectancy of these patients improved dramatically following the introduction of (safe) red cell transfusions, iron chelating agents, treatment of (blood-borne) infections and screening and treatment of heart complications.[4,5] In the 1950s, before regular (and safe) red cell transfusions were available, the median survival of children with thalassemia major was around 4-5 years. Upon introduction of regular transfusion schemes survival improved towards puberty; children still died young as a consequence of heart failure or endocrine failure.[6] Nowadays, these toxic effects of iron overload on heart and endocrine organs can be largely prevented or reversed by treatment with iron chelating agents, thanks to the ability to monitor cardiac iron loading by MRI and advances in iron chelating regimens.[7,8] Patients diagnosed with thalassemia major are currently living into their 50s or 60s.[7] Malignant transformation, not limited to the liver, as a consequence of decades of exposure to low levels of oxidatively active (labile) iron provides a new challenge nowadays.[6,9]

Iron loading in patients with transfusion-dependent hereditary anemias is linearly related to the number of red cell transfusions,[10] however iron loading is not limited to those receiving regular red cell transfusions. Patients with non-transfusion-dependent thalassemia do develop clinically relevant iron overload, which primarily results from increased intestinal iron absorption in response to ineffective erythropoiesis or stress erythropoiesis. It is unclear at which threshold of liver iron content (LIC), assessed by MRI, iron-related morbidity develops. Withal, in patients with non-transfusion-dependent thalassemia a LIC threshold of 3-5 mg Fe/g dry weight was associated with a significantly higher prevalence of iron-related morbidity, including vasculopathy, endocrine disturbances and osseous complications.[11] A yearly increase in liver iron of 0.5 mg Fe/g dry weight was reported in patients with non-transfusion-dependent thalassemia; this progressive accumulation will result in clinically relevant iron overload in adolescents.[12] Iron-loading in patients with thalassemia that are not transfusion-dependent is much slower than in patients receiving regular red cell transfusions (approximately 8 mg Fe/g dry weight per year with a transfusion burden of

one red cell transfusion every two weeks).[10] Instructions for prescription of iron chelating agents and advices for monitoring iron overload are incorporated in all modern guidelines for management of hemoglobinopathies,[13-16] of which the recommendations have been transferred to treatment guidelines for (or expert reviews on) other hemolytic or dyserythropoietic anemias.[17-20] Iron chelation therapy is currently the only available treatment modality for iron overload for the majority of anemic patients. Treatment-related toxicity is a major concern in daily clinical practice, despite improved gastro-intestinal tolerability of the newer film-coated formulation of deferasirox.[21,22] Also the treatment itself is expensive. In 2020 total expenses of deferasirox, the preferred iron-chelating agent, in the Netherlands were 6.8 million euro (± 7.9 million US dollar), divided over 769 patients. It has been estimated that at least eighty percent of these patients are diagnosed with hereditary anemia.[23]

Hence, the landscape of anemia has dramatically changed, in particular in high-income countries. We have been urged to advance from treating iron deficiency towards managing iron overload in the context of hereditary anemias. Significant advances in our understanding of iron physiology, and the ability to monitor iron loading in liver and heart by MRI, taught us that secondary hemochromatosis occurs in patients with transfusion-dependent and non-transfusion-dependent hereditary anemias.[24-27] Nevertheless, the clinical picture is not identical for all anemic disorders and strongly depends on bone marrow activity, duration of iron exposure and organ distribution of iron overload.[28] The fragile balance between iron and erythropoiesis in patients with hereditary anemias is the central theme of this thesis. In the next sections I will briefly introduce the basic knowledge of the main physiological and pathophysiological concepts of these hereditary anemias.

Erythropoiesis, ineffective erythropoiesis and hemolysis
Red blood cells are the primary carriers of oxygen in the peripheral circulation. This unique oxygen carrying capacity is carried out by one principal red cell component: hemoglobin. Red blood cells arise from lineage-committed progenitors during a process called erythropoiesis. At the end of this process, erythroblasts enucleate to form reticulocytes. These reticulocytes move out of the bone marrow to finish maturation in the peripheral circulation to become mature red blood cells that circulate until senescence.[29,30] The terminal phases of differentiation in the bone marrow, from the stage of colony-forming unit-erythroid (CFU-E) until enucleating erythroblast, take place in so-called erythroblastic islands.[31] The stages of CFU-E/proerythroblasts and early basophilic erythroblasts are erythropoietin (EPO)-dependent. EPO production is tightly regulated and inversely related to oxygen delivery to the renal cortex, where the majority of circulating EPO is produced by interstitial fibroblasts.[32] Survival of CFU-E/pro-erythroblasts depends on EPO: most cells will undergo programmed cell death by apoptosis in the presence of low EPO levels.[33] Under hypoxia, the number of erythroid progenitors surviving the EPO-dependent terminal differentiation period rapidly

increases as a consequence of increased EPO availability, and subsequently the erythroid progenitors advance towards circulating reticulocytes and ultimately mature red cells. EPO-independent mechanisms will contemporaneously provide for expansion of the CFU-E/pro-erythroblast population.[34]

The name 'rare hereditary anemias' refers to a highly heterogeneous subset of rare anemias caused by genetic defects along one of the several stages of erythropoiesis or in different cellular components that affect red blood cell integrity, and shorten life span. Each hereditary anemia is characterized by distinct amounts of hemolysis (red blood cell lysis) and/or ineffective erythropoiesis (failure to fulfill erythroid maturation).[34] Notably, patients diagnosed with in name the same disease, or even a similar mutation, may have completely different disease phenotypes ranging from mild, or even compensated, anemia, towards severe transfusion-dependent anemia.[e.g.35-38] Traditionally the hereditary anemias have roughly been divided in subcategories based on the origin of defect:

- disorders of hemoglobin synthesis (quantitative and qualitative defects including decreased solubility, instability, altered oxygen affinity and altered maintenance of the oxidation state of the heme-coordinated iron), e.g. thalassemia, sickle cell disease, Hb Köln;
- red cell membrane disorders, e.g. hereditary spherocytosis, hereditary xerocytosis;
- red cell enzymopathies, e.g. pyruvate kinase deficiency, glucose-6-phosphate dehydrogenase deficiency; and
- hypo-regenerative and hypo-productive anemias (genetic mutations disrupting the normal erythroid differentiation pathway), e.g. Diamond Blackfan anemia, congenital dyserythropoietic anemia.

Stress erythropoiesis describes the increased erythropoietic response observed in hemolytic anemias, morphologically characterized by expansion of the erythroid progenitors in an attempt to preserve the peripheral red blood cell pool. In cases of ineffective erythropoiesis there is a failure to differentiate and, despite the presence of chronic stress erythropoiesis in response to anemia, a failure to fill the peripheral red cell pool.[39] Hereditary anemias as hereditary spherocytosis and pyruvate kinase deficiency are classic examples of hemolytic anemias, whereas in β-thalassemia ineffective erythropoiesis dominates.

Iron
The regulation between **iron** and erythropoiesis is tightly connected. The importance of this interplay, and the consequences of dysregulation, are illustrated by two extremes: iron deficiency, the number-one cause of anemia word-wide,[40] and iron-overload in response to hereditary anemias.[24] Erythropoiesis strictly depends on the availability of iron to meet the demands for hemoglobin production. Under physiologic conditions 200 billion red cells are produced every day, containing 6 gram hemoglobin and 20 mg of iron.[29] The demand is mostly

supplied by iron recycling from phagocytosis of senescent autologous red blood cells (20-25 mg). Intestinal import of iron compensates for eventual iron loss (bleeding), or increased needs (e.g. pregnancy or hypoxia), and iron stores in hepatocytes serve as a buffer. Due to the absence of a renal or hepatic iron excretion mechanism, body iron levels are only controlled by intestinal absorption.[29,41]

EPO, erythroferrone (ERFE) and hepcidin are regarded as the core determining factors of the iron-erythropoiesis axis. Hepcidin has been entitled as the master regulator of systemic iron homeostasis. Its discovery in 2001 led to the description of the so-called iron-loading anemias.[42,43] Low hepcidin levels explain the mechanism of iron overload in patients with hereditary anemias with either, or both, hemolysis and ineffective erythropoiesis in the absence of red cell transfusions. Hepcidin regulates systemic iron availability by binding to the ferroportin transporter on enterocytes, macrophages and hepatocytes, which blocks its iron transporting canal and initiates its ubiquitination and degradation thereby providing a prolonged effect on iron transport.[42,44,45] Upon EPO stimulation, differentiating erythroblasts rapidly increase ERFE production, which downregulates hepcidin transcription.[46-48]

Notably, intracellular iron handling requires a highly coordinated process as ferrous iron (Fe(II)) is highly reactive and its presence may cause permanent protein and DNA damage. Normally, iron circulates in plasma as ferric iron (Fe(III)) occupying approximately 30% of the transport capacity of the main iron-transport protein transferrin. However, when the amount of total body iron is significantly elevated, the transferrin iron-binding ability is insufficient and nontransferrin-bound iron (NTBI) can be detected.[49-52] Highly reactive Fe(II) subspecies of NTBI are known as labile plasma iron (LPI). LPI has a chemically labile character and a high propensity for redox reactions.[53-55] The presence of high concentrations of oxygen and iron in red blood cells exposes them constantly to oxidative stress as reactive oxygen species (ROS) are formed. Both heme and iron catalyze the Fenton reaction that produces the highly reactive hydroxyl radical from the interaction between superoxide and hydrogen peroxides. Excessive ROS production leads to serious damage to organelles and DNA, or may induce programmed cell death. To provide protection against ROS, repair oxidative damage and to maintain a reducing intracellular environment, (developing) red cells are equipped with a broad variety of antioxidants.[56,57] In hereditary anemias, genetic disruptions of red cell development may disturb this fragile oxidative balance which may have a yet undefined role in the pathophysiology of these diseases. Additionally, iron-related toxicity in other cell types is expected in hereditary anemias. NTBI, present as a consequence of iron-loading will rapidly be cleared from the circulation primarily by the liver and various other organs as heart, endocrine glands and pancreas, where it contributes to tissue iron overload and related pathology.[58] This iron uptake is mediated by a range of NTBI transporters in a tissue-specific

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manner.[59] Notably, the coexistence of inflammation-related oxidative stress may amplify, or even catalyze, the toxic effects of Fe(II).[60] Sickle cell disease is an example of this potential deleterious interplay between iron and inflammation.[61]

Thesis rationale

Each disease, classified as a hereditary anemia, is unique. Still, the diseases share pathophysiological features such as hemolysis and ineffective erythropoiesis, and thereby are alike in their risk of iron-loading. The first aim of my thesis was to test the efficacy of proton pump inhibitors in the treatment of iron overload in patients with non-transfusion-dependent hereditary anemias *via* disruption of intestinal iron absorption. This was studied in the *PPI shine again* trial. While studying the iron-erythropoiesis balance in hereditary anemias, I formulated the following ambitions, which are:

1. to study disease-specific and individual risks of iron overload;
2. to explore connections between erythropoiesis, iron and other pathological pathways; and
3. to improve the understanding of the unique features of each anemia, and of each patient, that determines its clinical phenotype.

THESIS OUTLINE

Part 1: *New crossroads.*
The first part consists of three articles that describe two new concepts in an attempt to unravel novel pathophysiological features of hereditary anemias; both open an exciting, new area for future research. **Chapter 2** reviews the pathophysiological aspects of pyruvate kinase (PK) deficiency, focusing on the interplay between PK-activity and reticulocyte maturation, stretching the importance of adequate oxidative control for normal mitophagy, which forms the fulfilling step of reticulocyte maturation. We propose late-stage ineffective erythropoiesis as a key pathophysiological concept in pyruvate kinase deficiency. **Chapter 3** and **4** focusses on the EPO-fibroblast growth factor 23 (FGF23) signaling pathway in hereditary anemias. This, only briefly, previously described link between EPO and FGF23 may add to the understanding of (insufficient) EPO upregulation or responsiveness in hereditary anemias, as well as the bone mineralization disorders complicating these diseases. We speculate on a potential role for this pathway, not directly linked to ERFE, as a target for treatment with the opportunity to ameliorate ineffective erythropoiesis and development of disease complications.

Part 2: *The challenge called heterogeneity.*
This part includes three articles that discuss unique disease characteristics and unique phenotypic appearances. Sickle cell disease earned a unique place among the hemolytic anemias as an important part of hemolysis does not occur extravascular (e.g. in the spleen or liver), but in the (micro)circulation itself. The relative balance between intra- and extravascular hemolysis seems to determine one's risk of vasculopathic disease complications. In **chapter 5** we introduce a ratio of two laboratory markers that represents the contribution of intravascular hemolysis to total hemolysis in an individual sickle cell disease patient, and this simple ratio was clearly associated with pulmonary hypertension and early death. **Chapter 6** describes the unique monocyte transcriptome of sickle cell disease patients, sketching the balance of both pro- and anti-inflammatory pathways. In this chapter, we highlight the leading role of heme-oxygenase 1, an enzyme that breaks down heme, in determining this balance. **Chapter 7** describes our attempt to untangle the relation between genotypes and phenotypes in hereditary spherocytosis. Which turned out to be an unproductive attempt. This led us speculating on the importance of other (genetic and non-genetic) modifying factors in determining phenotypic profiles.

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Part 3: *About iron.*
The starting point of my trajectory, the interplay between iron and erythropoiesis, finalizes this thesis. **Chapter 8** functions as a bridge between part 2 and part 3, as it reports on disease-specific patterns of the EPO-ERFE-hepcidin signaling pathway regulating iron. However, with the notion that broad inter-individual variety exists in each disease. **Chapter 9** reflects on old, previously unpublished iron absorption and ferrokinetic data. This data provides an answer on the question what happens with intestinally absorbed iron in an already saturated portal circulation. And finally, the results of the previously mentioned clinical trial (*PPI shine again*) are described in **chapter 10**.
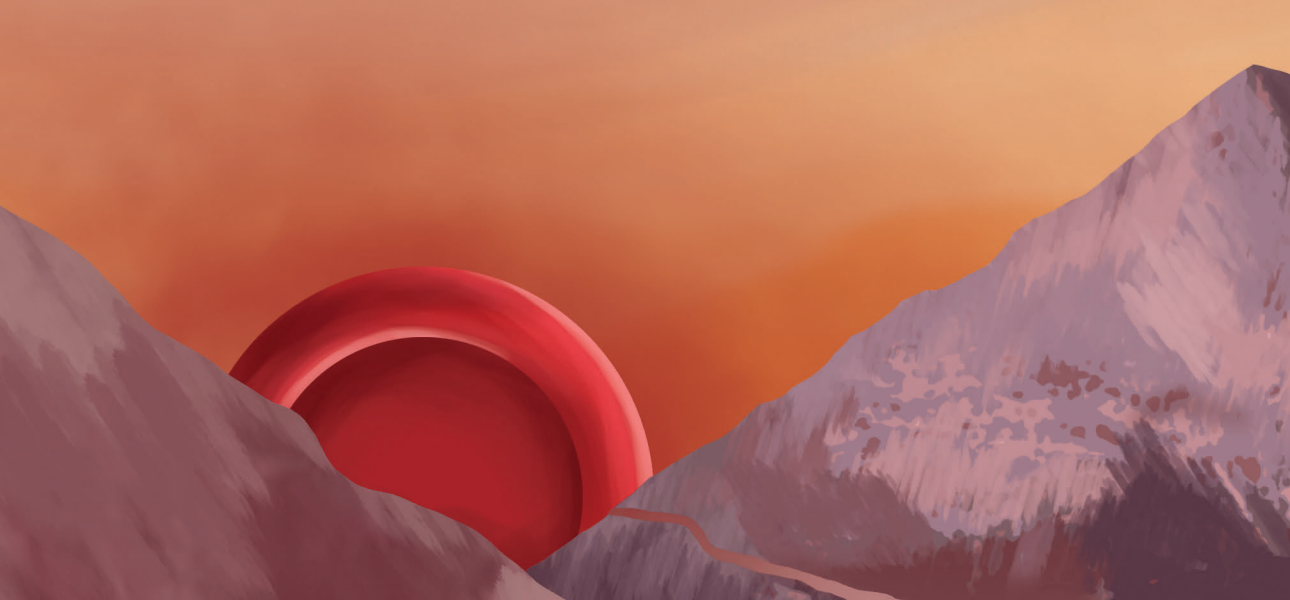
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PART 1

New crossroads

CHAPTER 2

A proposed concept for defective mitophagy leading to late stage ineffective erythropoiesis in pyruvate kinase deficiency

A.J. van Vuren[1], E.J. van Beers[1], R. van Wijk[2].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;

[2] Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

Pyruvate kinase deficiency (PKD) is a rare congenital hemolytic anemia caused by mutations in the *PKLR* gene. Here, we review pathophysiological aspects of PKD, focusing on the interplay between PK-activity and reticulocyte maturation in the light of ferroptosis, an iron-dependent process of regulated cell death, and in particular its key player glutathione peroxidase 4 (GPX4). GPX4 plays an important role in mitophagy, the key step of peripheral reticulocyte maturation and GPX4 deficiency in reticulocytes results in a failure to fully mature. Mitophagy depends on lipid oxidation which is under physiological conditions controlled by GPX4. Lack of GPX4 leads to uncontrolled auto-oxidation which will disrupt autophagosome maturation and thereby perturb mitophagy. Based on our review, we propose a model for disturbed red cell maturation in PKD. A relative GPX4 deficiency occurs due to glutathione depletion, as cytosolic L-glutamine is preferentially used in the form of α-ketoglutarate as fuel for the tricarboxylic acid cycle at the expense of glutathione production. The relative GPX4 deficiency will perturb mitophagy and, subsequently, results in failure of reticulocyte maturation, which can be defined as late stage ineffective erythropoiesis. Our hypothesis provides a starting point for future research into new therapeutic possibilities which have the ability to correct the oxidative imbalance due to lack of GPX4.

I. PYRUVATE KINASE DEFICIENCY

Pyruvate kinase deficiency: a rare hereditary hemolytic anemia

Pyruvate kinase deficiency (PKD) is a rare form of hereditary hemolytic anemia. It is the most common glycolytic enzymopathy and thereby an important cause of hereditary non-spherocytic hemolytic anemia,[1-6] with an estimated prevalence of clinically diagnosed PKD patients between 3.2 and 8.5 per million in Western populations.[7]

The disease is caused by compound heterozygous or homozygous loss of function mutations in the *PKLR* gene encoding for liver and red blood cell specific pyruvate kinase (PK-R).[5,8] Over the last decades more than 300 different pathogenic variants have been reported.[9] In the last step of glycolysis, PK converts phosphoenolpyruvate (PEP) into pyruvate, thereby generating ATP, the sole source of energy of the mature red blood cell. Consistent with the concomitant decrease in PK-R activity as a result of mutation, PKD leads to a loss of ATP and, retrograde accumulation of glycolytic intermediates.[10]

Under physiological conditions, both PK-R and PK-M2 are expressed in basophilic erythroblasts. The latter isozyme is produced from another gene, *PKM*. During further erythroid differentiation and maturation, a switch in isoenzymes occurs whereby progressively increased PK-R expression gradually replaces PK-M2.[11] Pyruvate in reticulocytes is ultimately destined for transport into mitochondria as master fuel for the tricarboxylic acid (TCA) cycle carbon flux. In reticulocytes pyruvate can also be derived from additional sources in cellular cytoplasm (e.g. oxidation of lactate, transamination of alanine). Pyruvate enters the TCA cycle as citrate or oxaloacetate. Modulation of pyruvate entrance balances anaplerotic carbon entrance and cataplerotic carbon exit to ensure continuous cycle of the TCA cycle carbon flux. Pyruvate drives ATP production in the mitochondria by oxidative phosphorylation and other pathways intersecting the TCA cycle.[11] Mature erythrocytes lack nuclei and mitochondria, and are therefore incapable to generate energy via the TCA cycle. Consequently, mature erythrocytes fully depend on anaerobic conversion of glucose by the Embden-Meyerhof pathway for generation of ATP.[12]

PKD is characterized by molecular as well as clinical heterogeneity. Clinical features of PKD vary widely and range from mild anemia to red cell transfusion dependency. The relationship between genotype and phenotype is still incompletely understood.[9,13] The presence of compound heterozygous pathogenic missense mutations may lead to the presence of several different combinations of PK tetramers each with its own kinetic, allosteric and structural properties.[14] Thereby, individual differences in metabolic or proteolytic activity, differences in splenic function, or variation in expression of (compensating) PK iso-enzymes and the activity of other compensating pathways may contribute to the disease

phenotype, as well as epigenetic factors and co-inheritance of other (red cell) diseases.[9] Treatment for PKD is generally supportive including splenectomy, blood transfusion and iron chelation as main therapies. However, new therapies are under investigation: For example, mitapivat, a small-molecule allosteric activator of PK has shown to induce a clinically relevant hemoglobin increase and improvement in hemolytic parameters in a phase II clinical trial.[15]

Remarkably, in PKD post-splenectomy reticulocyte counts tend to be extremely high, varying with hemoglobin response. Most of the patients (78%) were able to discontinue regular transfusions after splenectomy, and in this group the median post-splenectomy reticulocyte count was 32.3% (range 4.8%-65%). This brisk reticulocytosis is likely due to prolonged reticulocyte survival. Patients still requiring regular red cell transfusions after splenectomy tended to have lower reticulocyte counts post-splenectomy (20.9%, range 2.5%-44.1%).[16,17] Notably, the high reticulocyte counts post-splenectomy contrasts with the decrease in reticulocytes after splenectomy in other hemolytic anemias, such as hereditary spherocytosis.[18,19]

Reticulocyte features in PKD: historic observations
Radioisotope studies on PKD patients showed that red blood cells are heterogeneously affected, with a more seriously damaged population of younger cells which sequestrate in the spleen and are subsequently destructed by the reticuloendothelial system. Splenectomy improved survival of these younger cells, although destruction still took place on a slower rate in the liver. More mature PK-deficient red blood cells were able to survive longer.[20,21]

In PKD patients splenectomy resulted in considerable changes in red cell morphology. In sharp contrast with the paucity of red cell abnormalities before splenectomy, post-splenectomy samples from peripheral blood showed ecchinocytes, immature reticulocytes (with remaining organelles) and thin macrocytic discocytes.[18,19]

In another study on 17 PKD patients mutant PK was more susceptible to inhibition by ATP. Consumption of glucose and formation of lactate were lower in PK-deficient red blood cells when compared to PK-sufficient red cell populations with similar reticulocyte counts. In post-splenectomized PK-deficient patients with higher degrees of reticulocytosis more severe impairment of glucose metabolism was observed.[22]

These reports further underline selective destruction of relatively young PK-deficient reticulocytes by the spleen. The mechanism(s) involved in this unique pathophysiological feature of PKD is not understood. In an attempt to provide a rationale, we focus here on

the interplay between PK-activity and reticulocyte maturation in the light of ferroptosis, an iron-dependent process of regulated cell death, and in particular its key player glutathione peroxidase 4 (GPX4). Based on this we propose a model for disturbed red cell maturation in PKD that suggests that the main problem in PKD may not only be the PK-deficient erythrocyte, but for it to reach the mature red cell state.

II. GLUTATHIONE PEROXIDASE 4

Red cells and oxidative stress

Oxidative stress is defined as an imbalance in oxidants and reductants in favor of the oxidants. The presence of high concentrations of oxygen and iron in red blood cells exposes them constantly to oxidative stress as reactive oxygen species (ROS) are formed. Both heme and iron catalyze the Fenton reaction that produces the highly reactive hydroxyl radical from the interaction between superoxide and hydrogen peroxides. Excessive ROS production leads to serious damage to organelles and DNA, or induces programmed cell death (e.g. ferroptosis). To provide protection against ROS, repair oxidative damage and maintain a reducing intracellular environment, (developing) red cells are equipped with a variety of antioxidants including superoxide dismutase 1 and 2, catalase, glutathione peroxidase 1, peroxiredoxin I and II, and glutathione-synthesizing enzymes.[23;24]

Glutamine is an important metabolic fuel for rapidly proliferating cells to meet the cell's demand for ATP, biosynthetic precursors and reducing agents. When transferred to the mitochondrion, glutamine is converted to glutamate to serve as fuel for the TCA cycle when converted to α-ketoglutarate (anaplerotic carbon entrance). During hypoxia α-ketoglutarate may also be converted to citrate which is used for fatty acid synthesis, amino acids synthesis or production of the reducing agent NADPH. In the cytosol, glutamine can also be converted to glutamate by donation of its γ-nitrogen for nucleotide synthesis (pyrimidine metabolism). Cytosolic glutamate is critical for glutathione (GSH) synthesis, which in turn functions as reducing substrate for GPX4, an essential enzyme to protect the cell against oxidative damage.[25;26] (Figure 1A)

12/15-Lipoxygenases in mice, and its human homolog 15-lipoxygenase, and GPX4 act antagonistically. Lipoxygenases generate highly reactive peroxidation products of unsaturated fatty acids prone to further lipid peroxidation and GPX4 reduces lipid peroxidation products in membranes to stable hydroxyl-derivatives.[27] GPX4 plays an important role in red cell development, as two recently published papers showed that *Gpx4*-knockout mice are phenotypically characterized by ineffective erythropoiesis,[23;28] and disrupted reticulocyte maturation.[28]

The role of GSH depletion and GPX4 in ferroptosis

Ferroptosis has recently emerged as a new cell death modality. The importance of this newly recognized process for a variety of diseases, including neuron degenerative diseases, cancer and ischemic organ damage, has been shown. Ferroptosis is triggered by iron-dependent lipid peroxidation, in reactions catalyzed by iron and ROS. GPX4, and its cofactor GSH, function in the defense against ferroptosis by functioning as reducing agents required for clearance of lipid ROS. GSH requires cysteine for its synthesis from glutamine. Cellular import of cysteine is coupled to the export of glutamate via System X_C^- (SLC7A11; cysteine/glutamate antiporter). Upon blocking this system by the synthetic small-molecule erastin the import of cysteine is prevented. The resulting cysteine deprivation impairs synthesis of GSH, and thereby causes increased glutaminolysis. Glutaminolysis is a major source of anaplerosis; α-ketogluterate is the TCA cycle metabolite immediately downstream glutaminolysis. Mitochondrial TCA cycle activity (and subsequent mitochondrial hyperpolarization), and action of the electron transport chain are required for potent ferroptosis.[29-31] Oxidative phosphorylation requires a coordinated transfer of electrons through the complexes of the electron transport chain thereby generating a proton gradient across the inner mitochondrial membrane. Excessive promotion of glutaminolysis (e.g. by cysteine deprivation) stimulates TCA cycle activity and therefore potently enhances mitochondrial respiration leading to mitochondrial hyperpolarization and increased production of ROS, which ultimately promotes lipid peroxidation and, hence, accelerates ferroptosis. This accounts particularly for developing red cells: proximity of mitochondrial membranes to sources of free iron (heme-containing proteins) and ROS makes them important targets for lipid oxidation.[29-31]

Metabolic profiling has shown that GSH depletion is one of the mechanisms by which ferroptosis is initiated. Inactivation of GPX4, through depletion of GSH or by using GPX4 inhibitors, ultimately results in overwhelming lipid peroxidation and cell death in various non-erythroid cells.[32,33] N-acetylcysteine (NAC), a biosynthetic precursor to GSH, in turn has shown to prevent cell death induced by GSH-inhibiting agents.[32]

Several trials have been conducted with NAC that specifically focused on congenital red cell diseases. *In vitro* NAC amide, the -amide form of NAC with improved membrane permeability, possessed the capacity to replenish GSH in red cells from β-thalassemic patients and reduce the amount of ROS.[34] This reducing capacity of NAC was confirmed *in vivo* in children diagnosed with β-thalassemia major: parameters of oxidative stress declined and erythrocyte life span improved.[35] In sickle cell disease (SCD) NAC supplementation significantly lowered erythrocyte phosphatidylserine (PS) expression, measured as representative of erythrocyte membrane (oxidative) damage.[36] In red cells, extensive lipid peroxidative damage to the red cell inner membranes will lead to

abnormal PS externalization.[37-39] These, and several other studies on NAC, underlined the importance of maintaining cellular redox balance for red cell survival as imbalanced ROS production may lead to oxidative stress and (premature) cell death.[40,41]

In an attempt to reduce excessive oxidative stress in sickle red cells another compound gained interest: L-glutamine. Glutamine is a precursor for synthesis of GSH, nicotinamide adenine dinucleotide (NAD) and arginine, which all protect red cells against oxidative damage and (indirectly) maintain vascular tone.[42] Sickle red cells harbor a decreased NAD redox potential.[43,44] GSH consumption is increased in response to oxidative stress without evidence for substrate-limited synthesis of GSH.[45,46] In the presence of sufficient amounts of substrate, the rationale for L-glutamine supplementation to replenish GSH is limited. However, pharmaceutical doses of L-glutamine did increase the NAD redox potential in sickle red cells.[47] After several smaller trials, as summarized by others,[42] a phase 3 clinical trial led in 2017 to the approval of L-glutamine for patients with SCD in order to reduce sickle cell-related acute pain crises and hospitalizations.[48] Whether the efficacy of L-glutamine is related to modulation of GSH metabolism remains disputable, and various other unanswered questions remain regarding (long-term) efficacy and safety (including effects on non-erythroid cells), as well as its acceptance by SCD patients.[42]

Altogether, both the studies with NAC and L-glutamine in red cell disorders do underscore the importance of appropriate redox regulation in the (maturing) red cells which, in the presence of heme and iron, is continuously subjected to auto-oxidation, including lipid peroxidation.

III. RETICULOCYTE MATURATION

The role of mitophagy in reticulocyte maturation under physiological conditions
Enucleated reticulocytes exit the bone marrow, circulate in the blood for 2-3 days and then mature mostly in the spleen into mature erythrocytes. Throughout erythroid maturation mitochondria are progressively lost. The mechanism involved is a specialized form of macro-autophagy, called mitophagy: a selective process by which damaged and depolarized mitochondria are removed and degraded.[49] In mammalian reticulocytes mitophagy occurs in response to developmentally programmed changes in the cell.[49-51] In short, mitochondrial membrane depolarization in response to the mitochondrial protein BNIP3L (BCL2 interacting protein 3 like; NIX),[52] upregulated during terminal red cell differentiation, signals activation of canonical autophagy proteins (Atg). This initiates the formation of *de novo* double membranes, termed phagophores. Phagophores assemble around depolarized mitochondria which, upon closure, form the autophagosome. Finally, the autophagosome fuses with lysosomes to degrade their content or fuses with the plasma membrane for exocytosis. Several Atg proteins are involved

in the assembly of the phagophore. Inhibition of mammalian target of rapamycin (mTOR) is at the start of autophagosome formation, as it activates unc-51 like autophagy activating kinase 1 (ULK1), facilitator of the Atg5/Atg7 pathway, and releases beclin1.[53] Growth and maturation of the autophagosome is mediated by the Atg5-Atg12 pathway and by the microtubule-associated protein 1 light chain 3 (LC3, the mammalian homologue of yeast Atg8) pathway, both of which depend on Atg7.[54] When autophagy is inactive, Atg3 and Atg7 form a stable thioester with LC3. Maturation and growth of the autophagosome requires that phosphatidylethoanolamine (PE) is conjugated to LC3, a process called lipidation, this will occur in response to autophagic stimuli sensed by Atg3. In response to autophagic stimuli, the stable thioesters between LC3 and the catalytic thiols on both Atg3 and Atg7 become transient, which promotes LC3 lipidation.[53,55] The lipidated form of LC3 anchors to the phagophore membrane. Recognition of target mitochondria by the autophagosome occurs through LC3. BNIP3L on mitochondrion targeted for mitophagy has two lipidated LC3 binding sites, and thereby plays an important role in total engulfment of the mitochondrion and completion of autophagosome formation.[56,57] Besides this so-called Atg5/Atg7 pathway, several alternative pathways for initiation of the mitophagy process have been proposed with varying contributions to total mitophagy: 15-lipoxygenase is one of the central enzymes in an alternative pathway. 15-lipoxygenase is highly expressed in reticulocytes. However, its contribution to mitochondrial clearance seems to be only modest when compared to the classical Atg5/Atg7-dependent pathway as 12/15-lipoxygenase knockout mice have normal erythrocyte and reticulocyte counts.[49,58,59]

GPX4 deficiency results in ineffective erythropoiesis and reticulocytosis

Canli *et al.*[23] showed that loss of GPX4 causes erythroid precursor cell death and anemia in mice, due to ineffective erythropoiesis. Surprisingly, erythroid precursor cell death was triggered via RIP3-dependent necroptosis, and not via ferroptosis. Increased lipid peroxidation and oxidative stress in erythroid *Gpx4*-knockout cells did not impair their lifespan in the peripheral blood stage. However, it did increase the number of reticulocytes, suggesting defective maturation of reticulocytes. In contrast to these findings, GPX4 inhibition did not impact cell death during human erythroblast differentiation, as recently published by Ouled-Haddou *et al.*[60] This may suggest that human erythroblasts rely on other enzymes with GPX-activity that are able to overcome the effects of GPX4 inhibition. Notably, GPX4 inhibition in human erythroblasts did impair enucleation in a ferroptosis-, necroptosis- and mitophagy-independent manner by disrupting lipid raft clustering and myosin-regulatory light-chain phosphorylation required for contractile ring assembly and cytokinesis. Unfortunately, the influence of GPX4 on the reticulocyte stage of red cell development was not investigated.

In a mice model with exclusive knockout of *Gpx4* in the hematopoietic system, Altamura *et al.*[28] confirmed that lack of GPX4 resulted in a phenotype of ineffective erythropoiesis with increased numbers of immature reticulocytes in line with Canli *et al.*[23] The failure to

proceed towards maturation was associated with increased lipid peroxidation. (Remnants of) mitochondria were seen in GPX4 deficient reticulocytes. These studies were the first to show the link between GPX4 and impaired reticulocyte maturation, of which the following three key elements can be distinguished:

1. Lipid peroxidation. Lack of 12/15-lipoxygenase (or its human homolog 15-lipoxygenase) was not sufficient to disrupt reticulocyte maturation.[61] However, 12/15-lipoxygenase was shown to be involved in autophagy processes in murine macrophages.[62] In reticulocytes, taking into account that red cells carry high concentrations of iron and heme, highly expressed lipoxygenases may contribute to the initiation of lipid hydroxyperoxide production, but mainly sensitize the cell for iron-catalyzed spontaneous (non-enzymatic) peroxyl radical-mediated chain reactions called auto-oxidation.[28,63]

2. GPX4. Under physiologic conditions lipid peroxidation in reticulocytes is controlled by GPX4. Loss of GPX4 leads to uncontrolled lipid peroxidation with the highest levels of lipid peroxidation in immature reticulocytes when both GPX4 and vitamin E (known to antagonize peroxide production) were deficient.[23,28]

3. LC3 lipidation. Loss of GPX4 may lead to irreversible oxidation of Atg3 and Atg7. The loss of a stable covalent interaction of Atg3/Atg7, and LC3 in response to autophagy induction, makes their catalytic thiols more susceptible for oxidation. If catalytic thiols are available on both Atg3 and Atg7, oxidation will result in formation of a disulfide heterodimer; oxidation of a single catalytic thiol is more likely to result in formation of a stable S-gluthiolation. The irreversible oxidation of catalytic thiols on Atg3 and Atg7 prevents the lipidation process of LC3, and thereby inhibits its function in autophagosome maturation.[53,55] (Figure 1B)

Ultimately, this sequence of events will lead to severely perturbated mitophagy and therefore a defect in reticulocyte maturation.

IV. HYPOTHESIS: PKD LEADS TO DEFECTIVE RETICULOCYTE MATURATION

Based on the above we hypothesize that in PKD the young reticulocyte is fully dependent on its reduced PK-R activity to provide the cell with pyruvate to fuel the TCA cycle. In PKD, anaplerotic carbon entrance via other routes than pyruvate is essential. Glutaminolysis will be the major source of anaplerotic carbon entrance in the absence of sufficient amounts of pyruvate. As cytosolic glutamine is preferentially used in the form of α-ketogluterate to fuel the TCA cycle, there will be shortage of glutamine for synthesis of GSH and, subsequently, bioavailability of GPX4 will be impaired. The red cells are, in the presence of heme and iron,

continuously subjected to auto-oxidation, including lipid peroxidation. In developing red cells mitophagy pathways are triggered to fulfill the maturation process. Excessive auto-oxidation in the absence of GPX4 impairs the LC3 lipidation process when autophagy is stimulated by rendering Atg3 and Atg7 inactive as a consequence of catalytic thiol oxidation. Ultimately, this process will perturbate mitophagy, and subsequently halts the reticulocyte maturation process, as was observed in models of *Gpx4* loss.[23,28] (Figure 1)

In line with the observations of Ouled-Haddou *et al.* described above,[60] a shortage of GPX4 bioavailability in erythroblasts may impair enucleation, which fits with the presence of nucleated red cells in the peripheral blood of PKD patients. However, persistent expression of PK-M2, a suggested phenotypic modifier,[64] may limit disrupted enucleation in PK-R-deficient erythroid cells as a relevant reduction in GSH bioavailability in the presence of PK-M2 is not expected.
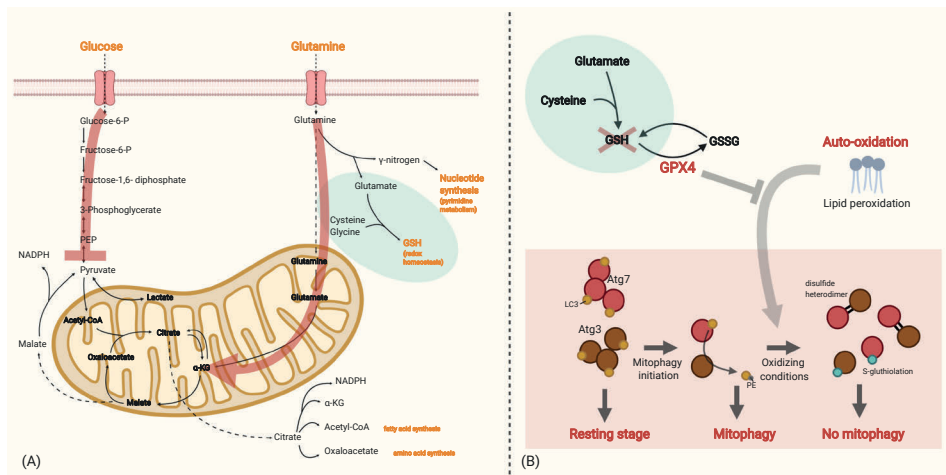
Figure 1. The interplay between GPX4, PKD and reticulocyte maturation.

(A) Schematic overview of glucose and glutamine uptake and metabolism in reticulocytes. The red arrows indicate the alterations in PKD. PK facilitates the final step of glycolysis converting PEP into pyruvate. In PKD anaplerotic carbon entrance via other routes than pyruvate is essential to continue TCA cycling. Therefore, cytosolic glutamine is preferentially used to form α-ketoglutarate at the expense of GSH production. (B) Under physiologic conditions lipid peroxidation in reticulocytes is controlled by GPX4. GPX4 depletion may lead to irreversible oxidation of Atg3 and Atg7. This impedes the LC3 lipidation process, essential for autophagosome formation and maturation. The red cross illustrates the presumed lack of GSH in PKD. As a consequence GPX4 function is blocked.

Created with BioRender.com. α-KG α-ketoglutarate; GPX4 glutathione peroxidase 4; GSH glutathione; GSSG glutathione disulfide; PEP phosphoenolpyruvate; PKD pyruvate kinase deficiency.

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Depending on the underlying mutation and other (non-)genetic factors, as discussed in section I, *Pyruvate kinase deficiency: a rare hereditary hemolytic anemia*, residual activity of PK will vary among patients, and even between individual cells in one patient. This is anticipated to lead to varying degrees of glutaminolysis required for anaplerosis, and a variable reduction of GSH synthesis and GPX4 bioavailability per cell. As a consequence, some reticulocytes will mature whereas in other reticulocytes the maturation process is blocked. Interestingly, earlier studies did not report an absolute decline in either GSH or GSSG in mature PK-deficient erythrocytes, but a decline in GSH/GSSG ratio was reported to result in a acquired reduction in PK-activity.[65-67] Notably, glutaminolysis for anaplerotic carbon entrance is not required in mature red cells that are incapable of generating energy via the TCA cycle.[12] In PKD, besides induction of a maturation defect, lack of GSH in PK-deficient reticulocytes may further disrupt the already reduced PK-activity by oxidation of the enzyme, thereby initiating a deleterious downward spiral.[65-67]

As described in section I, *Reticulocyte features in PK deficiency: historic observations*, earlier observations showed that (non-maturing) young reticulocytes in PKD are degraded by the red pulp macrophages of the spleen. The three most commonly recognized signals in the interaction between red pulp macrophages and red cells, band 3, PS and CD47-SIRPα, are known to be influenced by oxidative stress. Thus, distinct oxidative-stress induced modifications of these molecules on PK-deficient reticulocytes may facilitate their accelerated clearance by the spleen.[37-39,68-70] Splenectomy abrogates this process, resulting in a slower rate of removal of immature reticulocytes in the liver, thereby extending the longevity of non-maturing reticulocytes, which provides an explanation for the profound reticulocytosis that is observed post-splenectomy in PKD patients. Individual variation in the degree of reticulocytosis however may exist, depending (at least partly) on the individual hepatic capacity to substitute for splenic clearance of non-maturing reticulocytes.[16]

In conclusion, we postulate that defective reticulocyte maturation is a new key pathophysiological aspect of PKD, that can be classified as late stage ineffective erythropoiesis. The here proposed hypothesis provides an exciting new area of research on the pathophysiology of PKD and can offer new therapeutic possibilities aimed at correcting the oxidative imbalance due to lack of GSH. Interventions that enhance PK-activity, e.g. mitapivat, could (partially) correct reticulocyte maturation. Other potentially attractive therapeutic strategies that could target the disrupted redox balance underlying halted reticulocyte maturation are amino-acid supplementation with L-glutamine, vitamin E supplementation and NAC.

Supplementation of the amino acid L-glutamine may correct for the increased fuel demand of the TCA cycle. Additional supply of L-glutamine may restore GSH production in PKD, as we expect GSH synthesis in PKD to be substrate-limited. Supplementation of vitamin E could be beneficial because vitamin E interposes in lipid membranes, and acts as antagonist of lipid peroxide production through its high affinity for unpaired electrons. Vitamin E synergizes with GPX4 in correcting the action of lipid hydroxyperoxides, and as a consequence may therefore limit perturbation of mitophagy in PKD. Repletion of vitamin E deficiency was shown to partly correct lipid peroxidation in the presence of GPX4 deficiency; the role of supra-normal vitamin E doses remains to be explored however.[28] N-acetylcysteine may be a third way to restore disrupted mitophagy. Its supplementation has previously shown to prevent cell death induced by GSH-inhibiting agents.[32] Trials in β-thalassemia and SCD patients provided prove for its capacity to replenish the amount of GSH and, accordingly, reduce levels of oxidative stress.[34-36] Notably, catabolism of other amino acids may also correct for the lack of anaplerotic carbon entrance into the TCA cycle in PKD, or replenish the amount of glutamate. Both alanine aminotransferase (alanine catabolism) and aspartate aminotransferase (aspartate catabolism) may contribute to glutamate biosynthesis[71,72] Thereby, glutamate may also be derived from histidine, proline and arginine catabolism.[73] Future studies are warranted to investigate our hypothesis on the existence of late stage ineffective erythropoiesis in PKD as well as targeting it for therapeutic purposes.

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CHAPTER 3

The EPO-FGF23 signaling pathway in erythroid progenitor cells: opening a new area of research

A.J. van Vuren[1], C.A.J.M. Gaillard[2], M.F. Eisenga[3], R. Van Wijk[4], E.J. van Beers[1].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;
[2] Department of Internal Medicine and Dermatology, University Medical Center Utrecht, Utrecht University, The Netherlands;
[3] Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, University of Groningen, The Netherlands;
[4] Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

We provide an overview of the evidence for an erythropoietin-fibroblast growth factor 23 (FGF23) signaling pathway directly influencing erythroid cells in the bone marrow. We outline its importance for red blood cell production, which might add, among others, to the understanding of bone marrow responses to endogenous erythropoietin in rare hereditary anemias. FGF23 is a hormone that is mainly known as the core regulator of phosphate and vitamin D metabolism and it has been recognized as an important regulator of bone mineralization. Osseous tissue has been regarded as the major source of FGF23. Interestingly, erythroid progenitor cells highly express FGF23 protein and carry the FGF receptor. This implies that erythroid progenitor cells could be a prime target in FGF23 biology. FGF23 is formed as an intact, biologically active protein (iFGF23) and proteolytic cleavage results in the formation of the presumed inactive C-terminal tail of FGF23 (cFGF23). *FGF23*-knockout or injection of an iFGF23 blocking peptide in mice results in increased erythropoiesis, reduced erythroid cell apoptosis and elevated renal and bone marrow erythropoietin mRNA expression with increased levels of circulating erythropoietin. By competitive inhibition, a relative increase in cFGF23 compared to iFGF23 results in reduced FGF23 receptor signaling and mimics the positive effects of *FGF23*-knockout or iFGF23 blocking peptide. Injection of recombinant erythropoietin increases FGF23 mRNA expression in the bone marrow with a concomitant increase in circulating FGF23 protein. However, erythropoietin also augments iFGF23 cleavage, thereby decreasing the iFGF23 to cFGF23 ratio. Therefore, the net result of erythropoietin is a reduction of iFGF23 to cFGF23 ratio, which inhibits the effects of iFGF23 on erythropoiesis and erythropoietin production. Elucidation of the EPO-FGF23 signaling pathway and its downstream signaling in hereditary anemias with chronic hemolysis or ineffective erythropoiesis adds to the understanding of the pathophysiology of these diseases and its complications; in addition, it provides promising new targets for treatment downstream of erythropoietin in the signaling cascade.

3

INTRODUCTION

At a concentration of 5 million red blood cells (RBC) per microliter blood, RBCs are the most abundant circulating cell type in humans.[1] Normal erythropoiesis yields 200 billion RBCs every day, an equivalent of 40 mL of newly formed whole blood.[2] Regulation of erythropoiesis in the bone marrow (BM) microenvironment depends on systemic and local factors controlling differentiation, proliferation and survival of the erythroid progenitor cells (EPC). Inherited RBC abnormalities might result in chronic hemolysis with an increased erythropoietic drive, or ineffective erythropoiesis, thereby challenging the erythropoietic system. Systemic erythropoietin (EPO) production plays a critical role in maintaining erythropoietic homeostasis under physiologic and pathologic conditions.[1] Increasing evidence links EPO and erythropoiesis to skeletal homeostasis.[1] First, there is a longstanding observation that patients with hemolysis have increased risk of skeletal pathology such as osteoporosis and osteonecrosis. [1,3-5] Second, removal of osteoblasts in mice resulted in increased loss of erythroid progenitors in the BM, followed by decreased amounts of hematopoietic stem cells with recovery after reappearance of osteoblasts, pointing to a critical role of osteoblasts in hemato- and erythropoiesis.[6]

EPO, the core regulator of erythropoiesis, is an important regulator of fibroblast growth factor 23 (FGF23) production and cleavage.[7-12] FGF23 is originally known as a bone-derived hormone and key player in phosphate and vitamin D metabolism. FGF23 seems to provide a link between bone mineralization and erythropoiesis.[1,7] FGF23 was first discovered as a regulator of phosphate metabolism, due to the association between hereditary phosphate wasting syndromes and *FGF23* mutations.[13] FGF23 induces phosphaturia, directly suppresses parathyroid hormone and the amount of $1,25(OH)_2D_3$ (active vitamin D).[14,15] FGF23 is secreted by osteocytes in response to vitamin D, parathyroid hormone and elevated levels of serum phosphate. Due to important alterations in phosphate balance in chronic kidney disease (CKD), most research on FGF23 up until now was focused on CKD (see section *EPO, Iron, CKD, and Inflammation Are Important Regulators of iFGF23 Cleavage*).[16] However, a new, important role for FGF23 seems to exist as regulator of erythropoiesis.

Here, we review the interplay of EPO and FGF23 in the erythroid cells of the BM. We discuss that the action of FGF23 not only depends on the amount of intact FGF23 available, but also on the amount of FGF23 cleavage which is an important factor determining its efficacy. Elucidation of the role of the EPO-FGF23 signaling pathway in hereditary anemia and chronic hemolytic diseases will add to the understanding of the pathophysiology of the diseases, of bone mineralization disorders complicating chronic hemolytic diseases, and might provide new targets for treatment downstream of EPO. An overview of FGF23 production, cleavage and signaling is provided in Figure 1.
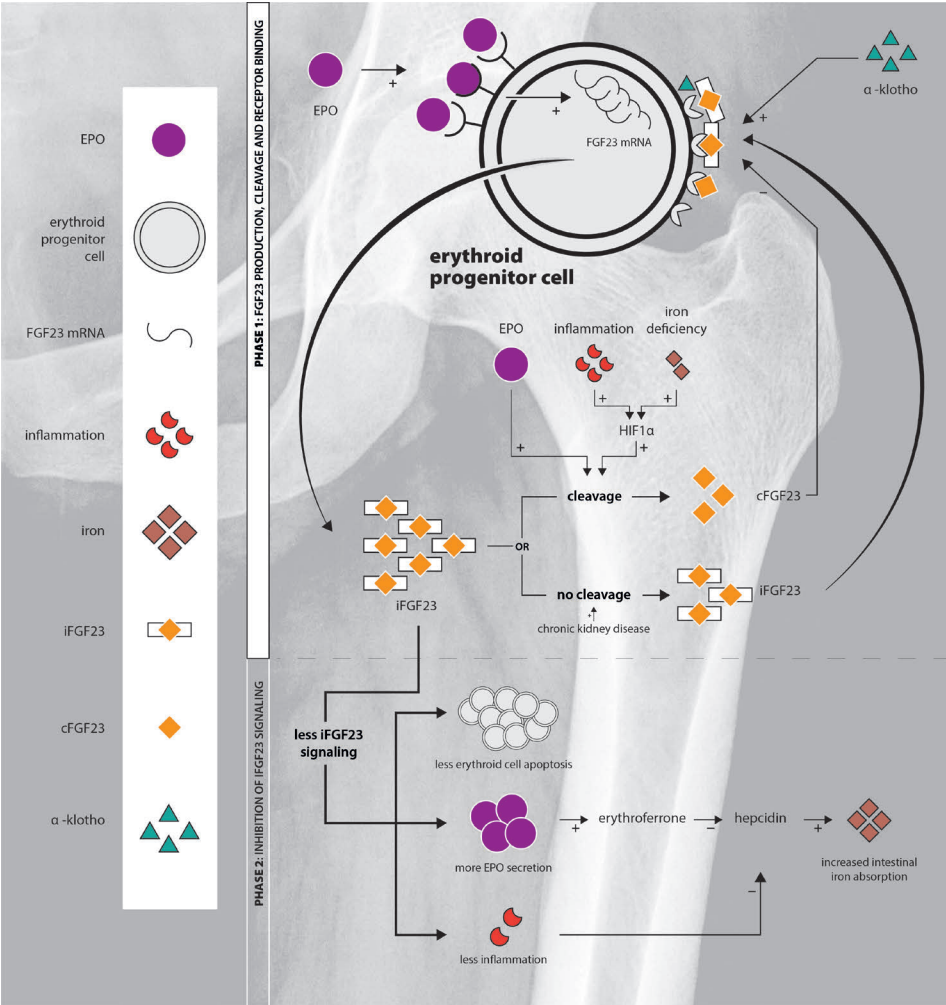
Figure 1. Schematic overview of the EPO-FGF23 signaling pathway in the erythroid lineage in the BM. Phase 1 displays FGF23 production, the secretory process and FGFR binding; phase 2 summarizes the effects of inhibition of iFGF23 signaling.

1. ANEMIA AND THE EPO SIGNALING CASCADE

Erythropoietin production by renal interstitial cells, and in a smaller amount by hepatocytes, plays a critical role in maintaining erythropoietic homeostasis. The primary physiological stimulus of increased EPO gene transcription is tissue hypoxia, which can augment circulating EPO up to a 1000-fold in states of severe hypoxia.[17,18] Under hypoxic conditions, EPO transcription is augmented by binding of hypoxia inducible factor (HIF)-2 to the EPO gene promoter. Under normoxic conditions prolyl hydroxylases (PHD) hydroxylate HIF1α and HIF2α, which associate with the von Hippel-Lindau tumor suppressor protein, targeting this complex for proteasomal degradation. Low iron or oxygen conditions inhibit hydroxylation by PHD2.[17,19] EPO exerts its effect on early erythroid progenitors via the EPO receptor (EPOR), with a peak receptor number at the CFU-E (Colony Forming Unit-Erythroid) stage and a decline until absence of the receptor in late basophilic erythroblasts. EPOR signaling results in survival, proliferation, and terminal differentiation.[1,2,20]

Besides kidney and liver, EPO expression has also been reported in brain, lung, heart, spleen, and reproductive organs. Besides kidney and liver, only EPO produced by the brain was capable to functionally regulate erythropoiesis.[21,22] More recently, it was discovered that local production of EPO by osteoprogenitors and osteoblasts in the BM microenvironment, under conditions of constitutive HIF stabilization, results in selective expansion of the erythroid lineage.[1,23] The role of osteoblastic EPO in the BM microenvironment under physiologic conditions is still under investigation.[24] The amount of circulating EPO is normal or elevated in most forms of hereditary anemia, although the amount is often relatively low for the degree of anemia.[25-27] EPO levels were generally elevated in β-thalassemia patients with large interpatient differences partly related to age.[28-32] Sickle cell disease (SCD) patients had elevated serum EPO concentrations ranging from the low end of expected for the degree of anemia to lower than expected.[33,34] Off-label application of recombinant EPO (rhEPO) has been tried in selected patients to reduce transfusion requirements and improve quality of life. Responses varied and were unpredictable.[28,35-37] Insight in components downstream of EPO in its signaling cascade might lead to insights in the EPO responsiveness in individual patients. FGF23 has shown to be one of those downstream components directly affecting erythropoiesis and providing feedback on EPO production, as outlined in section *Blockade of iFGF23 Signaling Results in More Erythropoiesis*.

2. ERYTHROID PROGENITORS EXPRESS FGF23 IN RESPONSE TO EPO

Osseous tissue has been regarded as the major source of FGF23. Selective deletion of *FGF23* in early osteoblasts or osteocytes in a murine model demonstrated that both cell types significantly contribute to circulating FGF23. However, FGF23 was still detectable in serum after deletion of the *FGF23* gene in both osteoblasts and osteocytes: other, non-osseous, tissues contribute to circulating FGF23.[38] It was shown that BM, specifically the early erythroid lineage, does significantly contribute to total circulating FGF23. In wild-type (WT) mice treated with marrow ablative carboplatin followed by a 3-day course of rhEPO, serum FGF23 was 40% lower compared to controls.[7] In WT mice, baseline FGF23 mRNA in BM was comparable with osseous tissue, but the amount of FGF23 protein in BM tissue was significantly higher. Hematopoietic stem cells and EPCs, including BFU-E (Burst Forming Unit-Erythroid), CFU-E and proerythroblasts, showed more than fourfold higher amounts of FGF23 mRNA compared with whole BM including lineage specific cells. FGF23 mRNA was shown to be transiently expressed during early erythropoiesis.[10] EPCs do express FGF23 mRNA under physiologic conditions, however significant increases are observed in response to EPO.[7,10,11] RhEPO induced FGF23 mRNA expression in BM cells 24 hours after injection.[11] Indirect immunofluorescence staining with anti-mouse FGF23 antibodies and lineage specific markers showed intense staining of erythroid progenitors and mature erythroblasts (CD71+ cells) of EPO-treated mice compared to controls.[11]

Thus, erythroid cells of the BM significantly contribute to FGF23 production and FGF23 production is increased in response to EPO. As will be discussed in sections *FGF23 Signaling Is Regulated by Cleavage of Intact FGF23* and *EPO, Iron, CKD, and Inflammation Are Important Regulators of iFGF23 Cleavage*, the amount of cleavage of FGF23 is equally important and EPO has a strong effect on this as well.

3. FGF23 SIGNALING IS REGULATED BY CLEAVAGE OF INTACT FGF23

FGF23 is formed as a full-length, biologically active protein (iFGF23). Intact FGF23 is cleaved into two fragments: the inactive N-terminal fragment of FGF23 fails to co-immunoprecipitate with FGFR (FGF receptor) complexes, which suggests that the C-terminal fragment (cFGF23) mediates binding to the FGFR.[39-41] Only intact FGF23 (iFGF23) suppresses phosphate levels in mice through the FGF receptor 1 (FGFR1).[42,43] cFGF23 competes with iFGF23 for binding to the FGFR, and thereby antagonizes iFGF23 signaling in mice and rats.[40,44] Treatment with cFGF23 increased the number of early and terminally differentiated BM erythroid cells and the colony forming capacity of early progenitors to the same amount as rhEPO. These data suggest that the outcome of rhEPO treatment resembles the effects of more cFGF23. Recently, it was shown that the cFGF23 fragment itself was able to induce heart hypertrophy in SCD patients,[41] probably via FGFR4 and independent from a costimulatory signal (see section *Presence of α-Klotho Is Essential for Normal Erythropoiesis*).[45]

Currently, two assays are available to measure iFGF23 and cFGF23: one assay that detects the C-terminal of FGF23 which measures both cFGF23 and (full-length) iFGF23 (Immunotopics/Quidel) and one assay that only detects iFGF23 (Kainos Laboratories).[12] Serum half-life time is approximately identical for both iFGF23 and cFGF23 ranging from 45-60 minutes.[46]

So, although still subject of debate, proteolytic cleavage of iFGF23 seems to abrogate its activity by two mechanisms: reduction of the amount of iFGF23 and generation of an endogenous inhibitor, cFGF23.[40] Therefore, measurement of both iFGF23 and cFGF23 is important: alterations in the iFGF23 to cFGF23 ratio lead to alterations of iFGF23 signaling efficacy.

Regulation of FGF23 secretion includes intracellular processing in the Golgi apparatus in which iFGF23 is partially cleaved within a highly conserved sutilisin-like proprotein convertase (SPC)-site by furin or prohormone convertase 1/3, 2, and 5/6. (Figure 2) Cleavage of iFGF23 generates two fragments: the C- and N-terminal peptide fragments (20 and 12 kDa).[47-49] Competition between phosphorylation and O-glycosylation of the SPC-site in the secretory pathway of FGF23 is an important regulatory mechanism of cleavage.[48] Secretion of iFGF23 requires O-glycosylation: the glycosyltransferase N-acetylgalactosaminyltransferase 3 (GalNT3) selectively exerts O-glycosylation of amino acid residues within or in the proximity of the SPC-site and blocks cleavage of iFGF23.[50] In contrast, phosphorylation of the SPC-site promotes FGF23 proteolysis indirectly by blocking O-glycosylation. The kinase Fam20C phosphorylates iFGF23 within the SPC-site, consequently reduces glycosylation and subsequently facilitates iFGF23 cleavage.[49]

Summarizing, a proportion of synthesized iFGF23 will be cleaved intracellularly before secretion, the amount of intracellular cleavage is determined by competition between glycosylation (GalNT3) and phosphorylation (Fam20C).[48,49,51] Various factors regulate post-translational modification, these are described in section *EPO, Iron, CKD, and Inflammation Are Important Regulators of iFGF23 Cleavage.*
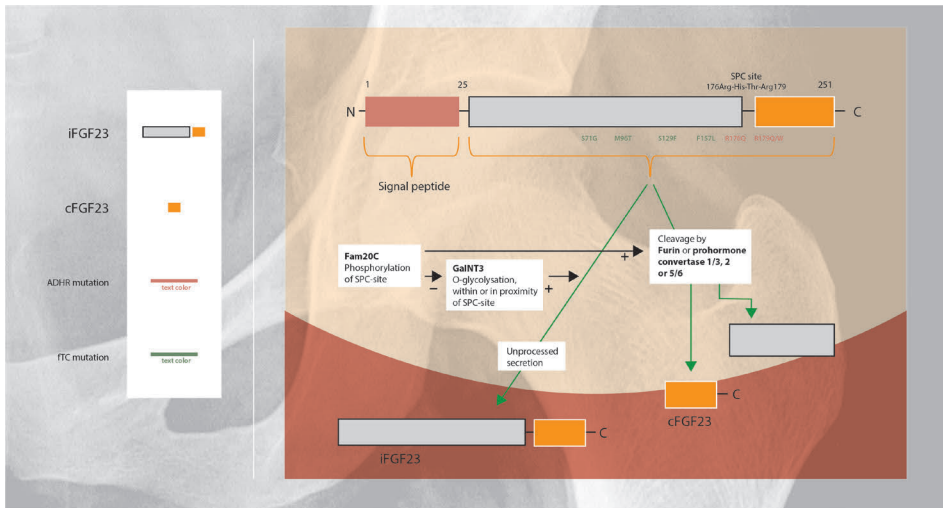

Figure 2. Schematic overview of the regulation of FGF23 protein cleavage and secretion.[141,160,167,168] FGF23 harbors a naturally-occurring proteolytic site at Arg176-XX-Arg179. O-glycosylation within or in the proximity of this sutilisin-like proprotein convertase (SPC) site of FGF23 by GalNT3 results in increased secretion of intact FGF23. Phosphorylation of the SPC-site by Fam20C indirectly promotes FGF23 cleavage by blocking O-glycosylation. ADHR is caused by mutations near the proteolytic site, that impairs proteolytic inactivation of FGF23 resulting in high levels of iFGF23 (Arg176Gln or Arg179Gln/Trp). FTC, is an autosomal recessive disorder, resulting from mutations in the FGF23 gene which lead to destabilization of the tertiary structure of FGF23 and rendering it susceptible to degradation (Ser71Gly, Met96Thr, Ser129Phe, and Phe157Leu).
FTC familial tumoral calcinosis; ADHR autosomal dominant hypophosphatemic rickets.

4. EPO, IRON, CKD AND INFLAMMATION ARE IMPORTANT REGULATORS OF IFGF23 CLEAVAGE

EPO, iron, inflammation and CKD have been identified as modifiers of iFGF23 cleavage. Notably, all these factors might co-exist in patients with hereditary anemia. The amount of cleavage is determined by alterations in GalNT3 and furin. Furin plays an important role in regulation of FGF23 cleavage in iron deficiency and inflammation, [52,53] whereas under conditions of high EPO GalNT3 inhibition might augment cleavage.[12]

4.1 EPO
Several studies report alterations of iFGF23 and cFGF23 after administration of rhEPO or under high endogenous EPO conditions, a summary is provided in Table 1.[7-12] Most experiments were carried out in animal models (rats and mice). Less information is available about the influence of EPO on the iFGF23/cFGF23 ratio in man.

In all animal studies one single injection or multiple-day regimen of rhEPO resulted in a significant increase in circulating cFGF23.[7,9-12] Increases in iFGF23 were less pronounced,[9,10,12] or absent,[11] after a single injection of rhEPO. Multiple-day regimens resulted in small rises in iFGF23, less pronounced than the increase in cFGF23.[7,11] EPO directly increased *FGF23* gene expression in murine hematopoietic cells.[9] Treatment of mice with a hematopoietic equipotent dose of a HIF-proline hydroxylase inhibitor (HIF-PH inhibitor) also led to a significant rise in plasma cFGF23, without an increase in circulating iFGF23. Increases in FGF23 expression after HIF-PH inhibitor treatment were mediated indirectly via EPO, as pre-administration of anti-EPO antibodies opposed upregulation of circulating FGF23.[9,53]

Effects of overexpression of endogenous EPO were investigated in a transgenic human EPO-overexpressing murine model. Results were in line with responses on rhEPO in mice: circulating cFGF23 and iFGF23 were significantly higher in EPO-overexpressing mice than in WT mice.[12] Acute blood loss in mice, as a surrogate model for high endogenous EPO, also significantly increased circulating cFGF23, but not iFGF23.[8]

Only four studies [7,8,11,12] explored effects of EPO on FGF23 in man. In all studies, rhEPO or a condition resulting in high endogenous EPO, increased circulating cFGF23, without [11,12] or with only minimal [7] rise in circulating iFGF23. In a large cohort of 680 kidney transplant recipients higher EPO values were associated with increased cFGF23 values and not with iFGF23 values, independent of renal function.[12]

Table 1. Overview studies on the effects of erythropoietin (EPO) on FGF23.

Study	Model
Studies in animals	
Clinkenbeard *et al.* 2017	WT C57BL/6 mice
Rabadi *et al.* 2018	C57BL/6 mice with and without 10% loss of total blood volume
Flamme *et al.* 2017	Male Wistar rats
Toro *et al.* 2018	WT C57BL/6 mice
	Sprague-Dawley rats, hemorrhagic shock with 50-55% loss of total blood volume
Daryadel *et al.* 2018	WT C57BL/6 mice
Hanudel *et al.* 2018	WT C57BL/6 mice with and without 0.2% adenine diet-induced CKD
	Transgenic Tg6 mice overexpressing human EPO
Studies in man	
Clinkenbeard *et al.* 2017	4 patients with unexplained anemia
Rabadi *et al.* 2018	131 patients admitted to ICU, categorized based on number of RBC transfusions in 48h before admission
Daryadel *et al.* 2018	28 healthy volunteers
Hanudel *et al.* 2018	680 adult kidney transplant patients

Together, these data show that EPO (endogenous or exogenous) increases the total amount of circulating FGF23 (iFGF23 and cFGF23) and alters the iFGF23/cFGF23 ratio in favor of cFGF23.

rhEPO	iFGF23/cFGF23
3-day regimen with increasing doses rhEPO	Max. ±40x increase in serum cFGF23; ±2x increase in serum iFGF23. Increases in cFGF23 in dose-dependent way.
None	6h: ±4x increase in plasma cFGF23; no increase in iFGF23. cFGF23 values remained increased 48h after blood loss.
Single injection rhEPO	4-6h: > 10x increase in plasma cFGF23 (extrapolated); ±2x increase in plasma iFGF23 (extrapolated).
Single injection high dose HIF-PH inhibitor	4-6h: comparable with rhEPO. Pretreatment anti-EPO: cFGF23 response almost absent.
Single injection rhEPO	4h: ±4x increase in plasma cFGF23; ±2.5x increase in plasma iFGF23.
None	24h: ±5x increase in plasma cFGF23; ±3.5x increase in plasma iFGF23.
Single injection rhEPO	24h: ±2x increase in plasma cFGF23; no increase in plasma iFGF23.
4-day regimen rhEPO	4d: increase in cFGF23 and iFGF23.
Single injection rhEPO	6h: *non-CKD* cFGF23 207 → 3289pg/mL; *CKD* cFGF23 2056 → 9376pg/mL. *Non-CKD* iFGF23 187 → 385pg/mL; *CKDI* no significant rise in iFGF23.
Transgenic EPO overexpression	cFGF23 *WT* 340pg/mL; *Tg6* 3175pg/mL. iFGF23 *WT* 317pg/mL, *Tg6* 589pg/mL.
Single injection rhEPO	6-18h: ±2x increase in serum cFGF23; ±1.5x increase in serum cFGF23.
None	Number of blood transfusions was associated with plasma cFGF23.
Single injection rhEPO	24h: significant increase in plasma cFGF23; plasma iFGF23 unchanged.
None	Higher EPO values were significantly associated with increased cFGF23 and not with iFGF23; independent of renal function.

It is uncertain which proteins mediate increased intracellular cleavage in the secretion pathway of iFGF23 in response to EPO. In mice, experiments investigating alterations in BM mRNA expression of GalNT3 after rhEPO injection were inconclusive.[11] Meanwhile, in EPO-overexpressing mice, compared to WT mice, GalNT3 and prohormone convertase 5/6 mRNA expression were significantly decreased in bone and BM, no differences were observed in Fam20c and furin mRNA expression.[12] Decreases in GalNT3 mRNA and absence of changes in furin and Fam20c mRNA expression were also observed in whole BM of mice after acute blood loss. However, the amount of GalNT3 mRNA expression in isolated erythroid precursors and mature erythroblasts (Ter119+ cells) of these mice was unchanged.[8] So, decreased GalNT3 expression might increase cleavage in response to high EPO, although further study is needed to elucidate the contributory of GalNT3 and other, yet unknown, mechanisms in response to EPO.

4.2 Iron deficiency

Iron deficiency in WT mice resulted in a significant increase of cFGF23, with a less pronounced or even absent increase in iFGF23.[53-56] Treatment of iron deficiency in CKD mice resulted in a significant decrease in whole bone FGF23.[7] Iron deficiency induced by iron chelation stabilized pre-existing HIF1a and increased FGF23 transcription.[53,55] HIF1a inhibition partially blocked elevations in total FGF23, and inhibited cleavage of iFGF23.[53] HIF1a stabilization under conditions of iron deficiency has been associated with upregulation of furin in liver cells.[52]

Two large cohort studies support the relevance of the observations in mice in men. In a cohort of 2.000 pre-menopausal women serum iron was inversely correlated with cFGF23, but not with iFGF23.[57] And, associations between low iron parameters and high cFGF23 and iFGF23 values were found in a cohort of 3.780 elderly, with a more pronounced increase in cFGF23.[58]

Multiple studies examined the effects of distinct formulations of iron, oral and intravenous, in CKD patients on circulating cFGF23 and/or iFGF23.[59-69] Results have been inconclusive: interacting effects of rhEPO or endogenous high EPO might have influenced results. Moreover, the carbohydrate moieties of parenteral iron formulations themselves might lead to increased amounts of iFGF23.[70,71]

In summary, iron deficiency leads to increased amounts of cFGF23 fragments. HIF1a stabilization plays an important role in upregulation of intracellular iFGF23 cleavage. Due to co-existence of anemia, erythropoiesis-related factors might influence the iron deficiency-FGF23 pathway. Observed differences in expression of proteins directly involved in the secretory process of FGF23, furin and GalNT3, suggest that EPO is not simply an intermediary between iron deficiency and FGF23: furin plays an important role in the upregulation of iFGF23 cleavage in iron deficiency, whereas EPO might act via GalNT3 inhibition a as discussed in section *Erythropoietin*.[12]

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4.3 CKD

Circulating total FGF23 rises progressively during early and intermediate stages of CKD and reaches levels of more than 1.000-times normal in advanced CKD. Elevated iFGF23 levels are considered as a compensatory mechanism for hyperphosphatemia, however regulation of FGF23 in CKD remains incompletely understood.[12,72,73] Elevated total FGF23 is associated with progression of CKD,[73-75] left ventricular hypertrophy,[45] expression of IL-6,[76] impaired neutrophil recruitment,[77] cardiovascular morbidity,[45,78,79] and overall mortality.[74,80,81]

Besides the role of the kidney in clearance of iFGF23, CKD has also been identified as regulator of iFGF23 cleavage. Acute bilateral nephrectomy resulted in an immediate two-until three-fold increase in iFGF23 levels with concomitant increase in iFGF23/cFGF23 ratio.[82] In a murine CKD model, CKD was associated with less proteolytic cleavage of iFGF23 independent of iron status.[56] Notably, iron deficiency, high endogenous EPO, or administration of rhEPO still resulted in increased total FGF23 production and cleavage in CKD.[12]

So, CKD is associated with increased total FGF23 and alteration of the iFGF23/cFGF23 ratio in favor of iFGF23. As CKD progresses towards end-stage renal disease, the iFGF23/cFGF23 ratio will approximate 1:1.[83] Co-existence of iron deficiency or rhEPO administration still influence FGF23 secretion in CKD.

4.4 Inflammation

The association between FGF23 and inflammation has been reported in many diseases.[84-92] Multiple inflammatory signaling pathways seem to interact closely to regulate FGF23 production and cleavage during acute or chronic inflammation. Additionally, other regulators of FGF23 expression and cleavage might develop under inflammatory conditions as inflammation-induced functional iron deficiency.

Regulation of FGF23 depends on chronicity of inflammation.[53,91] In two murine models of acute inflammation, bone FGF23 mRNA expression and serum cFGF23 concentrations increased tenfold, without changes in iFGF23.[53] Increases in FGF23 mRNA were absent in the presence of NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells, a canonical protein complex regulating many proinflammatory genes) inhibitor, which underlines the importance of the NFkB signaling pathway in regulation of FGF23 mRNA by pro-inflammatory stimuli.[93] Co-treatment of bone cells with TNF or IL-1β and furin inhibitors resulted in increased levels of iFGF23, which suggests that increased cleavage of iFGF23 during acute inflammation is mediated by furin.[53,93,94] HIF1a was identified as an intermediate in FGF23 mRNA upregulation: iron deficiency and hypoxia only stabilized pre-existing HIF1a, where inflammation also led to increased cellular expression of HIF1a in bone cell lines.[53]

Chronic inflammation resulted in increased amounts of total FGF23 with increased amounts of iFGF23. Chronic inflammation seems to exhaust or downregulate the FGF23 cleavage system.[91]

In the presence of inflammation, development of functional iron deficiency,[95] discussed in section *Iron Deficiency*, might contribute to increased cleavage of iFGF23.[53] The inflammatory cytokine IL-6 promotes hepcidin transcription in hepatocytes via the IL-6 receptor and subsequent activation of JAK tyrosine kinases and signal transducer and transcription activator 3 complexes that bind to the hepcidin promotor. Additionally, activin B stimulates formation of hepcidin transcriptional complexes via the BMP (bone morphogenetic protein)/ SMAD signaling pathway.[2,96-98] Hepcidin controls the inflow of iron from enterocytes, the reticuloendothelial system and hepatocytes into the circulation via regulation of the expression of iron exporter ferroportin.[99] Upregulation of hepcidin redistributes iron to the reticuloendothelial system at the expense of FGF23 producing cells including RBC precursor cells, osteocytes and osteoblasts. Moreover, inflammation induces proteins that scavenge and relocate iron, including lactoferrin, lipocalin 2, haptoglobin and hemopexin. These proteins contribute to inflammation-induced functional iron deficiency.[100]

Summarizing, inflammation does augment both FGF23 expression and its cleavage, by increased HIF1a expression and stabilization and increased furin activity, but also via hepcidin-induced functional iron deficiency and subsequent non-hypoxic HIF1a stabilization.

5. BLOCKADE OF IFGF23 SIGNALING RESULTS IN MORE ERYTHROPOIESIS

The effects of iFGF23 signaling have been studied by direct infusion of rh-iFGF23,[11] and by blockage of iFGF23 signaling by knockout,[101] or rh-cFGF23 injection.[44] *FGF23*-knockout mice displayed severe bone abnormalities, reduced lymphatic organ size, including spleen and thymus and elevated erythrocyte counts with increased RBC distribution width and reduced mean cell volume, and mean corpuscular hemoglobin.[101] Knockout of the *FGF23* gene in mice resulted in a relative increase in hematopoietic stem cells, with decreased apoptosis, increased proliferative capacity of hematopoietic stem cells *in vitro* to form erythroid colonies, and an increased number of immature (pro-E, Ter119^{+med}, CD71$^{=hi}$) and mature erythroid cells (Ter119^{+hi}) in BM and peripheral blood. Hematopoietic changes were also observed in fetal livers, underlining the importance of FGF23 in hematopoietic stem cell generation and differentiation during embryonic development independent of the BM microenvironment. EPO, HIF1a and HIF2a mRNA expression were significantly increased in BM, liver and kidney of *FGF23*-knockout mice, and the EPO receptor was upregulated on isolated BM mature erythroid cells. On the other hand, EPO, HIF1a and HIF2a mRNA expression in osseous tissue was decreased; which might be explained by the remarkably lower osteoblast numbers in *FGF23*-knockout mice. Administration of rh-iFGF23 in WT mice resulted in a rapid decrease in erythropoiesis and a significant decrease in circulating EPO. *In vitro* administration of iFGF23 to *FGF23*-knockout BM-derived erythropoietic cells normalized erythropoiesis, normalized HIF and EPO mRNA abundance and normalized EPOR expression.[101] Alterations of EPO expression in response to iFGF23 were also observed by others: injection of rh-iFGF23 in mice reduced kidney EPO mRNA levels with 50% within 30 minutes, persisting over 24 hours.[11]

Inhibition of iFGF23 signaling with rh-cFGF23 in CKD mice resulted in decreased erythroid cell apoptosis, upregulation of renal and BM HIF1a and subsequent EPO mRNA expression, elevated serum EPO levels and amelioration of iron deficiency. Inflammatory markers and liver hepcidin mRNA expression declined after iFGF23 blockage.[44] Lower hepcidin expression might have followed directly from decreases in inflammation, however might also have resulted from increased EPO expression.[102]

Interestingly, the increase in erythropoiesis after iFGF23 inhibition resembles the effects of α-klotho inhibition as outlined in the next paragraph.[103] In summary, current studies underline the importance of FGFR signaling by FGF23 for early erythropoiesis.

6. PRESENCE OF A-KLOTHO IS ESSENTIAL FOR NORMAL ERYTHROPOIESIS

Murine BM erythroid cells (Ter119^{+}) express the FGF23 receptors FGFR1, 2 and 4, and a small amount of FGFR3.[101] The FGFR1, that among others regulates phosphaturia, needs three components to be activated: the FGFR itself, iFGF23 and α-klotho (α-KL). A-KL, first described as an aging suppressor,[104] forms a complex with FGFR1 subgroup c, FGFR3 subgroup c or FGFR4 thereby selectively increasing the affinity of these FGFRs to FGF23.[105,106] A-KL simultaneously tethers FGFR and FGF23 to create proximity and stability.[107] Membrane-bound α-KL is predominantly expressed in kidney, parathyroid gland and brain choroid plexus, however shed α-KL ectodomain seems to function as an on-demand cofactor.[107] There is expression of α-KL mRNA in BM, including BM erythroid cells (Ter119^{+}), spleen and fetal liver cells.[101,108] The importance of α-KL for hematopoietic stem cell development and erythropoiesis was demonstrated in *a-KL*-knockout mice. Knockout of the *a-KL* gene resulted in a significant increase in erythropoiesis with significant increases in immature pro-erythroblasts and a relatively mature fraction of erythroblasts. *In vitro a-KL*-knockout BM cells generated more erythroid colonies than BM cells of WT mice. EPO mRNA expression was significantly upregulated in *a-KL*-knockout mice kidney, BM and liver cells, along with upregulation of HIF-1a and HIF-2a.[108] Effects of *a-KL*-knockout are remarkably similar to effects of iFGF23 blockade or knockout. This suggests that α-KL is indeed an essential cofactor for FGF23 signaling in the regulation of erythropoiesis. However, if the link between less α-KL and more EPO involves less iFGF23 signaling remains to be proven. Besides EPO, iron load seems to influence α-KL. Iron overload decreased renal expression of α-KL at mRNA and protein level; iron chelation suppressed the downregulation of α-KL via angiotensin II.[109]

Recent studies showed that FGF23 has various effects on many tissues in an α-KL-dependent way, but might also act in an α-KL-independent way especially under pathological conditions. The mechanism by which FGF23 activates the FGFR2 independent of α-KL on leukocytes and the FGFR4 independent of α-KL on cardiomyocytes is still unclear.[77,110,111]

In conclusion, α-KL seems to be essential for FGF23 signaling in erythropoiesis, as *a-KL*-knockout resembles the effects of iFGF23 blockade or knockout on erythroid cell development.

7. FGF23 EXPRESSION IN HEREDITARY ANEMIA

Currently, information about the abundance of the EPO-FGF23 pathway in hereditary anemia is limited to two studies: one study in β-thalassemia mice and one study in SCD patients. β–thalassemia intermedia mice are characterized by anemia, iron overload and high endogenous EPO. FGF23 mRNA expression in bone and BM of thalassemia intermedia mice were elevated, reaching expression levels of endogenous EPO-overexpressing, polycythemic mice. The amount of circulating iFGF23 was significantly elevated compared to WT mice (436 versus 317 pg/mL), although the increase in iFGF23 was small compared to the increase in total circulating FGF23 (3129 versus 340 pg/mL in WT mice).[12] Circulating FGF23 levels were measured in 77 SCD patients, no EPO measurements were available.[41] Serum ferritin concentrations and estimated glomerular filtration rate were significantly higher in SCD patients than in the control group. Mean plasma cFGF23 concentrations were significantly higher in SCD patients than in healthy controls (563 versus 55 RU/mL). The magnitude of multiplication of cFGF23 in SCD patients compared to healthy controls was comparable with the multiplication of cFGF23 observed after rhEPO. (Table 1) In 75% of the SCD patients cFGF23 values were above the upper limit of normal, whereas in only ten percent of the SCD patients iFGF23 values were above the upper limit of normal. Unfortunately, the association between the iFGF23/cFGF23 ratio, EPO and the extent of erythropoiesis was not evaluated.

The first study underlines that the EPO-FGF23 pathway is upregulated in β-thalassemia intermedia and can be upregulated under iron-overloaded conditions. The second study suggests that FGF23 production and cleavage are increased in SCD, if EPO or inflammation, or another factor, is the most important driving force remains to be investigated.

The activity of the EPO-FGF23 pathway in other hereditary anemias, including BM failure syndromes, with distinct amounts of hemolysis and ineffective hematopoiesis, accompanied by distinct elevations in circulating EPO, remains to be investigated. Besides activity of the pathway, the contribution of other factors influencing FGF23 signaling in hereditary anemias, including inflammation and iron load, remains to be investigated. Moreover, the role of the individual FGFRs and α-KL in FGF23 signaling in hereditary anemia is currently unknown.

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8. IFGF23 DIRECTLY IMPAIRS BONE MINERALIZATION

The mineral ultrastructure of bone is crucial for its mechanical and biological properties. Non-collagenous proteins, as osteocalcin and osteopontin, are secreted during osteoid mineralization.[112] Loss of function of either or both osteocalcin and highly phosphorylated osteopontin significantly reduces crystal thickness and results in altered crystal shape.[113] Tissue non-specific alkaline phosphatase (TNAP) is anchored to the membranes of osteoblasts and chondrocytes and to matrix vesicles released by both cells, and degrades pyrophosphate (PPi) to Pi. Pyrophosphate is an inhibitor of bone mineralization, and the regulation of pyrophosphate by TNAP controls continuous extracellular mineralization of apatite crystals. TNAP deficiency leads to accumulation of pyrophosphate, thereby decreasing mineralization.[114]

FGF23 and EPO, are known regulators of bone mineralization, and are discussed in section *Fibroblast Growth Factor 23*. Finally, we discuss the contribution of these factors to defective bone mineralization in chronic diseases of erythropoiesis.

8.1 Fibroblast growth factor 23

Both gain and loss of function mutations in the *FGF23* gene result in bone mineralization disorders. (Table 2) Gain of function mutations in *FGF23* cause autosomal dominant hypophosphatemic rickets (AHDR), a disease marked by severe decreased bone mineral density.[115-117] The metabolic mirror of ADHR is familial tumoral calcinosis, which is associated with pathologic increase of bone mineral density and is caused by loss of function mutations in the *FGF23* or *GalNT3* gene.[116,117] So, disturbances in FGF23, either primary (congenital) or secondary (e.g. in response to high EPO), ultimately result in bone mineralization deficits.

FGF23 seems to act auto- and/or paracrine in the bone environment.[118] A model has been proposed for a local role of FGF23 signaling in bone mineralization, independent of α-KL, via FGFR3. Local FGF23 signaling in osteocytes results in suppression of TNAP transcription, which leads to decreased degradation, and subsequent accumulation, of pyrophosphate and suppression of inorganic phosphate production. Both directly reduce bone mineralization. Osteopontin secretion is indirectly downregulated by FGF23 signaling: lower availability of extracellular phosphate suppresses osteopontin expression. [118] Although, acting locally, also high systemically circulating FGF23 could modulate pyrophosphate metabolism.[118-120] Moreover, alterations in vitamin D metabolism contribute to impaired bone mineralization in response to iFGF23. $1,25(OH)_2D_3$ inhibits bone mineralization locally in osteoblasts and osteocytes via stimulation of transcription and subsequent expression of presumably inadequately phosphorylated osteopontin.[118,121]

So, iFGF23 signaling results directly in impaired bone mineralization via TNAP suppression. Notably, current knowledge is based on *FGF23*-knockout models, thereby not reflecting the interplay of iFGF23 and cFGF23.[118-120]

8.2 EPO

In addition to its role in erythropoiesis, EPO regulates bone homeostasis. Mice overexpressing endogenous EPO developed severe osteopenia.[122] Treatment of WT mice with rhEPO for ten days resulted in a significant reduction in trabecular bone volume and increased bone remodeling. Similar changes in bone volume were observed after increased endogenous EPO expression due to induction of acute hemolysis.[123,124] Despite these observations, the action of EPO on bone homeostasis remains controversial. Effects might be dose-dependent: supraphysiologic EPO concentrations induced mineralization,[1,125-131] whereas low endogenous overexpression or moderate exogenous doses of EPO impaired bone formation via EPOR signaling.[122,124,128,132] Whether excess cFGF23, in response to EPO, is capable to neutralize α-KL-independent osseous signaling of iFGF23, is currently unknown. We hypothesize that supraphysiologic EPO concentrations suppress the iFGF23/cFGF23 ratio to a level where the amount of cFGF23 is sufficient to fully prevent signaling of iFGF23 by competitive inhibition at the FGFR3. This resembles the hypermineralization observed in patients with elevated cFGF23 in familial tumoral calcinosis based on a *GaLNT3* mutation.[133]

8.3 Bone mineralization in disorders of erythropoiesis

Impaired bone mineralization, osteoporosis, is an important complication of chronic disorders affecting erythropoiesis.[134] The etiology of low bone mass is multifactorial including marrow expansion, various endocrine causes, direct iron toxicity, side effects of iron chelation therapy, lack of physical activity and genetic factors.[135,136] In SCD and thalassemia bone abnormalities have been attributed mainly to marrow expansion,[134] although a linear correlation between circulating EPO levels and degree of bone demineralization in patients with identical diseases lacked.[137] Eighty percent of adult SCD patients had an abnormal low bone mineral density,[138] and up to 90% of β-thalassemia patients had an elevated fracture risk.[139,140] More recently, among children and young adults receiving regular transfusions and adequate iron chelation therapy Z-scores were within the normal range.[139,140] The role of transfusions in correction of bone mineral density underlines the importance of EPO signaling in the etiology of bone disease.

Table 2. FGF23-related disorders. Summary of laboratory parameters and clinical characteristics of disorders associated w

Disease	Locus	Inheritance pattern	Genetic defect	FGF23 function	iFGF23	cFGF23	TmP/ GFR	Serum calcium
ADHR (OMIM 193100)	12p13.3	AD	R176Q, R179Q/W	GoF	= or ↑	↑ or =	↓	=
fTC (OMIM 211900)	12p13.3	AR	S71G, M96T, S129F, F157L	LoF	= or ↓	↑	↑	=

AD autosomal dominant; ADHR autosomal dominant hypophosphatemic rickets; AR autosomal recessive; FGF23 fibroblast growth factor 23; fTC familial tumoral calcinosis; GoF gain of function; IDA

Currently, it is unknown what the extent is of the contribution of high EPO and subsequent lowering the iFGF23/cFGF23 ratio, to impaired bone mineralization in patients with chronic disorders of erythropoiesis. We suggest that iFGF23 excreted by bone marrow erythroid cells might act on the surrounding osteocytes and osteoblasts in an auto- and/or paracrine way which will impair bone mineralization via TNAP suppression, subsequent pyrophosphate accumulation and indirect downregulation of ostopontin.[118-120] Hypothetically, rhEPO therapy in selected patients might increase EPO levels towards adequately elevated EPO levels, with further decline in the iFGF23/cFGF23 ratio, ultimately turning the balance towards increased bone mineralization.

SUMMARY AND FUTURE DIRECTIONS

We have outlined the importance of the EPO-FGF23 signaling pathways in erythroid cell development and bone mineralization. Both the amount of iFGF23 and its cleavage product cFGF23 determine signaling capacity. Insight in the activity of the EPO-FGF23 signaling pathway in rare hereditary anemias with varies degrees of hemolysis and ineffective erythropoiesis and varying circulating EPO concentrations, will add to the understanding of the pathophysiology and bone complications of these diseases.

with gain of function (ADHR) [13,141,156-161] and loss of function (fTC) mutations [141,159,160,162-166] in the *FGF23* gene.

	Serum phosphate	Urinary phosphate	PTH	$1,25(OH)_2D$	Bone features	Erythropoiesis
	↓	↑	= or ↑	= or ↓	Bone deformities including varus deformity lower extremities, rachitic rosary, craniosynostosis, short stature; bone pain, bone fractures.	IDA, or low serum iron, associated with elevated FGF23 in ADHR.
	↑	↓	= or ↓	= or ↑	Tumoral calcinosis, or ectopic calcifications, hyperostosis, vascular calcifications.	Not reported.

iron deficiency anemia; LoF loss of function; PTH parathyroid hormone; TmP/GFR tubular maximum reabsorption rate of phosphate per glomerular filtration rate.

Currently, two therapeutic agents are under development, or already registered, interfering with the EPO-FGF23 axis: FGF23 antagonists (KRN23; a therapeutic antibody against the C-terminus of FGF23) and FGFR1 inhibitor (BGJ-398; a small molecule pan-FGF kinase inhibitor).[141] Both agents have been tested for disorders characterized by high iFGF23 concentrations: tumor-induced osteomalacia (iFGF23 secreting tumors), or x-linked hyperphosphatemia (*PHEX* mutation results in high iFGF23).

Administration of rhEPO decreases the iFGF23/cFGF23 ratio, inhibiting apoptosis in erythroid cells. However, both EPO and an increase in the absolute amount of iFGF23 impair bone mineralization. Hypothetically, application of selective iFGF23 antagonists, or cFGF23 agonists, might bypass non-FGF23 related side-effects of rhEPO by regulating a more downstream component of the EPO-FGF23 pathway.

Uncertainties exist regarding (long-term) application of FGF23 antagonists or FGFR1 inhibitors in human. Thereby, the influence of FGF23, and pharmacological manipulation of FGF23, on energy metabolism is unclear. FGF23 is along with FGF21 and FGF19, both clearly associated with energy metabolism, grouped as endocrine FGFs.[141]

Moreover, iFGF23 serves as a proinflammatory paracrine factor, secreted mainly by M1 proinflammatory macrophages.[44,84,85,142,143] Oxygen supply in inflamed tissues is often very limited.[144,145] This inflammation-induced hypoxia leads to increased expression of EPOR in

macrophages, suppresses inflammatory macrophage signaling and promotes resolution of inflammation.[146,147] In response on EPO, a substantial increase in cFGF23 compared to iFGF23 might antagonize the pro-inflammatory effects of iFGF23 or even promote development of a M2-like phenotype, characterized by immunoregulatory capacities.[1,146,148] Several forms of hemolytic hereditary anemias present with chronic (low-grade) inflammation, which might play an important role in the vascular complications of these diseases.[149-155] Theoretically, cFGF23 agonists might diminish inflammation in these patients and improve clinical outcomes.

In conclusion, although first discovered as phosphate regulator, FGF23 is an important regulator of erythropoiesis being part of the EPO-FGF23 signaling pathway. A new area of research is open to extent our knowledge about FGF23 biology beyond the kidney. Experimental research is required to identify the molecular and cellular players of the EPO-FGF23 signaling pathway and the role of the various FGFRs in erythropoiesis. Thereby, to determine the clinical relevance of the pathway in patients with alterations in erythropoiesis, we propose measuring iFGF23, cFGF23 and EPO levels in patients with various forms of dyserythropoietic or hemolytic anemia, and relating these values to inflammation, bone health and vasculopathic complications.

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CHAPTER 4

Interplay of erythropoietin, fibroblast growth factor 23 and erythroferrone in patients with hereditary hemolytic anemia

A.J. van Vuren[1], M.F. Eisenga[2], S. van Straaten[1], A. Glenthøj[3], C.A.J.M. Gaillard[4], S.J.L. Bakker[2], M.H. de Borst[2], R. van Wijk[5], E.J. van Beers[1].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;
[2] Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, University of Groningen, The Netherlands;
[3] Department of Hematology, Herlev and Gentofte Hospital, Denmark;
[4] Division of Internal Medicine and Dermatology, University Medical Center Utrecht, Utrecht University, The Netherlands;
[5] Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

Recently, erythropoietin (EPO) was identified as regulator of fibroblast growth factor 23 (FGF23). Proteolytic cleavage of biologically active intact FGF23 (iFGF23) results in the formation of C-terminal fragments (cFGF23). An increase in cFGF23 relative to iFGF23 suppresses FGF receptor signaling by competitive inhibition. EPO lowers the i:cFGF23 ratio, thereby overcoming iFGF23-mediated suppression of erythropoiesis. We investigated EPO-FGF23 signaling and levels of erythroferrone (ERFE) in 90 patients with hereditary hemolytic anemia (www.trialregister.nl [NL5189]). We show, for the first time, the importance of EPO-FGF23 signaling in hereditary hemolytic anemia: there was a clear correlation between total FGF23 and EPO levels (r = +0.64; 95% confidence interval [CI] [0.09, 0.89]), which persisted after adjustment for iron load, inflammation, and kidney function. There was no correlation between iFGF23 and EPO. Data are consistent with a low i:cFGF23 ratio. Therefore, as expected, we report a correlation between EPO and ERFE in a diverse set of hereditary hemolytic anemias (r = +0.47; 95% CI [0.14, 0.69]). There was no association between ERFE and total FGF23 or iFGF23, which suggests that ERFE does not contribute to the connection between FGF23 and EPO. These findings open a new area of research and might provide potentially new druggable targets with the opportunity to ameliorate ineffective erythropoiesis and the development of disease complications in hereditary hemolytic anemias.

KEY POINTS

- EPO plays an important role in FGF23 signaling in hereditary hemolytic anemias.
- There is a clear correlation of EPO and ERFE in patients with a variety of congenital hemolytic anemias other than hemoglobinopathies.

INTRODUCTION

Systemic erythropoietin (EPO) production plays a critical role in maintaining erythropoietic homeostasis.[1] The amount of circulating EPO is elevated in most forms of hereditary anemia, although the amount is often still relatively low for the degree of anemia. Insight into components of the signaling cascade downstream of EPO might lead to a greater understanding of EPO responsiveness in individual patients. Recently, EPO was identified as an important regulator of production and cleavage of fibroblast growth factor 23 (FGF23).[2-7] Because erythroid progenitor cells express high levels of FGF23 and carry the FGF receptor, it is likely that they are involved in the FGF23 metabolic pathway.

FGF23 was originally identified as a bone-derived hormone, a key player in phosphate and vitamin D metabolism, and a regulator of bone mineralization. It was shown that bone marrow, specifically the early erythroid lineage, contributes significantly to total circulating FGF23.[2,5,8] Erythroid progenitor cells express FGF23 messenger RNA (mRNA) under physiologic conditions, and significant increases are observed in response to EPO.[2,5,6] FGF23 is formed as intact biologically active protein (iFGF23). Proteolytic cleavage results in the formation of an assumed inactive C-terminal tail FGF23 (cFGF23). By competitive inhibition, an increase in cFGF23 relative to iFGF23 leads to suppression of FGF receptor signaling.[9,10] (Figure 1A-1)

FGF23 knockouts or animals treated with iFGF23-blocking peptides show increased erythropoiesis, reduced erythroid cell apoptosis, and increased *EPO* mRNA and EPO levels.[6,10,11] *In vitro* administration of iFGF23 to *FGF23*-knockout bone marrow–derived erythropoietic cells normalizes erythropoiesis, and administration of cFGF23 in chronic kidney disease (CKD) mice decreases erythroid cell apoptosis and increases serum EPO levels. Recombinant EPO stimulates a proportional increase in *FGF23* mRNA transcription and iFGF23 cleavage, which translates into increased levels of circulating cFGF23 with only a modest increase in iFGF23 levels. Consequently, EPO alters the i:cFGF23 ratio in favor of cFGF23, overcoming the suppressive effects of iFGF23 on erythropoiesis.[2-7] Thereby, iron deficiency and acute inflammation also decrease the i:cFGF23 ratio.[12-15] (Figure 1A-2) On the other hand, an excess of circulating iFGF23 and a high i:cFGF23 ratio are observed in CKD as a consequence of impaired FGF23 cleavage in the presence of increased *FGF23* transcription.[16]

In addition to FGF23, erythroferrone (ERFE) is a known hormone produced by erythroblasts in response to EPO, and it acts as a negative regulator of hepcidin during stress erythropoiesis.[17] ERFE expression is expected to be increased in hereditary anemias with ineffective erythropoiesis (characterized by increased EPO levels), which will contribute to hepcidin suppression and subsequent iron loading. It is unknown whether ERFE interferes with or mediates EPO-FGF23 signaling.

As recently discussed,[18] we hypothesize that EPO plays an important role in FGF23 signaling in hereditary hemolytic anemia. We explored this hypothesis in the current study, also taking into account ERFE, as a negative regulator of hepcidin.

STUDY DESIGN

Total FGF23, iFGF23, and EPO were measured in 90 patients and 10 healthy controls from the ZEbRA trial, a cross-sectional observational study on clinical sequelae and pathophysiology of rare congenital hemolytic diseases, which was conducted at the University Medical Center Utrecht (Netherlands Trial Register [NL5189]).

Laboratory parameters related to erythropoiesis, hemolysis, iron metabolism, kidney function, and calcium-phosphate metabolism were measured using routine laboratory procedures. An overview of relevant patient characteristics is provided in Table 1.

Two FGF23 enzyme-linked immunosorbent assays were used for FGF23 measurements in stored plasma samples: 1 that detects an epitope in the C-terminal part of the FGF23 molecule and, therefore, quantifies cFGF23 fragments and (full-length) iFGF23 (total FGF23; Immunotopics/Quidel, San Clemente, CA) and 1 that detects only iFGF23 (KAINOS Laboratories, Tokyo, Japan).

Plasma ERFE levels were quantified using an Intrinsic ERFE IE ELISA Kit (Intrinsic LifeSciences, San Diego, CA), according to the manufacturer's instructions.

In addition, a broad range of chemokines and cytokines were measured (interleukin-1α [IL-1α], IL-1β, IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-21, IL-22, IL-23, IL-27, IL-31, tumor necrosis factor-α, tumor necrosis factor-β, interferon-γ, monocyte chemoattractant protein-1, macrophage inflammatory protein 1α [MIP-1α], MIP-1β, MIP-3α, eotaxin, monocyte chemoattractant protein-4, MDC, IP-10, PIGF, VEGF, soluble [s]CD163, HSP70, soluble vascular endothelial growth factor receptor 1, sICAM, and sVCAM) with a Luminex assay.

Correlations among total or iFGF23, ERFE, and EPO, as well as between iFGF23 and calcium, phosphate, 25-OH vitamin D, and CKD-EPI values (using the Chronic Kidney Disease Epidemiology Collaboration equation), were determined by linear regression analyses. In the case of parametric testing, bootstrapping was performed to confirm significance. Statistical significance for all tests was set at a 2-sided $P < 0.05$. Statistical analysis was performed using IBM SPSS software, version 25.0.0.2 (SPSS Inc., Chicago, IL).

The study was approved by the Medical Research Ethics Committee of the University Medical Center Utrecht in accordance with the Dutch Medical Research Involving Human Subjects Act and other applicable Dutch and European regulations.

RESULTS AND DISCUSSION

We observed a clear correlation between levels of total FGF23 and EPO in patients with various hereditary hemolytic anemias (r = +0.64, 95% confidence interval [CI] [0.09, 0.89]) that persisted after adjustment for iron load (transferrin saturation), inflammation (C-reactive protein), and kidney function (CKD-EPI). (Figure 1B) As expected, there was not a correlation between iFGF23 and EPO values or between iFGF23 and total FGF23. These data are consistent with a low i:cFGF23 ratio in response to EPO. To exclude a significant influence of calcium phosphate metabolism and kidney function on FGF23 regulation in our patients, we evaluated the relationship between biologically active FGF23 and serum calcium, phosphate, 25-OH vitamin D, and CKD-EPI measurements. iFGF23 did not correlate with serum calcium, phosphate, 25-OH vitamin D, or CKD-EPI (P > 0.05).

Although not an unexpected finding, we are the first to report a strong correlation between EPO and ERFE in a diverse set of patients with rare hereditary hemolytic anemias other than hemoglobinopathies,[19-21] with and without transfusion dependency and with and without iron-reducing therapy (r = +0.47; 95% CI [0.14, 0.69]). There was no correlation between ERFE and total FGF23 or iFGF23. ERFE is known to inhibit induction of hepcidin; hepcidin values were unavailable, which limited the ability to study the link between erythropoiesis and iron metabolism in our cohort. Hepcidin itself was previously shown to be capable of inducing FGF23 production and its cleavage, independently of inflammation.[13] Our data underscore the importance of EPO-ERFE signaling in hemolytic anemia; however, ERFE is not simply an intermediary in EPO-FGF23 signaling. Our observation is in line with a previous study performed in mice.[2] Thereby, we confirm the persistence of a relationship between EPO and FGF23 regulation in the absence of iron deficiency.

The influence of inflammation on the EPO-FGF23 axis[22] was clearly illustrated by higher mean total FGF23 in patients with sickle cell disease (549 RU/L; standard deviation, 606 RU/L) compared with other patients (182 RU/L; standard deviation, 442 RU/L; mean difference, 367; 95% CI [64, 743]), without a significant difference in EPO or iFGF23.

Table 1. Demographic, laboratory and clinical characteristics per disease.

	HS N = 24	PKD N = 23
Gender male (%)	46	48
Age, years	47 (37, 59)	43 (29, 48)
Iron chelator use, %‡	0	36
Phlebotomies, %§	17	11
Regular transfusions, %	0	26
Splenectomy, %	42	65
total FGF23, RU/mL	83 (64, 113)	74 (65, 93)
iFGF23, pg/mL	42 (40, 52)	37 (29, 44)
Erythropoietin, IU/L	13 (6, 31)	25 (17, 32)
Erythroferrone, ng/mL	4 (2, 22)	14 (6, 33)
Hemoglobin, g/dL	13.5 (11.9, 15.3)	10.0 (8.2, 12.1)
MCV, fL	90 (88, 93)	104 (97, 111)
Reticulocytes, x10^9/L	285 (143, 489)	552 (252, 893)
Leukocytes, x10^9/L	6.6 (5.6, 10.1)	8.7 (6.9, 10.0)
Platelets, x10^9/L	235 (185, 407)	417 (283, 821)
Creatinine, μmol/L	68 (62, 81)	63 (49, 67)
CKD-EPI, ml/min/1.73m²	97 (92, 113)	110 (105, 120)
Calcium, μmol/L	2.34 (2.26, 2.36)	2.38 (2.32, 2.43)
Phosphate, μmol/L	NA	1.00 (0.85, 1.16)
25-OH vitamin D, nmol/L	49 (37, 107)	48 (28, 69)
Ferritin, μg/L	178 (104, 266)	521 (225, 781)
TSAT, %	31 (25, 38)	47 (31, 77)
CRP, mg/L	1.1 (0.5, 4.0)	0.9 (0.7, 1.8)
Total bilirubin, μmol/L	28 (20, 51)	55 (43, 83)
LD, IU/L	200 (186, 246)	191 (164, 347)
AST, IU/L	24 (19, 33)	32 (27, 43)

Unless otherwise indicated, data are median (interquartile range). * "Other" includes patients with Piezo-1–mutated hereditary xerocytosis (N = 8), other hemoglobinopathies (N = 6), and other enzymopathies (N = 2). † Not reported values were not available. ‡ Iron chelator use was defined as

SCD N = 15	β-thalassemia N = 12	Other* N = 16	Healthy controls N = 10†
40	67	50	33
30 (27, 35)	30 (27, 39)	50 (32, 65)	
27	83	27	0
33	10	46	0
33	75	13	0
7	75	20	0
451 (153, 616)	169 (116, 361)	119 (78, 179)	77 (63, 91)
46 (29, 50)	51 (38, 70)	50 (44, 55)	45 (38, 50)
32 (21, 68)	58 (38, 137)	30 (21, 34)	3 (3, 4)
7 (5, 12)	62 (40, 73)	16 (11, 40)	1 (0, 2)
9.8 (8.7, 12.3)	9.0 (8.1, 9.7)	13.1 (10.1, 14.0)	14.2 (13.7, 15.7)
92 (84, 112)	80 (76, 83)	96 (81, 103)	90 (86, 92)
178 (107, 260)	142 (63, 221)	303 (96, 630)	59 (44, 72)
7.8 (5.2, 13.3)	11.8 (8.6, 13.9)	6.5 (5.9, 7.6)	6.2 (4.7, 8.0)
385 (318, 481)	595 (298, 774)	255 (184, 447)	266 (239, 315)
57 (44, 76)	49 (43, 68)	61 (55, 70)	
139 (126, 149)	132 (117, 146)	107 (91, 128)	
2.30 (2.23, 2.36)	2.32 (2.29, 2.45)	2.43 (2.31, 2.48)	
1.09 (0.84, 1.26)	1.28 (1.17, 1.34)	1.04 (0.94, 1.22)	
34 (21, 65)	44 (20, 51)	64 (48, 77)	
562 (62, 481)	591 (394, 1300)	147 (101, 428)	
43 (25, 74)	92 (80, 120)	51 (23, 68)	
5.4 (3.0, 9.5)	1.6 (0.6, 6.9)	2.4 (1.7, 3.3)	
31 (22, 47)	35 (33, 77)	28 (21, 85)	
313 (263, 393)	190 (153, 284)	209 (172, 403)	
38 (34, 43)	29 (20, 37)	26 (18, 28)	

prescription of an iron chelating agent during the last 12 months. § Phlebotomies apply to a regular phlebotomy scheme in the past. AST aspartate transaminase; CRP C-reactive protein; HS hereditary spherocytosis; LD lactate dehydrogenase; MCV mean corpuscular volume; TSAT transferrin saturation.

We identified a correlation between total FGF23 and MIP-1β (r = +0.49; 95% CI [0.15, 0.72]) and between total FGF23 and sICAM (r = +0.54; 95% CI [0.16, 0.75]), markers of endothelial dysfunction and immune players in pulmonary hypertension.[23,24] Moreover, we speculate on the contribution of a decline in the i:cFGF23 ratio to vasculopathic complications. Our hypothesis is in line with previous observations that too little FGF signaling activates tumor growth factor-β signaling, leading to endothelial-to-mesenchymal transition and smooth muscle cell proliferation, which contribute to the development of pulmonary hypertension.[25,26]

In conclusion, we show, for the first time, a strong correlation between EPO and FGF23 signaling in a broad range of hereditary hemolytic anemias. Additionally, we show a clear, but not previously reported, correlation between EPO and ERFE in patients with a variety of hemolytic congenital anemias. These findings open up a new area for research and hold promise for potential future treatment in hereditary hemolytic anemia, because therapeutic targeting of the FGF23 pathway might provide an opportunity to ameliorate ineffective erythropoiesis and the development of complications.
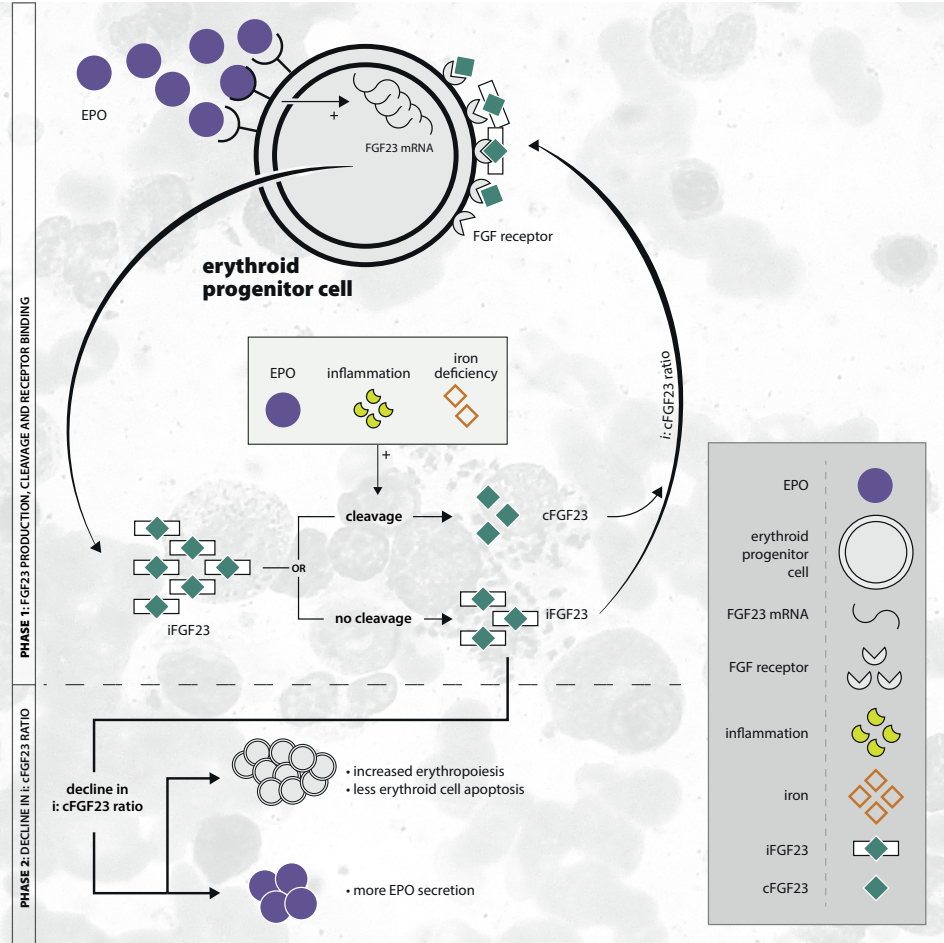
Figure 1. The relationship between EPO and FGF23 metabolism.

(A) Schematic overview of the EPO-FGF23 signaling pathway in the erythroid lineage in bone marrow. Phase 1 shows FGF23 production, the secretory process, and FGF receptor binding; phase 2 summarizes the effects of a decrease in the i:cFGF23 ratio.
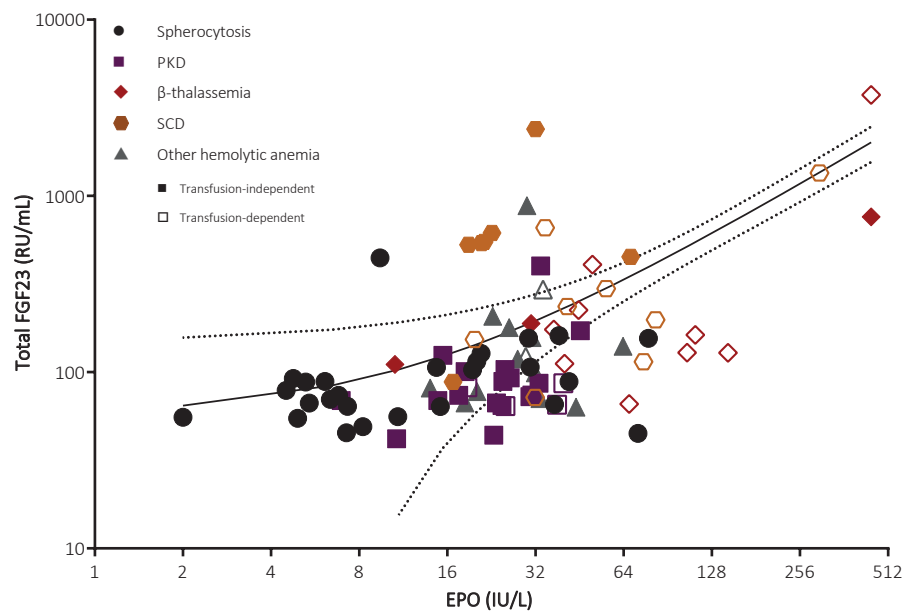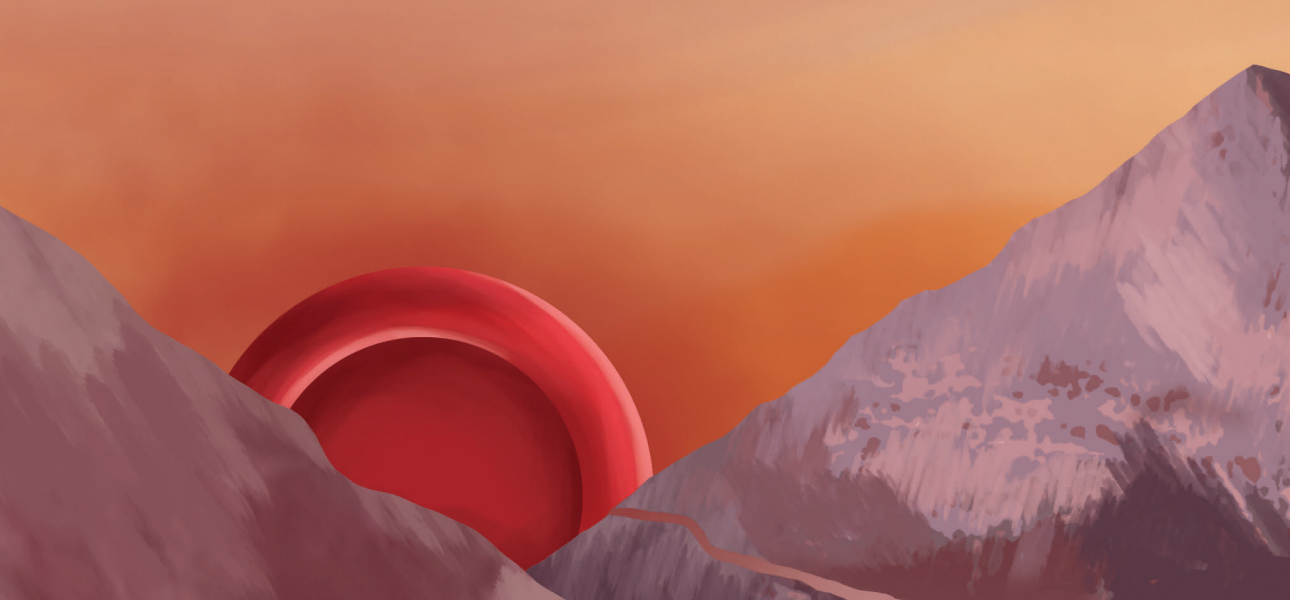
Figure 1. Continued.

(B) Correlation between C-terminal FGF23 and EPO. Individual patient data are represented as disease and transfusion requirement. Correlation between total FGF23 and EPO in patients with various hemolytic anemias: r = +0.64; 95% CI [0.09, 0.89]. The solid line represents the linear regression line; the dashed lines are the 95% CI bands.

PKD pyruvate kinase deficiency; SCD sickle cell disease.

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PART 2

The challenge called heterogeneity

CHAPTER 5

Lactate dehydrogenase to carboxyhemoglobin ratio as a biomarker of heme release to heme processing is associated with higher tricuspid regurgitant jet velocity and early death in sickle cell disease

A. J. van Vuren[1], C.P. Minniti[2], L. Mendelsohn[3], J.H. Baird[4], G.J. Kato[5] and E.J. van Beers[1].

[1]   Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;

[2]   Division of Hematology, Montefiore Medical Center/Albert Einstein College of Medicine, Bronx, NY, USA;

[3]   Sickle Cell Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA;

[4]   Division of Hematology, Department of Medicine, Stanford University, Stanford, USA;

[5]   Heart, Lung and Blood Vascular Medicine Institute and the Division of Hematology–Oncology, Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA.

ABSTRACT

Hemolysis is a hallmark of sickle cell disease (SCD), occurring in intravascular and extravascular compartments. Intravascular hemolysis releases free hemoglobin, heme and lactate dehydrogenase (LDH), and is with vasculopathic consequences. Overall hemolysis generates the heme breakdown product carbon monoxide (CO), transported in blood as carboxyhemoglobin (HbCO). End-alveolar CO (EACO) in exhaled breath is an accepted proxy marker. We explored the use of these, as potential biomarkers of the relative contribution of intra- and extravascular hemolysis, to the risk of cardiopulmonary complications and death in SCD.

We investigated the relationship between EACO to HbCO plasma levels (N = 37). After establishing a good correlation, we evaluated the relation of the ratio of LDH/HbCO as representatives of intravascular and total hemolysis respectively to echocardiography parameters and mortality rates in adults with SCD at the NIH, Bethesda, USA (N = 157).

EACO and HbCO were strongly correlated to each other, and to indicators of total hemolysis. HbCO did not correlate to LDH. LDH/HbCO ratio was positively correlated with echocardiographic markers of cardiopulmonary risk, tricuspid regurgitation velocity (TRV; r = 0.38, p < 0.01). This ratio was significantly higher in patients with TRV ≥3.0m/s (p = 0.02). Survival was worse in the high LDH/HbCO group at 10 and 15 years (76% vs. 93%, and 69% vs. 88%). In multivariable analysis the LDH/HbCO ratio was associated with all-cause mortality (p < 0.01) alone and when adjusted for age, CRP and ferritin (p = 0.02).

So, a ratio of two readily available laboratory markers, LDH and HbCO, represents a promising prognostic biomarker associated with vasculopathic complications in SCD. [NCT01547793; NCT00011648]

INTRODUCTION

Chronic hemolysis is a hallmark of sickle cell disease (SCD). Prior estimates of intravascular hemolysis as a proportion of the total hemolytic rate in SCD ranged from less than 10% to more than 30%.[1] Chronic intravascular hemolysis is associated with severe vasculopathic complications including pulmonary hypertension and early mortality in SCD.[2-4] Intravascular hemolysis leads to release of cellular free hemoglobin and heme.[5-7] Normally, these are scavenged by respectively haptoglobin and hemopexin. However, in SCD patients the availability of scavenging proteins in plasma is typically decreased because of rapid consumption by the prodigious amount of intravascular hemolysis.[8-11] Both cellular free hemoglobin and heme, devoid of anti-oxidant buffering mechanisms normally present in red cells,[12-14] are recognized as erythroid damage-associated molecular pattern molecules (e-DAMPs) and contribute to development of chronic vasculopathy, platelet activation and pulmonary hypertension in SCD.[1,15-20] Cell free hemoglobin has the capacity to scavenge nitric oxide by a deoxygenation reaction and contributes to formation of oxidants via the Fenton reaction, whereas free heme also attenuates pro-inflammatory Toll-like receptor 4 signaling in response to endogenous ligands.[2] Intravascular heme release is associated with elevated levels of serum lactate dehydrogenase (LDH).[2,4,21-23] Free plasma hemoglobin and aspartate transaminase (AST) are, combined with LDH, commonly used as biomarkers for intravascular hemolysis in SCD. The individual correlation of these biomarkers to hemolytic rate is limited and improves only marginally when the markers are combined.[2]

All heme metabolized either via extravascular hemolysis or cleared from the plasma via scavenger proteins, will lead to endogenous carbon monoxide (CO) production by heme-oxygenase 1 (HO-1).[24] In contrast to heme, CO has many protective effects in SCD including inhibition of polymerization of sickle hemoglobin, increased red blood cell hydration, anti-oxidative and anti-inflammatory effects.[25] Theoretically, the balance of detrimental intravascular hemolysis to beneficial CO production (total hemolysis) could be a prognostic biomarker in SCD. Under physiological conditions the breakdown of hemoglobin accounts for 80% of endogenous CO production.[26,27] Because the vast majority of endogenous CO is cleared by exhalation,[28,29] end-alveolar CO (EACO) in morning first breath is an accepted reflection of endogenously produced CO, and thereby an accepted proxy marker of overall hemolysis. Before exhalation, CO is transported primarily as the conjugate carboxyhemoglobin (HbCO). HbCO levels up to 4.3% are reported in SCD.[30-32] Several small studies have reported correlations between EACO and other hemolytic biomarkers.[33-40]

Here we explore whether HbCO can be a convenient surrogate marker of endogenous CO production as a byproduct of hemolysis.[41] In addition, we hypothesize that the ratio of LDH to CO may give an estimate of the relative contribution of intravascular heme release to total heme processing and relates to clinical endpoints, and therefore could be a new readily available biomarker of interest in SCD.[42,43]

METHODS

Study population

The study population consisted of two cohorts, in which all patients provided written informed consent, in accordance with the Declaration of Helsinki.

[cohort A] We investigated the relationship between HbCO and EACO in breath samples in 37 adult SCD patients (homozygous S or heterozygous hemoglobin S combined with C, D or β⁺-thalassemia) enrolled from January 2012 until November 2015 [NCT01547793]; research protocols approved by the institutional review board of the National Institutes of Health (National Heart, Lung and Blood Institute - NHLBI, Bethesda, USA).

Breath samples were obtained during steady-state disease, defined as no transfusions in the preceding eight weeks and no acute painful crises or other complications in the preceding four weeks. Subjects were asked to avoid smoke exposure at least 24 hours prior to breath collection. Patients were provided with a gas sampling bag that was used by the patient to sample exhaled breath directly upon awakening at home. Samples were analyzed on an Ametrek TA7000 Gas Purity Monitor. CO measurements were corrected for environmental CO. Blood samples were collected on the same day. HbCO was determined using CO-oximetry and methemoglobin using electron paramagnetic resonance spectroscopy. Other biomarkers of erythropoiesis or hemolytic activity were measured with commercial assays used in clinical practice.

[cohort B] We investigated the correlation between HbCO in routinely taken blood samples, and biomarkers for hemolysis in 157 adult SCD patients (homozygous S or heterozygous hemoglobin S combined with C, D or β⁺-thalassemia) included from April 2002 until June 2012 in a study on Secondary Pulmonary Hypertension in Adults with Sickle Cell Anemia; [NCT00011648], research protocols approved by the institutional review board of the National Institutes of Health (NHLBI, Bethesda, USA). The individual LDH/HbCO ratio was calculated from the median HbCO and median LDH values from samples routinely obtained during follow-up.

In patients of cohort B that had tricuspid regurgitation velocity (TRV) and mortality data available (N = 92), we investigated the relation of HbCO with TRV and with mortality. Mortality data were based on death certificates with data cut-off for this manuscript occurring in October 2018. Patients with self-reported smoking were excluded from the analyses.

Statistical analysis

Correlation between EACO and HbCO was determined by linear regression analysis. Correlations of EACO or HbCO and other hematological and hemolytic parameters were determined by Pearson's correlation. Differences between groups were tested with a Mann-Whitney U test. Differences in survival between two groups were calculated with a Log Rank test. Cox proportional hazards regression models were used to examine the association of the LDH/HbCO ratio while adjusting for other factors. In case of parametric testing, bootstrapping was performed to confirm significance.[44] Statistical significance for all tests was set at a two-sided p < 0.05. All analyses were performed with IBM SPSS Statistics version 25.

5

RESULTS

An overview of the cohorts' demographics, hematological and hemolytic parameters is provided in Table 1. In both cohorts, we confirmed the expected correlation among surrogate biomarkers of intravascular heme release LDH and AST (cohort A: r = 0.70, 95% confidence interval [CI] [0.10, 0.83]; and cohort B: r = 0.44, 95% CI [0.29, 0.63]).

Table 1. Overview of markers of erythropoiesis and hemolysis.

	Cohort A[*]	Cohort B[†]
Number of patients	37	157
Age (yr)	NA	NA
Erythropoiesis		
Hb, g/dL	8.7 (8.3, 9.6)	8.4 (7.4, 9.6)
Absolute reticulocytes, x10^9	166 (106, 271)	-
Intravascular hemolysis		
LDH, U/L	370 (309, 459)	320 (261, 431)
AST, U/L	32 (25, 45)	42 (30, 60)
Methemoglobin, %	1.5 (1.2, 1.8)	1.2 (0.9, 1.6)
Other hemolysis		
HbCO, g/dL	0.19 (0.11, 0.25)	0.23 (0.19, 0.29)
Bilirubin, total, mg/dL	1.4 (0.8, 3.1)	2.1 (1.4, 3.1)
Other SCD		
HbS, %	78 (72, 83)	79 (71, 87)
HbF, %	16 (11, 22)	9 (4, 15)

Median values (IQR) of the hematological and hemolytic parameters per cohort.
[*]Results of blood samples collected on day of EACO measurement.
† Routine blood analyses with results on HbCO values collected during follow-up.
Hb hemoglobin; LDH lactate dehydrogenase; AST aspartate transaminase; HbCO carboxyhemoglobin; IQR interquartile range.

EACO and HbCO strongly correlated to each other

We identified a clear positive correlation of HbCO with EACO (r = 0.66, 95% CI [0.36, 0.81]). (Figure 1) However, in both cohorts, there was no consistent correlation of EACO or HbCO with either LDH or AST. On the other hand, in cohort A, both EACO and HbCO were significantly, positively correlated with absolute reticulocyte counts (respectively r = 0.46, 95% CI [0.20, 0.70] and r = 0.58, 95% CI [0.34, 0.80]). (Table 2) Additionally, we explored variability in CO production in individual patients in whom more than four blood samples were available (N = 21). (Figure S1 and Table S1) The observations suggest that every patient has a finite HbCO range with minor variation over time and not significantly associated with changes in LDH in

this limited dataset. Thus, the serum level of LDH (known to reflect intravascular hemolysis and other tissue injury, but not extravascular hemolysis) appears to be heterogeneous among SCD patients and does not directly represent total hemolysis as represented by HbCO. This interpretation led us to formulate that the fraction of total hemolysis that is intravascular might more directly correlate with vascular health outcomes.
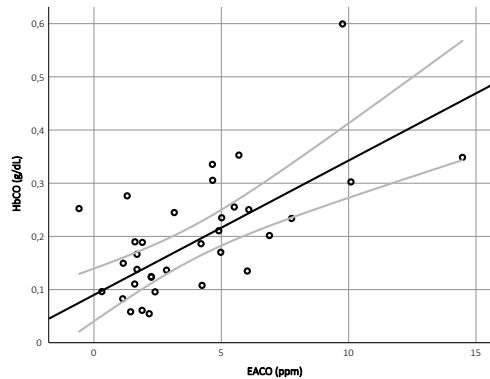

Figure 1. Relation between HbCO and EACO in non-smoking steady-state SCD patients (cohort A). Scatterplot of HbCO (*y-axis*) and end-alveolar CO (EACO; *x-axis*). Fitline represents Pearson's correlation; r = 0.66, 95% CI [0.36, 0.81].

Higher LDH/HbCO ratios are associated with an increased risk of pulmonary hypertension

Next, we investigated whether an increased fraction of intravascular hemolysis, an increased ratio of LDH to HbCO, was related to clinical endpoints. Clinical data were available in 92 individual patients with sickle cell syndromes (86 HbSS, 3 HbSb[+], 3 HbSC) with a median age at the time of echocardiography of 30 years (interquartile range [IQR] 22, 40).

Previously, tricuspid regurgitation velocity (TRV) ≥ 3.0m/s was associated with the highest risk of development of pulmonary hypertension and increased mortality in SCD.[45-49] Patients in this high-risk category (N = 13) had a significantly higher LDH/HbCO ratio compared to the other patients (p = 0.02). (Figure 2A) There was no significant difference in LDH values between the two groups. Individual median LDH/HbCO ratios and TRV were significantly correlated (r = 0.38, 95% CI [0.12, 0.60]. However, LDH and TRV were not correlated (r = 0.22, 95% CI [-0.10, 0.52]). (Figure 2B) Of interest, a cutoff value of LD/HbCO ratio of 1,200 could be used to exclude patients with high risk TRV values: all patients (N = 25) with LDH/HbCO ratio < 1,200 had a TRV < 3.0m/s. The LDH/HbCO ratio was greater than 1,200 in 15/16 patients (94%) with catheterization-proven pulmonary hypertension. (Supplemental section)

Table 2. Association between EACO/HbCO and hematological and hemolytic parameters.

| CO-measurement | Cohort A | | Cohort B |
	EACO	HbCO	HbCO
Erythropoiesis			
Hb, g/dL	-0.07	0.22	0.29[**]
Absolute reticulocytes, x10^9	0.46[**]	0.58[**]	-
Intravascular hemolysis			
LDH, U/L	-0.07	-0.22	-0.03
AST, U/L	0.07	-0.05	-0.07
Methemoglobin, %	-0.12	0.39[*]	-0.06
Other hemolysis			
Bilirubin, total, mg/dL	0.42[**]	0.34[*]	0.19[**]
Bilirubin, direct, mg/dL	0.40[*]	0.25	0.09[*]
Bilirubin, indirect, mg/dL	0.41[*]	0.34[*]	0.19[**]

Correlation between EACO (ppm) or HbCO (g/dL), and other parameters of erythropoiesis and hemolysis.

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

Hb hemoglobin; LDH lactate dehydrogenase; AST aspartate transaminase; HbCO carboxyhemoglobin.

Increased LDH/HbCO ratios are associated with all-cause mortality

Median follow-up of the included 92 patients was 12.1 years (IQR 10.3, 16.2). During follow-up, 25% (23/91) of the patients died; 48% (11/23) of these patients were initially diagnosed with pulmonary hypertension based on cardiac catheterization.

All-cause mortality predictability by LDH/HbCO ratio and LDH was analyzed in a Cox proportional hazard model. (Table 3) The LDH/HbCO ratio was significantly associated with mortality in the unadjusted analysis ($p < 0.01$). Age, CRP and ferritin were previously reported to be related to early-mortality in SCD.[50,51] In our cohort age ($p = 0.03$), CRP ($p = 0.05$) and ferritin ($p < 0.01$) were associated with mortality, as was TRV ($p < 0.01$). In the multivariate-adjusted analysis including age, CRP and ferritin, LDH/HbCO ratios remained significantly associated with all-cause mortality ($p = 0.02$). There was a clear trend toward association between LDH and all-cause mortality in the unadjusted analysis ($p = 0.10$), however, it did not reach statistical significance in our cohort. Kaplan-Meier survival probabilities for patients stratified by LDH/HbCO ratios greater than and less than 1,200 are shown in Figure 3 ([A] for all patients and [B] for patients with TRV values < 3.0 m/s). Five-year, 10-year and 15-year overall survival in the group with LDH/HbCO ratio > 1,200 were respectively 92.1%, 76.0% and 69.1%, whereas 5-year, 10-year and 15-year overall survival in the group with LDH/HbCO ratio < 1,200 were respectively 100%, 92.9% and 88.0%. In summary, the LDH/HbCO ratio predicts survival probability independent of age, ferritin and CRP.
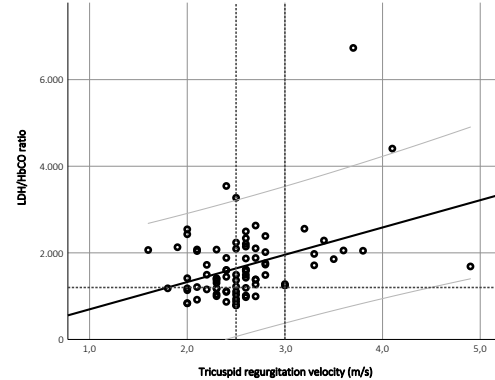
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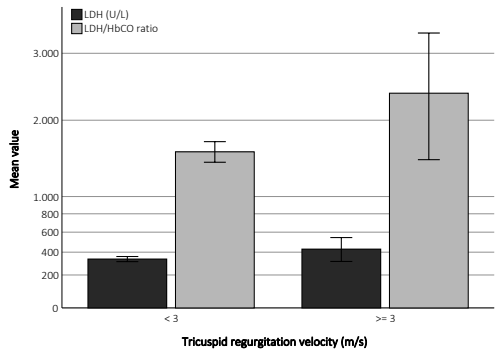
B

Figure 2. Association between LDH/HbCO and tricuspid regurgitation velocity (TRV).

(A) Patients were divided based on peak TRV in a high risk (N = 13) and lower risk category (N = 72). Bars represent mean values of individual median LDH values, and individual median LDH/HbCO ratios. (B) Median LDH/HbCO ratio of individual patients (*y-axis*) plotted against TRV (*x-axis*). Fitline represents Pearson's correlation; r = 0.38, 95% CI [0.12, 0.60]. Two vertical lines are set at a TRV of 2.5 and 3.0 m/s. The horizontal line is set at a LD/HbCO ratio of 1,200.

Table 3. Association between LDH/HbCO ratio and mortality.

	Variable	Hazard ratio	95% CI	P-value
LD/HbCO ratio *Unadjusted*	LDH/HbCO ratio / 1000	1.88	1.28-2.78	< 0.01
Adjusted	LDH/HbCO ratio / 1000	1.74	1.05-2.88	0.02
	Age	1.03	0.98-1.09	0.25
	Ferritin	1.00	0.99-1.00	0.75
	CRP	1.13	1.04-1.22	0.01

Output of Cox proportional hazards model. Association between LDH/HbCO ratio and all-cause mortality. The analysis was adjusted for age, ferritin and CRP.

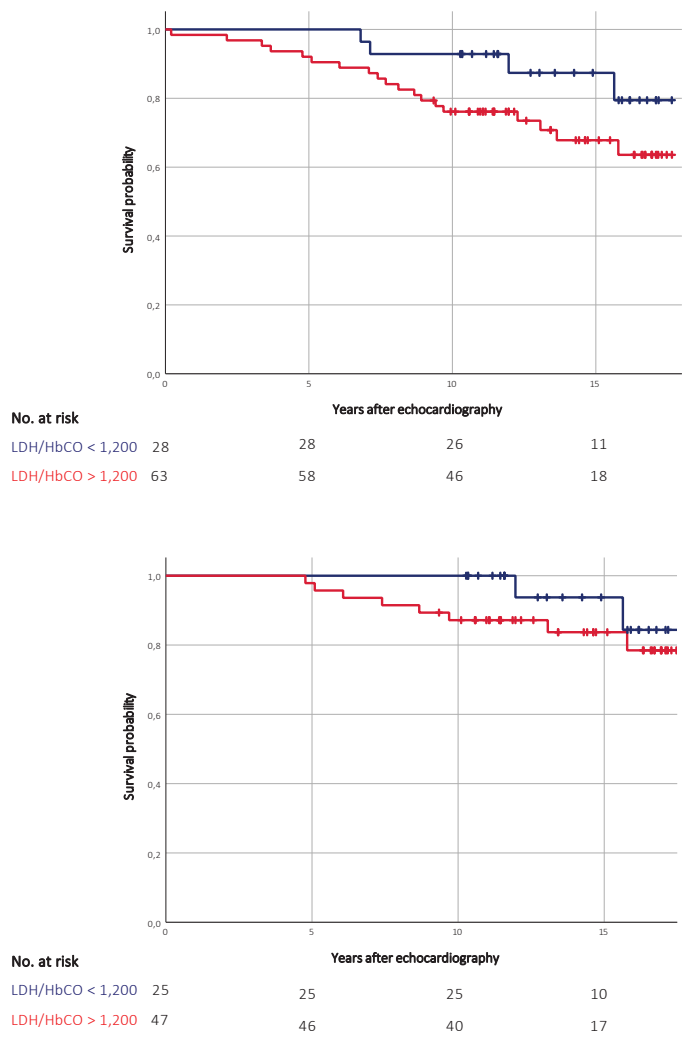LDH lactate dehydrogenase; CRP C-reactive protein.

Figure 3. Survival analysis.

Kaplan–Meier estimates of overall survival for patients with LD/HbCO ratios > 1200 and patients with LD/HbCO ratios < 1200. (A) Survival probability of all patients independent of tricuspid regurgitation velocity (TRV). In the group patients with LD/HbCO values < 1200 5-year overall survival (OS) was 100%, 10-year OS 92.9% and 15-year OS 88.0%. In the group patients with LD/HbCO values > 1200 5-year OS was 92.1%, 10-year OS 76.0% and 15-year OS 69.1%. (B) Survival probability in the group of patients with TRV values < 3.0 m/s. In the group patients with LD/HbCO values < 1200 5-year and 10-year OS was 100%, 15-year OS was 94.4%. In the group patients with LD/HbCO values > 1200 5-year OS was 97.9%, 10-year OS 87.1% and 15-year OS 84.1%.

DISCUSSION

In this paper we have shown that HbCO is related to the accepted biomarkers of global hemolysis and EACO, and thereby appears to be a readily available marker for total hemolysis. As intravascular hemolysis is detrimental and CO production is protective in SCD, we explored the ratio of LDH to HbCO as biomarker in this study. The fraction of intravascular hemolysis is heterogeneous among SCD patients and not driven by total hemolysis. The intravascular hemolysis fraction of total hemolysis predicts clinical outcome in SCD patients: a LDH/HbCO ratio > 1,200 identifies SCD patients with a high probability to develop pulmonary hypertension and at high-risk of early death. Based on these findings, we suggest further investigation of serum LDH and HbCO concentrations in larger cohorts, to further characterize the added value of assessment of the convenient LDH/HbCO ratio in individual patients.

The products of intravascular red cell lysis have shown to be of major importance in the pathophysiology of vasculopathy in SCD. Infusion of high dose hemoglobin or heme in SCD mice induces lethal vaso-occlusive events.[52,53] In contrast, low-dose heme and subsequent induction of HO-1 and production of the gasotransmitter CO is protective against vaso-occlusion in SCD mice.[52-54] Therefore, it is biologically plausible that an increase in the amount of CO, as product of heme processing, relative to the amount of intravascular heme, provides useful information on characterization of hemolysis in SCD and its vasculotoxicity.

Excess cell free hemoglobin and heme, not cleared from the circulation via scavenger proteins, are excreted in the urine, exposing the kidney to substantial heme toxicity.[55,56] Indeed, hemoglobinuria is detectable in many patients with SCD, and is associated with biomarkers of intravascular hemolysis.[57] This urinary excretion of heme somewhat confounds the interpretation of CO release as an indication of total hemolysis. However, the amount of excreted heme is limited compared to total red cell turnover. Therefore, we consider that HbCO is generally reflective of the total amount of CO production due to adaptive heme catabolism.

Multiple studies have established the association of pulmonary hypertension with hemolytic severity; serum LDH has been the most widely used marker of intravascular hemolysis associated with catheterization-proven pulmonary hypertension or elevated TRV in SCD cohorts.[46,48,49,58-66] Elevated TRV in SCD was significantly associated with mortality in a recent meta-analysis, but no consistent significant association was observed between LDH and mortality.[51] Notably, the current cohort on whom we have CO, LDH and mortality data is a subset with less statistical power than some of the prior datasets. As such, it may be underpowered to replicate the prior LDH-mortality association. Although all patients

diagnosed with SCD were eligible for enrollment in these trials our cohort was enriched for SCD patients at risk for vasculopathic complications and/or early death. In this relatively small cohort, we show that the ratio of LDH to HbCO is superior to predict either elevated TRV or mortality as compared to LDH alone. Furthermore, SCD patients with low LDH/HbCO values (< 1,200) had a low risk of pulmonary hypertension and all-cause mortality. These results suggest that the LDH/HbCO ratio may enhance the utility of LDH to identify patients at risk for vasculopathic complications and early death in SCD. Based on these findings, we suggest further investigation of serum LDH and HbCO concentrations in larger cohorts in concert with other risk factors, or predicting factors, such as hemoglobin, CRP, NT-proBNP and TRV, to further characterize the added value of assessment of the convenient LDH/HbCO ratio in individual patients.

One limitation of our study is the presentation of HbCO values in relation to other hemolytic parameters in two cohorts that differ in regard to patient selection and size. The EACO cohort (A) was prospective and smaller; and, the HbCO cohort (B) was larger but retrospective and, therefore, may be biased toward a more acutely or chronically affected subpopulation for whom HbCO data were available. Altogether, HbCO results must be considered exploratory and in need of additional validation. The results here do not invalidate previously published correlations of serum LDH from larger studies with greater statistical power. Our new results highlight the potentially greater strength of the LDH/HbCO ratio.

In conclusion, a ratio of two readily available clinical laboratory markers, LDH and HbCO, representative of the intravascular fraction of the total hemolytic rate, is promising as a biomarker in SCD. Increased LDH/HbCO ratios are strongly associated with increased risk for pulmonary hypertension, the pathophysiologic complication of intravascular hemolysis, and all-cause mortality. And thereby may add to the individual risk prediction in SCD patients. We suggest inclusion of these markers in future prospective trials in SCD for validation.

SUPPLEMENTAL FILES

Table S1. Individual HbCO and LDH variation.

Patient number	Samples available	Mean HbCO (mg/dL) (SEM)	Mean LDH (U/L) (SEM)
1	9	213 (23)	347 (22)
2	8	112 (10)	210 (7)
3	14	183 (14)	372 (13)
4	17	251 (43)	306 (10)
5	9	241 (14)	257 (15)
6	6	183 (9)	352 (13)
7	17	207 (17)	229 (13)
8	11	219 (15)	263 (10)
9	7	206 (27)	451 (61)
10	9	218 (18)	449 (26)
11	10	264 (7)	303 (6)
12	6	266 (24)	564 (89)
13	9	260 (16)	275 (8)
14	13	268 (14)	494 (29)
15	21	268 (12)	497 (28)
16	10	299 (12)	266 (20)
17	5	260 (31)	427 (14)
18	10	363 (15)	292 (21)
19	8	296 (31)	336 (26)
20	4	617 (29)	185 (11)
21	5	604 (59)	469 (49)

Individual mean HbCO and mean LDH values including SEM of the patients of cohort B with 4 or more samples with results on HbCO and LDH levels.

SEM standard error of the mean.

Supplemental paragraph
Cardiac catheterization was performed in a subgroup of 25 patients. In 16 patients pulmonary hypertension was diagnosed defined as a mean pulmonary artery pressure over 25 mmHg (median age 39 years; IQR 30, 48). 94% (15/16) of the patients with catheterization proven pulmonary hypertension had a LDH/HbCO ratio over 1,200. In the subgroup of patients with intermediate-risk TRV values, pulmonary hypertension was only diagnosed in individuals with LDH/HbCO ratios exceeding 1,200.
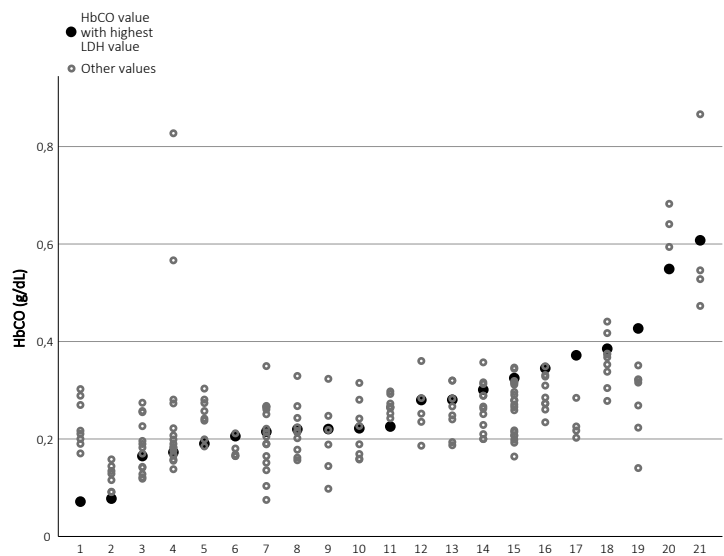
Figure S1. Individual variability in CO production. Individual HbCO data of patients of cohort B with 4 or more samples with results on HbCO and LDH levels. Scatter plot with HbCO values (*y-axis*) of individual patients (*x-axis*) sorted by median HbCO value. Every dot represents a single HbCO measurement. The HbCO value with the highest LDH value is colored black.

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CHAPTER 6

A unique monocyte transcriptome discriminates sickle cell disease from other hereditary hemolytic anemias and shows the particular importance of lipid and interferon signaling

A.J. van Vuren[1]; S. van Straaten[1]; M. Mokry[2]; R. van Wijk[3]; E.J. van Beers[1].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;
[2] Division Heart and Lungs, Department of Cardiology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[3] Central Diagnostic Laboratory - Research, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

Sickle cell disease (SCD) is a relatively common, hereditary hemolytic anemia characterized by complex pathophysiology, including chronic inflammation and oxidative stress.[1] Ischemia reperfusion injury and hemolysis have been recognized as potent triggers of inflammation, and the inflammatory marker C-reactive protein was identified as an independent predictor of early mortality.[2] Emerging studies highlight the importance of pro-inflammatory Toll like receptor 4 (TLR4) signaling in acute and chronic SCD complications.[3-5]

In general, TLR signaling is dependent on reactive oxygen species (ROS) production by production by nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase highly expressed on monocyte membranes. Chronic intravascular hemolysis leads to release of damage-associated molecular pattern (DAMP) molecules like high mobility group box 1 (HMGB1) protein.[4,6] HMGB1 plays a predominant role in TLR4 activity in SCD and synchronization with heme attenuates the pro-inflammatory TLR4 response.[4] Therefore, heme is currently designated as an erythroid-derived (e-)DAMP molecule.[7,8] The contribution of heme seems to be highly dependent on the pro-oxidant effect of hemin-bound iron, and iron is known to catalyze redox signaling by participating in the Fenton reaction. ROS production by heme has shown to indirectly activate the NF-κB pathway.[6,9]

Free heme, product of intravascular hemolysis, therefore serves as a potent modulator of TLR4 signaling in SCD, and TLR4⁺ cells, monocytes, play an important role in the pathophysiology of SCD.[10] The aim of our study was to identify the main activated pathways in monocytes in response to intravascular hemolysis in SCD patients. In our analyses, we compared SCD to a group of other hemolytic diseases characterized almost exclusively by extravascular hemolysis, and thus not subjected to large amounts of intravascular cell-free heme. We investigated gene expression profiles of TLR4⁺ cells, by positive selection of its co-receptor CD14, from patients with SCD and other hereditary hemolytic anemias to identify differential regulated genes and pathophysiological pathways. Our results illustrate the importance of the relative contribution of pro- and anti-inflammatory signaling in SCD monocytes, which may contribute to the SCD phenotype.

Data were available from 14 individual SCD patients (i.e. 11 HbSS, 3 HbSC), obtained during steady-state disease defined as no acute SCD complications in the preceding month. Table S1 highlights the main clinical characteristics of the SCD patients without (N = 11) or on deferasirox (DFX) therapy (N = 3), healthy controls (N = 10) and patients with various forms of hereditary hemolytic anemia (N = 46). The latter group consists of patients diagnosed with pyruvate kinase deficiency (N = 14), β-thalassemia (N = 2), hereditary xerocytosis (N = 7) and hereditary spherocytosis (N = 23). None of these patients was treated with DFX at the time of blood sampling. In the SCD patient group, 5 patients required regular exchange red cell transfusions, including 3 patients treated with hydroxyurea.

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For visualization of the data, a plot was generated based on a principal component analysis of the 3000 most variable genes in the dataset. (Figure 1) The cluster of CD14$^+$ cells of SCD patients not treated with DFX clustered apart from both CD14$^+$ cells derived from healthy controls and cells from patients with various hemolytic anemias. Whereas CD14$^+$ cells of healthy controls and patients with other hemolytic anemias overlapped in the principal component analysis. DFX treatment seemed to correct the transcriptome alterations of SCD patients towards normal.
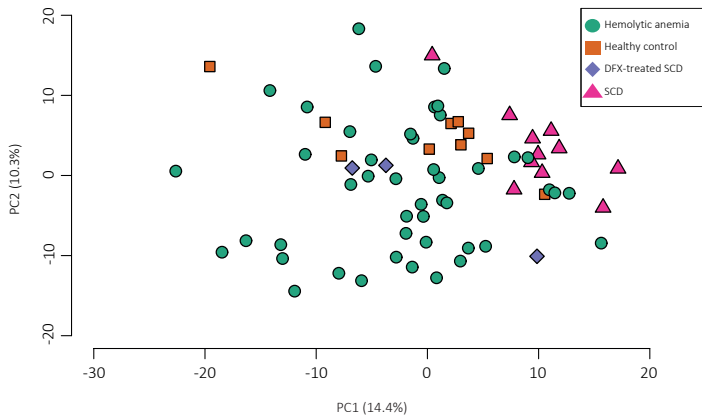

Figure 1. Principle component analysis (PCA). PCA is a technique to reduce dimensionality and emphasize variation in a dataset. New variables are constructed as weighted averages of the original variables. These new variables are called the principal components. The plot of the two most important principal components (explaining the variance or dimensionality of gene expression) illustrates distinct patterns in our dataset. The percentages provided on *x-axis* and *y-axis* (...) are relative but display the variance that the principal component accounts for in the analysis. Gene expressions profiles of patients that are more closely correlated cluster together. Input data is regularized log transformed. Each dot represents one patient, and the location of a dot is determined from the top 3000 most variable genes in the dataset. The dots are colored according to disease category.
DFX deferasirox; SCD SCD without DFX treatment.

Next, we analyzed the differentially expressed genes (DEGs) from CD14$^+$ cells of non-DFX SCD patients when compared to either healthy controls, or patients with other hemolytic anemias. The analysis rendered 744 genes differentially expressed in the comparison of non-DFX SCD and healthy controls, of which 505 were upregulated in SCD. In the comparison of non-DFX SCD and other hereditary hemolytic anemias 593 genes were differentially expressed, including 248 genes higher expressed in SCD.

Pathway enrichment analysis showed significant enrichment of genes involved in interferon (IFN) type I and II signaling in the set of individual genes that were differentially expressed in non-DFX SCD when compared with healthy controls (adj. p = 4.4E-16). (Table S2-A) We observed a profound upregulation of all components of IFN signaling, including IFN receptors (e.g. *IFNGR1*, *IFNGR2*), signal transduction molecules (e.g. *JAK2*, *STAT1*, *STAT2*, *IRF7*), and a broad range of IFN-stimulated genes (e.g. *IFI27*, *IFIT3*, *OAS1*, *ISG15*). Furthermore, pathway enrichment analysis showed significant enrichment of pathways involved in chemokine signaling (adj. p = 0.06) and TLR2/4 signaling (adj. p = 0.06) with profound upregulation of the individual genes in CD14$^+$ cells derived from SCD patients.

In addition, pathway enrichment analysis in the set of individual genes differentially expressed between non-DFX SCD and other hereditary hemolytic anemias underlined the importance of chemokine signaling (adj. p = 0.01) with the most profound upregulation of IFNg-inducible genes *CXCL11* and *CXCL9*. (Table S2-B) Our analysis also showed enrichment of genes involved in cholesterol biosynthesis (the mevalonate pathway; adj. p = 0.09) which were all upregulated in SCD, as well as upregulation of genes involved in immune interactions between lymphoid and non-lymphoid cells (adj. p = 0.09).

HMOX1 in SCD

Next, we aimed to define a core list of protein-encoding genes that represents the specific features of CD14$^+$ cells in SCD. For this purpose, we made a more stringent selection of the individual DEGs (see *Statistical analysis* in section S1) and selected those protein-encoding genes that were differentially expressed when comparing CD14$^+$ cells of SCD patients with CD14$^+$ cells of healthy controls and patients with other hereditary hemolytic anemias. This analysis rendered 29 genes, as presented in Figure 2. Several of the encoded proteins in the selection have previously been related to SCD. For the other proteins an important role in SCD could be presumed based on protein characteristics and actions in other disease models. Again, the list highlights the importance of two processes related to immune signaling: CXCR3 (chemokine) signaling by CXCL9 and CXCL11, and lipid metabolism (*STARD4*, *DLC1*, *SQLE*, *ME1*). Thereby, a role for CD14$^+$ cells in development of vasculopathy in SCD is supported by upregulation of *PPARG*, *GUCY1A1*, *KLF5*, *CTSL* and CXCR3 signaling (*CXCL9* and *CXCL11*), which all have previously been associated with vascular remodeling and development of pulmonary hypertension.

Interestingly, but not unexpected, heme oxygenase-1 (*HMOX1*) was one of these genes (versus healthy controls adj. p = 5.6E-13; versus other hemolytic anemias adj. p = 3.3E-15). Profound upregulation of *HMOX1* in CD14$^+$ cells of SCD patients is in line with the hypothesis that intravascular free heme is an important effector of gene regulation in monocytes.[11] Heme-oxygenase 1 (HO-1) mediates heme detoxification. Transcriptional regulation is highly complex and upregulation is mediated by multiple pathways involved in stress and inflammation, as

extensively reviewed by others.[12] Monocyte HO-1 has known to be important in prevention of vasculopathic injury in SCD. HO-1 induction required crosstalk with endothelial cells damaged by toxic heme.[10] A large proportion of the immunomodulatory effectivity of HO-1 is contributed to one of the end products of heme degradation, carbon monoxide (CO).[13] So, the strong upregulation of HO-1 in SCD monocytes suggests an essential preventive response to counterbalance continuous pro-inflammatory signaling initiated by a broad range of e-DAMPs.

IFN metabolism, TLR4 and HO-1

The regulation of gene expression of many pro-inflammatory genes relies on integration of signals from TLR4 and IFN signaling pathways. Combined action of STAT1-containing transcription factor complexes and NFkB provides a robust platform for transcriptional activation of a broad range of pro-inflammatory genes.[14] These processes are tightly controlled by HO-1. HO-1 seemed to be required for early activation of the type I IFN-inducing pathway. However, CO suppressed the capacity to secrete pro-inflammatory cytokines, including IFN-stimulated genes, in response to TLR4 activation.[15]

Moreover, we identified a profound upregulation of IFNg-inducible cytokines *CXCL9-CXCL11* mRNA expression in SCD patients not on DFX, which are associated with Th1 polarization and activation. Monocytes do have a pivotal role in directing T cell fate: heme induced a relative regulatory T cell proliferation via monocyte HO-1, and subsequent CO production, and inhibited antigen-independent activation, proliferation and maturation of naïve Th1 and CD8^{+} T cells in SCD.[16]

Hence, the extent of pro-inflammatory signaling in SCD depends on crosstalk between various pathways including type I and II IFN, TLR4, and the inhibitory effects mediated by HO-1.

Cholesterol metabolism, TLR4 and HO-1

Disruption of cellular cholesterol homeostasis occurs as part of the innate immune response.[17] Of particular interest is the upregulation of various enzymes involved in the mevalonate pathway (*SQLE, DHCR24, MSMO1, SC5D, HMGCS1*), and of genes presumably involved in lipid raft formation or preservation (*ABCG1, STARD4*).[18] Mevalonate is crucial for induction of trained immunity in monocytes, a feature characterized by increased expression of cytokines (TNF-α, IL-6 and IL-1β) and genes in the glycolytic pathway, and for a hyperinflammatory phenotype in specific diseases.[19] Inhibition of cholesterol synthesis with statins (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which prevent conversion of HMG-CoA into mevalonate) could be an effective therapy for hyperinflammatory disorders in which trained immunity plays a role.[19] Upregulation of expression of the enzymes of mevalonate pathway in SCD suggests that this pathway contributes to the hyperinflammatory profile in SCD. Various reports on the efficacy of statins in prevention of vascular complications in SCD support this hypothesis.[20]
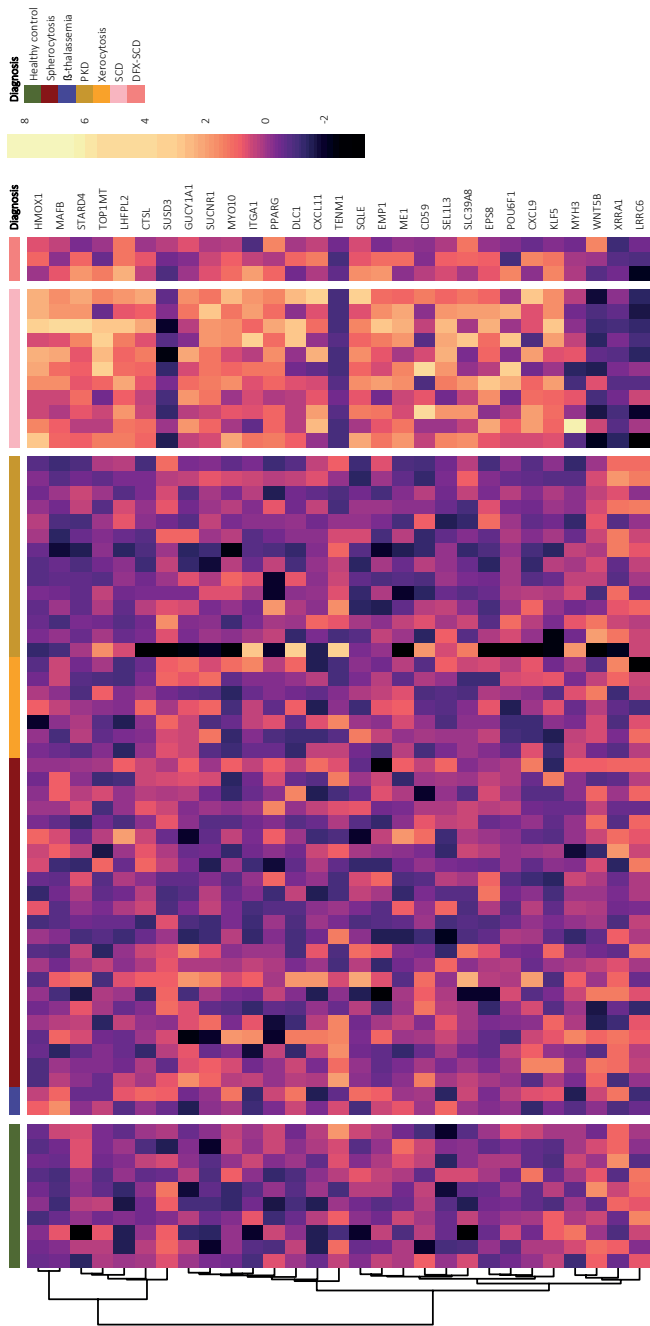
Figure 2. Heatmap of top-29 differentially expressed genes in SCD not treated with DFX compared to other hemolytic anemias and healthy controls. The heatmap is constructed with Z-scores of quantile normalized, log2 transformed data. Hierarchical, unsupervised clustering of the genes (rows) was applied by the complete linkage method. This mathematical method defines the distance between two classes as the maximum (Euclidean) distance between two elements from each class. The pair of elements which have the smallest distance between each other are aggregated in a new element. The dendrogram provides a graphical representation of the aggregations of the clustering analysis. The columns of the heatmap are colored by diagnosis.

DFX-SCD DFX-treated SCD; PKD pyruvate kinase deficiency; SCD SCD without DFX treatment.

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Generally, the acute inflammatory response inhibits reverse cholesterol transport back to the liver as a way to amplify inflammation. In our cohort we observed in SCD monocytes a reduction of *ABCG1* transcript expression, encoding a cholesterol exporter. TLR4 signaling itself is known to suppress *ABCG1* expression in oxidized LDL-induced inflammation (one of the most identifiable toxic end products of intravascular heme release). Expression of the cholesterol exporters ABCA1 and ABCG1 was further decreased in response to trained immunity induction and could be counteracted with statin therapy.[19] Moreover, our data hints towards a previously suggested role for lipid rafts or caveolae formation in TLR4 signaling.[21] Remarkably, CO both inhibited translocation of TLR4 to lipid rafts,[22] and attenuated interaction between Caveolin-1 and TLR4 which dampens TLR4 signaling.[23]

In summary, both the mevalonate pathway and lipid rafts are known enhancers of TLR4 signaling. Our data shows the importance of both processes in pro-inflammatory signaling in SCD monocytes.

In *conclusion*, our analysis of the CD14$^+$ cell transcriptome in patients with hereditary hemolysis shows that patients with SCD have a characteristic gene expression pattern. This pattern includes upregulation of *HMOX1*, a signature of high intracellular iron and oxidative stress, and thereby underlines previous observations on the importance of e-DAMP molecules (including heme) in initiating pro-inflammatory signaling in SCD. Moreover, it shows that lipid metabolism and IFN signaling are important differentiating pro-immune signaling pathways. The unique SCD monocyte transcriptome also underlines the importance of both pro- and anti-inflammatory pathways. Coexistence of anti- and pro-inflammatory transcriptional activity has previously been shown in monocytes from sepsis patients.[24] And, in line with sepsis and sepsis recovery the balance might shift in response to, for example, vaso-occlusive crises. We hypothesize that the relative upregulation of both pathways is associated with disease complications, especially vasculopathic complications, in SCD and that HO-1 has an important role in determining this balance. Importantly, all discussed pathways yield potentially druggable targets that possibly could reduce the pro-inflammatory phenotype in SCD and related (vasculopathic) complications.

MATERIALS & METHODS

Patients

Analyses were performed on blood samples from patients enrolled in the ZEbRA study, designed as a cross-sectional observational study on clinical sequelae and pathophysiology of rare congenital hemolytic diseases, registered in the Netherlands Trial Register [NTR], identifier NL5189. The ZEbRA cohort consists of adult patients diagnosed with a form of congenital hemolytic anemia. Patients were included from January 2016 until July 2017 in a cross-sectional, observational study at the University Medical Center Utrecht, The Netherlands; member of the collaborative network EuroBloodNet. All patients provided written informed consent, in accordance with the Declaration of Helsinki, approved by the institutional review boards of the METC Utrecht.

Patients treated with systemic anti-inflammatory drugs were excluded (N = 1). Deferasirox (DFX)-treated SCD patients (N = 3) were analyzed separately, given the above described effects of DFX on TLR4 signaling. Patients on hydroxyurea were not analyzed separately, as it was shown that hydroxyurea does not prevent NLRP3 inflammasome- and TLR-dependent inflammation.[25]

Monocyte isolation & RNA sequencing

Peripheral blood mononuclear cells were isolated from venous blood samples, CD14^{+} cells were isolated using anti-CD14 microbeads (Miltenyi Biotech) to reach a purity over 95%.

Total RNA was isolated from monocytes using the RNA-Bee (Bio-Connect Life Sciences) according to the manufacturer's instructions. The concentration of RNA was quantified using Qubit RNA HS assay and Qubit fluorometer (ThermoFisher). Polyadenylated mRNA was isolated using Poly(A) beads (NEXTflex). Sequencing libraries were prepared by using the Rapid Directional RNA-seq kit (NEXTflex).

The library was sequenced at the Utrecht Sequencing Facility (USEQ) on a Nextseq500 platform (Illumina) using a single-end 75-base pair high-output run. Reads were aligned to the human reference genome (GRCh37) using STAR version 2.4.2a. Read groups were added to the BAM files with Picard's AddOrReplaceReadGroups (v1.98). The BAM files were sorted with Sambamba v0.4.5, and transcript abundances were quantified with HTSeq-count version 0.6.1p117 using the union mode. Subsequently, reads per kilobase of transcript per million reads sequenced were calculated with edgeR's rpkm function.

Statistical analysis

Differentially expressed genes (DEGs; p-value < 0.1 and absolute log2fold change > 0.5) were identified by using the DESeq2 package version 1.24.0 in R. To correct for presence of reticulocytes and lymphocytes, data was corrected for expression of four hemoglobin genes (*HBA1, HBB, HBG1, HBG2*) and four T cell specific genes (*CD3E, CD3D, CD3G, CD247*).

RLog transformed data (DESeq2 package) was used as input for the principal component analysis (PCA). A core list was constructed from a more stringent selection (adj. p < 0.01; absolute log2fold change > 1) of the protein-encoding genes differentially expressed in both the comparisons of SCD without DFX versus other hemolytic anemias, and of SCD without DFX versus healthy controls. Quantile normalized, log2 transformed data was used as input for the heatmap.

Pathway enrichment analysis (ReactomePA package version 1.28.0 in R) was performed with the DEGs from the DESeq2 analysis (adjusted p-value < 0.1 and absolute log2fold change > 0.5).[26]

In the DESeq2 analysis and pathway enrichment analysis, p-values were adjusted with the Benjamini-Hochberg procedure.

SUPPLEMENTAL FILES

Table S1. Baseline characteristics of patients of the ZEbRA cohort included in this analysis.

	Non-DFX-SCD (N = 11)	DFX-SCD (N = 3)	Other hemolytic anemia (N = 46)	Healthy controls (N = 10)
Clinical characteristics				
Gender male (fraction)	0.55	0	0.48	0.33
Age (median (Q1, Q3)	31 (27, 39)	27 (21, NA)	46 (37, 54)	NA
Regular transfusions (fraction)	0.18	1	0.10	0
Hydroxyurea therapy (fraction)	0.55	0.66	0	0
Laboratory parameters Median value (Q1, Q3)				
Hemoglobin (mg/dL)	9.4 (8.7, 12.3)	10.3 (9.8, NA)	13.1 (11.1, 14.0)	14.2 (13.7, 15.7)
MCV (fL)	92 (84, 126)	91 (95, NA)	95 (89, 103)	90 (86, 92)
Reticulocytes (x10^9/L)	173 (107, 250)	260 (178, NA)	285 (152, 564)	59 (44, 72)
Leukocytes (x10^9/L)	7.8 (5.2, 11.3)	14.9 (6.4, NA)	7.3 (5.7, 10.0)	6.2 (4.7, 8.0)
Monocytes (x10^9/L)	0.71 (0.31, 1.07)	1.05 (0.68, NA)	0.52 (0.38, 1.00)	0.51 (0.37, 0.56)
Platelets (x10^9/L)	396 (322, 486)	377 (260, NA)	279 (223, 445)	266 (239, 315)
Ferritin (mg/L)	470 (62, 658)	2209 (960, NA)	186 (101, 393)	NA
TSAT (fraction)	0.36 (0.22, 0.51)	0.86 (0.80, NA)	0.35 (0.24, 0.45)	NA
CRP (mg/L)	7.4 (4.6, 9.5)	3.8 (0.9, NA)	1.6 (0.7, 3.6)	NA
Total bilirubin (mmol/L)	31 (22, 43)	87 (28, NA)	41 (25, 62)	NA
LD (U/L)	350 (291, 413)	305 (225, NA)	179 (200, 297)	NA
AST (U/L)	39 (34, 43)	38 (34, NA)	26 (19, 37)	NA

The cohort included 14 SCD patients
(3 DFX-treated, 11 non-DFX-treated); 46 patients with various forms of hemolytic anemia
(pyruvate kinase deficiency N = 14; β-thalassemia N = 2; Piezo 1-mutant hereditary xerocytosis N = 7; hereditary spherocytosis N = 23). None of the patients in the other hemolytic anemia group were treated with DFX or other iron chelating agents.
AST aspartate transaminase; CRP C-reactive protein; LD lactate dehydrogenase; MCV mean corpuscular volume; TSAT transferrin saturation.

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Table S2. Pathway enrichment analysis in non-DFX SCD patients compared to healthy controls (A) or patients with various other hemolytic anemias (B).

A. Non-DFX-SCD versus Healthy Controls

Description	Adjusted p-value	Genes
Interferon Signaling (R-HSA-913531)*	4,4E-16	IFI27/IFIT3/IFIT1/USP18/IFIT2/RSAD2/IFITM1/ GBP1/NCAM1/IFI6/FCGR1B/ HERC5/MX1/IFITM3/OASL/ISG15/OAS3/XAF1/ FCGR1A/STAT1/OAS2/GBP4/GBP2/CAMK2D/ OAS1/FLNB/DDX58/NEDD4/JAK2/IRF7/IFI35/ EIF2AK2/MX2/TRIM21/TRIM5/TRIM22/STAT2/ RANBP2/IFNGR1/IFNGR2/PLCG1
MyD88 deficiency (TLR2/4)†	0.06	TLR1/TLR2/LY96/TLR6
Chemokine receptors bind chemokines	0.06	CXCL11/CXCL3/CXCL2/CXCL10/CCR5/CXCL9/ CCR1/CX3CR1

B. Non-DFX-SCD versus Patients with Various Hemolytic Anemias

Description	Adjusted p-value	Genes
Chemokine receptors bind chemokines (R-HSA-380108)	0.01	CXCL11/CXCL9/CXCL3/CXCL10/CCR5/CCR1/PF4/ CXCR5
Cholesterol biosynthesis (R-HSA-191273)	0.09	SQLE/DHCR24/MSMO1/SC5D/HMGCS1
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell (R-HSA-198933)	0.09	SIGLEC11/LILRB5/NCR1/LAIR1/CLEC4G/FCGR2B/ CD247/SH2D1A/ SIGLEC12/CD3G/CD8A

Genes upregulated in SCD are colored red, genes downregulated in SCD are colored green.

* Interferon alpha/beta signaling (R-HSA-909733; adj. p-value 2.6E-12), Interferon gamma signaling (R-HSA-877300; adj. p-value 5.2E-07), Antiviral mechanism by IFN-stimulated genes (R-HSA-1169410; adj. p-value 1.8E-06), and ISG15 antiviral mechanism (R-HSA-1169408; adj. p-value 5.9E-4) were also significantly enriched in non-DFX SCD patients.

† IRAK4 deficiency (TLR2/4) (R-HSA-560304; adj. p-value 0.07) was also significantly enriched in non-DFX SCD patients.

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CHAPTER 7

The complexity of genotype-phenotype correlations in hereditary spherocytosis: a cohort of 95 patients

A.J. van Vuren[1], B. van der Zwaag[2], R. Huisjes[3], N.S.M. Lak[4], M.B. Bierings[5], E.J. Gerritsen[6], E.J. van Beers[1], M. Bartels[7], R. van Wijk[3].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;

[2] Department of Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;

[3] Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;

[4] Princess Maxima Center for Pediatric Oncology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands;

[5] Department of Stem cell transplantation, Princess Maxima Centre for Paediatric Oncology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands;

[6] Department of Pediatrics, ADRZ Medical Center, Goes, The Netherlands;

[7] Pediatric Hematology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

Introduction: Hereditary spherocytosis (HS) is a phenotypically and genetically heterogeneous disease. With the increased use of Next-Generation-Sequencing (NGS) techniques in the diagnosis of red blood cell disorders, the list of unique pathogenic mutations underlying HS is growing rapidly.

Aim: Explore genotype-phenotype correlation in HS patients.

Methods: Analysis on a cohort of 95 HS patients genotyped by targeted-NGS as part of routine diagnostics (UMC Utrecht, Utrecht, The Netherlands).

Main findings: In 85/95 (89%) of patients a pathogenic mutation was identified, including 56 novel mutations. *SPTA1* mutations were most frequently encountered (36%, 31/85 patients), primarily in patients with autosomal recessive forms of HS. Three *SPTA1* (α-spectrin) mutations showed autosomal dominant inheritance. *ANK1* (ankyrin-1) mutations accounted for 27% (23/85 patients) and *SPTB* (β-spectrin) mutations for 20% (17/85 patients). Moderate or severe HS was more frequent in patients with *SPTB* or *ANK1* mutations, reflected by lower hemoglobin concentrations and higher reticulocyte counts. Interestingly, mutations affecting spectrin-association domains of *ANK1*, *SPTA1* and *SPTB* resulted in more severe phenotypes. Additionally, we observed a clear association between phenotype and aspects of red cell deformability as determined by the Laser-assisted Optical Rotational Cell Analyzer (LoRRca MaxSis). Both maximal deformability and area under the curve were negatively associated with disease severity (respectively $r = -0.46$, $p < 0.01$, and $r = -0.39$, $p = 0.01$).

Conclusion: Genotype-phenotype prediction in HS facilitates insight in consequences of pathogenic mutations for the assembly and dynamic interactions of the red cell cytoskeleton. In addition, we show that measurements of red blood cell deformability are clearly correlated with HS severity.

INTRODUCTION

Hereditary spherocytosis (HS) is the most common inherited hemolytic anemia in individuals from North Europe and North America, affecting approximately 1:2000 individuals.[1, 2] HS is genetically and phenotypically highly heterogeneous. The disease is characterized by altered red blood cell (RBC) membrane integrity due to mutations in genes encoding membrane or cytoskeletal proteins.[1] The RBC membrane is composed of approximately 20 major proteins and at least 850 minor proteins with differential expression and functionality, including transport proteins, adhesion proteins and signaling receptors.[2,3] The integral membrane proteins are organized into macromolecular complexes centered on band 3. The main components of the cytoskeleton are spectrin, actin, and its associated proteins, protein 4.1R and ankyrin.[3] Together, these proteins provide the RBC membrane with a high degree of flexibility and elasticity, allowing the cell to deform with linear extensions up to 250%.[1,4] The common hallmark of RBC in HS is disruption of the vertical association between the cytoskeleton and the overlying lipid bilayer.[5,6] HS mutations lead to reduced expression or impaired incorporation of one of the major proteins of the cytoskeleton or membrane, resulting in an imbalance in spatial protein configuration. The degree of imbalance depends on compensation by normal alleles in single heterozygotes, or by other mutations in compound heterozygotes.[7] Ultimately, destabilization of the lipid bilayer leads to loss of membrane lipids, and, therefore, loss of surface area.[8] This results in RBCs that become progressively spheroidal with reduced deformability, impeding traversing the narrow apertures of the splenic vascular walls. Spherocytes will be sequestered in the spleen leading to premature removal of RBCs.[9] The severity of HS is directly related to the extent of loss of surface area, and consequently the degree of spherocytosis: among the red cell indices the percentage of microcytes was the best indicator of disease severity.[10-12] While knowledge on the static structure of the RBC cytoskeleton markedly increased over the last decades, insights in the dynamic capacities, and the impact of genetic defects on the function of membrane proteins remain limited.[3]

HS is based on the pathophysiological effects of defects in genes encoding for one or more of the major RBC cytoskeleton and (trans)membrane proteins: ankyrin-1 (*ANK1*), band-3 (*SLC4A1*), α-spectrin (*SPTA1*), β-spectrin (*SPTB*) and protein 4.2 (*EPB42*).[6,13] We have previously demonstrated that using targeted NGS, a probable causative mutation (and 29 unique mutations) could be identified in these genes in 85% of HS patients (27/33).[14] Compared to Sanger sequencing, targeted-next-generation sequencing (NGS) of preselected gene panels has a higher diagnostic efficiency and, thereby, rapidly provides a thorough genetic analysis in patients suspected of RBC membrane disorders.[13,15,16] Currently, making the diagnosis of HS is multi-facetted: reports of clinical and family history, analysis of biochemical hemolysis parameters, analysis of RBC morphological features, and functional testing, including the osmotic fragility test, eosin-5-maleimide (EMA) binding test[17] and, more

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recently, osmotic gradient ektacytometry.[18,19] Overall, in 75% of the HS patients, there is an autosomal dominant (AD) inheritance pattern, whereas in the remaining 25% of patients HS is inherited in an autosomal recessive (AR) way, or is due to a de novo mutation.[6] Originally, in Northern Europe and the USA, *ANK1* mutations were shown to account for 40-65% of the cases, *SLC4A1* mutations for 20-35%, *SPTB1* mutations for 15-30%, and *SPTA1* and *EPB42* each for less than 5% of the HS cases.[6,20] Interestingly, in Japan, mutations in *EPB42* (45-50%) and *SLC4A1* (20-30%) were most abundant. [6,21]

More than ten years ago Iolascon and Avvisati already hypothesized on the existence of genotype/phenotype correlations in hereditary spherocytosis by stating that the biochemical and genetic heterogeneity of spherocytosis could represent the basis for clinical heterogeneity.[22] With the growing list of, mostly unique, HS mutations, attempts have been made to unravel the relationship between genotype and phenotype.[13,23-25] Mild, moderate, and severe forms of HS have been defined according to severity of anemia and degree of compensation for hemolysis.[5,6,9,26,27] (Table S1) From these studies, AR forms of HS due to mutations in the *SPTA1* gene combining low expression and null alleles seemed to be associated with a more severe phenotype. Among the mutations in other HS genes, there was a broad variability in phenotypic presentation.[13,23-25] So, until now, a clear genotype-phenotype correlation in HS has not been observed.

Here we present a large cohort consisting of 95 HS patients in whom 56 novel pathogenic mutations, including three apparently AD *SPTA1* mutations, were identified. Our data provide novel insights in the highly complex genotype-phenotype correlation in HS due to the complexity of the interactions in the RBC cytoskeleton. Based on our findings, we conclude that knowledge of underlying molecular defects as well as functional analysis of RBC deformability are required to understand phenotypic variability in HS. Future studies exploring the direct effects of genetic mutations on RBC protein expression and function will be necessary to further elucidate genotype-phenotype correlations.

RESULTS

Overview mutations and disease severity

Our cohort included 95 patients suspected to have HS. Clinical characteristics and median laboratory parameters are provided in Table 1. Cholecystectomy and splenectomy were simultaneously performed in 6/11 patients who underwent splenectomy in the last 10 years. Table 2 shows the identified HS mutations categorized per gene, including the pathogenicity classification for the novel mutations (according to recommendations for interpretation)[28] and phenotype per patient. In 85 patients a mutation was identified in one of the HS associated genes, 56 new pathogenic HS mutations were reported. Three disease-causing mutations were identified in two (*SPTA1* c.5791C>T p.(Gln2931); and *SLC4A1* c.37G>T p.(Glu13*))or three (*SPTA1* c.83G>A p.(Arg28His)) seemingly unrelated families.

Table 1. Patient characteristics.

	Without or before splenectomy	Post-splenectomy
Number of patients (N)	70	19
Age (years) – genetic analysis	30 (22)	28 (19)
Gender (% male)	49	47
Laboratory parameters [reference range]		
Hb (g/dL) [11.9-17.2]	11.6 (2.4)	14.2 (1.8)
MCV (fL) [80-97]	87 (9)	87 (9)
MCH (fmol) [1.75-2.25]	1.9 (0.2)	1.9 (0.2)
MCHC (mmol/L) [19-23]	21.9 (0.9)	21.6 (0.8)
Reticulocyte count; absolute (x10^9/L) [25-120]	381 (220)	222 (160)
Platelets (x10^9/L) [150-450]	243 (112)	486 (215)
Leukocytes (x10^9/L) [4-10]	7.4 (2.7)	9.4 (1.7)
LD (U/L) [0-250]	297 (199)	224 (60)
Bilirubin (μmol/L) [3-21]	48 (34)	21 (12)
AST (U/L) [0-35]	29 (11)	36 (15)
Ferritin (μg/L) [20-250]	339 (500)	129 (95)
HS diagnostics		
EMA (%)	78 (12)	75 (9)

82 of 95 included patients had hematological data available. Of 7 patients data obtained before and after splenectomy were available. Mean values are presented (± standard deviation).
EMA eosin-5-maleimide binding test.

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Table 2. Pathogenic mutations in the HS cohort. List of individual patients, apparent inheritance pattern, identified mutations and disease phenotype.

No	Family	Type mutation	Inheritance pattern	Gene	Allele 1
1	**A**	missense	AR	SPTA1	c.83G>A p.(Arg28His)
2	**A**	missense	AR	SPTA1	c.83G>A p.(Arg28His)
3		missense	AR	SPTA1	c.83G>A p.(Arg28His)
4		missense	AR	SPTA1	c.83G>A p.(Arg28His)
5		missense	AR	SPTA1	c.101G>C p.(Arg34Pro)
				SLC4A1	c.1162C>T p.(Arg388Cys)
6		other	AR	SPTA1	c.678G>A p.(Glu227Valfs*10)[†]
7		other	AR	SPTA1	c.1273C>T p.(Arg425*)
8	**B**	other	AD	SPTA1	c.1850dup p.(Ser618Glufs*10)
9	**B**	other	AD	SPTA1	c.1850dup p.(Ser618Glufs*10)
10	**B**	other	AD	SPTA1	c.1850dup p.(Ser618Glufs*10)
11	**B**	other	AD	SPTA1	c.1850dup p.(Ser618Glufs*10)
12		other	AD	SPTA1	c.1959T>A p.(Tyr653*)
13		other	AR	SPTA1	c.2353C>T p.(Arg785*)
14		other	AR	SPTA1	c.2755G>T p.(Glu919*)
15		other	AR	SPTA1	c.2806-13T>G p.(?)
				SLC4A1	c.118G>A p.(Glu40Lys)
16		other	AR	SPTA1	c.3257dup p.(Leu1086Phefs*5)
17		other	unknown	SPTA1	c.3569+2T>C p.(?)
18		other	unknown	SPTA1	c.4081del p.(His1361Metfs*15)
19		missense	AD	SPTA1	c.4240C>T p.(Arg1414Cys)
20		other	AR	SPTA1	c.4339-99C>T p.(?)[b]
21		other	AR	SPTA1	c.4339-99C>T p.(?)[b]
22		other	AR	SPTA1	c.4339-99C>T p.(?)[b]
23		other	AR	SPTA1	c.4339-99C>T p.(?)[b]
24		other	AR	SPTA1	c.5941C>T p.(Gln1981*)[‡]
25		other	unknown	SPTA1	c.4339-99C>T p.(?)[b]
26		other	unknown	SPTA1	c.4339-99C>T p.(?)[b]
				SLC4A1	c.2494C>T p.(Arg832Cys)
27		other	unknown	SPTA1	c.4738-1G>A p.(?)
28		other	AR	SPTA1	c.5791C>T p.(Gln1931*)
29		other	AR	SPTA1	c.5791C>T p.(Gln1931*)
30		other	AR	SPTA1	c.5941C>T, p.(Gln1981*)
31		other	AR	SPTA1	c.6843-2A>G
32	**C**	missense	AD	ANK1	c.245T>C p.(Leu82Pro)
33	**C**	missense	AD	ANK1	c.245T>C p.(Leu82Pro)
34		missense	AD	ANK1	c.341C>T p.(Pro114Leu)

Pathogenicity 1	Allele 2	Pathogenicity 2	LELY[a]	Phenotype
5-P			heterozygous	Severe
5-P			heterozygous	Moderate
5-P			heterozygous	Severe
5-P			heterozygous	Severe
5-P			heterozygous	Mild
3-VUS				
5-P			homozygous	Mild
5-P			normal	Mild
5-P			normal	Moderate
5-P			normal	Mild
5-P			normal	Mild
5-P			heterozygous	Mild
5-P			normal	Mild
5-P			heterozygous	Moderate
5-P			heterozygous	Mild
5-P	c.4339-99C>T p.(?)[b]	5-P	normal	Mild
3-VUS				
5-P			heterozygous	Moderate
5-P			homozygous	Mild
5-P			heterozygous	Mild
4-LP			normal	Severe
5-P	c.6769G>T (p.Glu2257*)	5-P	normal	Moderate
5-P	c.4339-99C>T p.(?)[b]	5-P	normal	Mild
5-P	c.4339-99C>T p.(?)[b]	5-P	normal	Mild
5-P	c.4339-99C>T p.(?)[b]	5-P	normal	Mild
5-P	c.[4339-99C>T[b];4347G>T] p.[(?);(Lys1449Asn)]	5-P; 3-VUS	normal	Mild
5-P			normal	Mild
5-P			normal	Mild
3-VUS				
5-P			normal	Moderate
5-P			homozygous	Mild
5-P			homozygous	Mild
5-P	c.[4339-99C>T;c.4347G>T] p.[(?);(Lys1449Asn)]	5-P; 3-VUS	normal	
5-P	c.6843-2A>G p.(?)	5-P	normal	Moderate
4-LP				
4-LP				
4-LP			homozygous	Severe

Table 2. Continued.

No	Family	Type mutation	Inheritence pattern	Gene	Allele 1
35	D	missense	AD	ANK1	c.344T>C p.(Leu115Pro)
36	D	missense	AD	ANK1	c.344T>C p.(Leu115Pro)
37		other	AD	ANK1	c.498C>G p.(Tyr166*)
38		other	AD	ANK1	c.841C>T p.(Arg281*)
39		other	AD	ANK1	c.856C>T p.(Arg286*)
40	E	missense	AD	ANK1	c.971T>C p.(Leu324Pro)
41	E	missense	AD	ANK1	c.971T>C p.(Leu324Pro)
42		other	AD	ANK1	c.1306-8T>G p.(?)
43		other	AD	ANK1	c.2559-2A>G
44		other	AD	ANK1	c.2625del p.(Glu876Serfs*40)
45		other	AD	ANK1	c.3649_3650insT p.(Pro1217Leufs*5)
46		other	AD	ANK1	c.3754C>T p.(Arg1252*)
47		other	AD	ANK1	c.3985G>T p.(Val1329Leu)§
48		other	AD	ANK1	c.4105-1G>A p.(?)
49		other	AD	ANK1	c.5005G>T p.(Glu1669*)
50	F	other	AD	ANK1	c.5044C>T, p.(Arg1682*)
51	F	other	AD	ANK1	c.5044C>T, p.(Arg1682*)
52		other	AD	ANK1	c.5079_5080insTCAG p.(Glu1694Serfs*48)
53	G	other	AD	ANK1	c.5201_5202insTCAG p.(Thr1735Glnfs*7)
54	G	other	AD	ANK1	c.5201_5202insTCAG p.(Thr1735Glnfs*7)
55		other	AD	SLC4A1	c.37G>T p.(Glu13*)
56		other	AD	SLC4A1	c.37G>T p.(Glu13*)
57		other	AD	SLC4A1	c.577_578del p.(Ser193Leufs*9)
58		other	AD	SLC4A1	c.620del p.(Gly207Alafs*24)
59		other	AD	SLC4A1	c.787del p.(Arg263Alafs*34)
60		missense	AD	SLC4A1	c.1421C>A p.(Ala474Asp)
61		other	AD	SLC4A1	c.1430C>A p.(Ser477*)
62		missense	AD	SLC4A1	c.1462G>A p.(Val488Met)
63	H	other	AD	SLC4A1	c.2057+1G>A p.(?)
64	H	other	AD	SLC4A1	c.2057+1G>A p.(?)
65		missense	AD	SLC4A1	c.2138G>T p.(Gly713Val)
66		missense	AD	SLC4A1	c.2348T>A p.(Ile783Asn)
67		missense	AD	SLC4A1	c.2423G>A p.(Arg808His)
68		other	AD	SPTB	c.1-?_6414+?del p.(?)$
69		other	unknown	SPTB	c.648-?_4002+?dup p.(?)¥
				SPTA1	c.2373C>A p.(Asp791Glu)
				EPB41	c.225T>G p.(Phe75Leu)
70		missense	AD	SPTB	c.544T>G p.(Trp182Gly)
				SLC4A1	c.118G>A p.(Glu40Gly)
71		missense	AD	SPTB	c.647G>A p.(Arg216Gln)

Pathogenicity 1	Allele 2	Pathogenicity 2	LELY[a]	Phenotype
4-LP				Mild
4-LP			heterozygous	Moderate
5-P				Moderate
5-P				Severe
5-P				Mild
5-P				
5-P				Mild
3-VUS				Mild
5-P				Moderate
5-P			heterozygous	Moderate
5-P				Severe
5-P			homozygous	Severe
4-LP			heterozygous	Mild
5-P				Severe
5-P				
5-P			normal	Moderate
5-P			heterozygous	Severe
5-P				Mild
5-P				Severe
5-P				Moderate
5-P				Mild
5-P				Mild
5-P				Mild
5-P				Moderate
5-P				Mild
4-LP				Mild
5-P				Mild
5-P				Severe
5-P				Mild
5-P				Mild
4-LP				
5-P				Mild
5-P				Moderate
5-P			heterozygous	Mild
5-P			normal	
5-P				
3-VUS				
5-P				Severe
3-VUS				
4-LP				Moderate

7

Table 2. Continued.

No	Family	Type mutation	Inheritence pattern	Gene	Allele 1
72		other	AD	SPTB	c.1069C>T p.(Gln357*)
73		other	AD	SPTB	c.1540C>T p.(Gln514*)
74		other	AD	SPTB	c.1912C>T p.(Arg638*)
75		other	AD	SPTB	c.2136_2137delinsTT p.(Gln713*)
76		other	AD	SPTB	c.2137C>T p.(Gln713*)
77	I	other	AD	SPTB	c.3449G>A p.(Trp1150*)
78	I	other	AD	SPTB	c.3449G>A p.(Trp1150*)
79	I	other	AD	SPTB	c.3449G>A p.(Trp1150*)
80	I	other	AD	SPTB	c.3449G>A p.(Trp1150*)
81		other	AD	SPTB	c.3581del p.(Leu1194Trpfs*32)
				SPTA1	c.4339-99C>T p.(?)[b]
82		other	AD	SPTB	c.4267C>T p.(Arg1423*)
83		other	AD	SPTB	c.4542dup p.(Leu1515Thrfs*12)
				SPTA1	c.3565C>T, p.(His1189Tyr)
84		missense	AD	SPTB	c.6203T>G p.(Leu2068Arg)
85		missense	AD	EPB42	c.554G>C p.(Arg185Pro)
86			AR	NONE	
87			AR	NONE	
88			AR	NONE	
89			unknown	NONE	
90			unknown	NONE	
91			AD	NONE	
92			AD	NONE	
93			AD	NONE	
94			AD	NONE	
95			AD	NONE	

Families are depicted with a letter. Mutation nomenclature is according to the HGVS variation nomenclature guidelines[53] and based on the following NCBI Reference Sequence transcriptnumbers *SPTA1: NM_003126.3; SPTB: NM_000347.5; SLC4A1: NM_000342.3; ANK1: NM_000037.3; RHAG: NM_000324.2; EPB41: NM_004437.3; and EPB42: NM_000119.2.* The presence of an a^LELY status was reported in all patients with a SPTA1 mutation, and in other patients if known. Sequence variants were interpreted according to the recommendations of the American College of Medical Genetics and Genomics using standard terminology to describe identified variants: 1-B Benign, 2-LB Likely Benign, 3-VUS Variance of Unknown Significance, 4-LP Likely Pathogenic, 5-P Pathogenic.[28]

[a] α^LELY mutation: low expression allele LYON. Composed of two *in cis* variants in *SPTA1*: c.[5572C>G;c.6531-12C>T] p.[(Leu1858Val);(?)] ultimately resulting in strongly reduced amounts of α-spectrin. Allele frequency in the European population 29% (http://gnomad.broadinstitute.org/variant/1-158597507-G-C), not associated with hemolysis on its own.[16,54]

Pathogenicity 1	Allele 2	Pathogenicity 2	LELY[a]	Phenotype
5-P			homozygous	Severe
5-P				
5-P				Moderate
5-P				Moderate
5-P			heterozygous	Moderate
5-P			homozygous	Severe
5-P			homozygous	Severe
5-P			homozygous	Moderate
5-P				Severe
5-P			normal	Severe
5-P				
5-P				Mild
5-P			normal	Moderate
3-VUS				
4-LP				Mild
4-LP				Mild
				Moderate
				Moderate
				Mild
				Moderate
				Mild
				Moderate
				Mild
				Mild

[b.]α^{LEPRA} mutation: low expression allele PRAGUE. c.4339-99C>T mutation in the *SPTA1* gene. The mutation enhances an alternative acceptor splice site, frameshift and ultimately premature termination of translation. Approximately 1/6 of the transcripts escapes alternative splicing, resulting in low α-spectrin production. Accounts for nearly 5% of SPTA1 alleles among white people.[16,36]

† The *SPTA1* c.678G>A p.(Glu227Valfs*10) mutation, tested on RNA level, resulted in a 5 base pair intron retention (r.678_678+1insGUGAG) and was subsequently identified as a frameshift mutation.

‡ De novo stop-gain mutation in index patient. DNA analysis was performed in both parents.

§ De novo mutation in index patient. DNA analysis was performed in both parents. The *ANK1* c.3985G>T p.(Val1329Leu) mutation results in alteration of the first nucleotide of exon 35 of *ANK1*. This alteration might negatively influence splicing of pre-mRNA, resulting in disturbed maturation of the *ANK1* transcript. Although, experimental evidence supporting this suggestion currently lacks.

$ The *SPTB* c.1-?_6414+?del p.(?) mutation results in deletion of the whole *SPTB* gene.

¥ The *SPTB* c.648-?_4002+?dup p.(?) mutation results in duplication of exons 6-18 of the *SPTB* gene.

SPTA1 mutations were identified in 31/85 (36.5%) of the HS cases in our cohort, *ANK1* in 23/85 (27.1%), *SPTB* in 17/85 (20.0%), *SLC4A1* in 13/85 (15.2%) and *EPB42* in 1/85 (1.2%) patients. (Figure 1A) In 10/95 (10.5%) of the patients no mutation could be identified. Missense mutations accounted for 23/85 (24.2%) of the identified mutations, the remaining mutations were nonsense, frameshift, indels or splice site mutations. In 65/95 patients (68.4%) HS was inherited in an apparent AD manner, AR inheritance was reported in 23/95 (24.2%) patients. In the remaining number of 7/95 (7.4%) patients, the inheritance mode could not be identified. Interestingly, all mutations in *ANK1*, *SLC4A1*, *SPTB* and *EPB42* showed an AD inheritance pattern, except one *SPTB* mutation in which the inheritance pattern was unknown.

We investigated the correlation between affected gene and disease severity, hematologic parameters, and EMA test results. The fraction of patients with moderate or severe phenotypes was highest among patients with *SPTB* (12/15, 80%) and *ANK1* mutations (13/19, 68.4%). (Figure 1B) Hemoglobin levels were available for 62 non-splenectomized patients (or obtained from patients before splenectomy) with an identified mutation, all values obtained post-splenectomy were excluded. (Table 3) Five of the six patients with phenotypically severe HS and who not underwent splenectomy required regular red cell transfusions. Mean hemoglobin levels were significantly lower in in HS patients with *SPTB* mutations, compared to patients with *SCL4A1* mutations ($p = 0.05$), and in patients with *ANK1* mutations compared to patients with *SLC4A1* mutations ($p = 0.04$). In agreement with mean hemoglobin levels, absolute reticulocyte counts, in patients without splenectomy or obtained before splenectomy, differed significantly between patients from genetic subgroups. Reticulocyte counts were significantly higher in patients with *ANK1* mutations than in patients with *SCL4A1* ($p < 0.01$) and *SPTA1* mutations ($p < 0.01$); and in patients with *SPTB* mutations compared to patients with *SCL4A1* ($p < 0.01$) and *SPTA1* mutations ($p = 0.02$). Bilirubin, lactate dehydrogenase, and aspartate aminotransferase concentrations did not differ statistically between the genetic subgroups. EMA values, available in 42 patients without or obtained before splenectomy, were significantly higher in HS patients with *SPTA1* mutations compared to patients with *ANK1* ($p < 0.01$), *SPTB* ($p < 0.01$) and *SLC4A1* ($p = 0.04$) mutations.
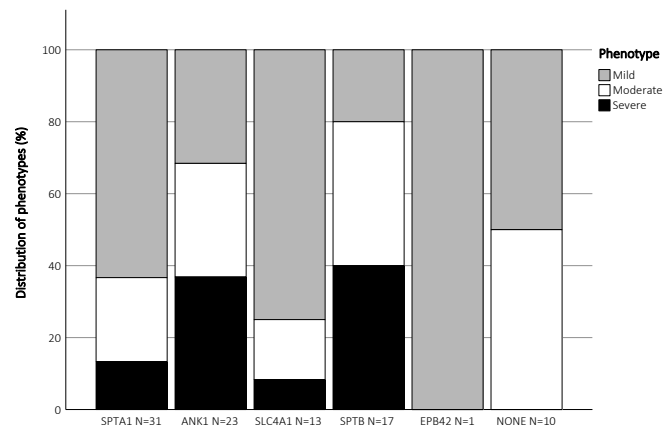
A
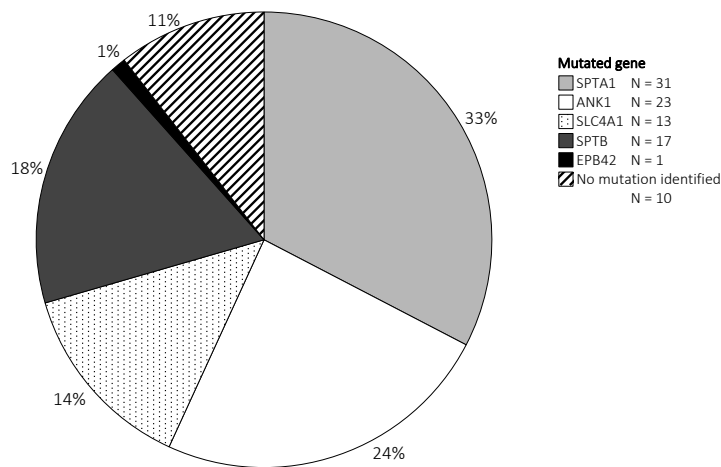
B

Figure 1. Overview of mutated genes and phenotype. The cohort included a total number of 95 patients diagnosed with HS. Patients were categorized according to gene with the HS mutation. (A) Graphic overview of the distribution of the cohort. (B) The distribution of HS phenotypes per gene category. N number of patients.

Table 3. Erythropoietic and hemolytic parameters per gene group.

	SPTA1 N = 26	ANK1 N = 15	SPTB N = 9	SLC4A1 N = 11	EPB42 N = 1
Gender (% male)	46	40	56	64	0
Laboratory parameters [reference range]					
Hb (g/dL) [11.9-17.2]	11.7 (2.3)	10.8 (2.2)	10.5 (2.7)	13.2 (1.9)	14.5 (-)
Reticulocyte count. absolute (x10^9/L) [25-120]	298 (185)	506 (203)	580 (214)	259 (84)	210 (-)
LD (U/L) [0-250]	274 (119)	291 (52)	495 (497)	214 (34)	247 (-)
Bilirubin (μmol/L) [3-21]	52 (53)	50 (17)	47 (20)	39 (14)	11 (-)
AST (U/L) [0-35]	33 (15)	31 (15)	27 (5)	24 (5)	NA
Ferritin (μg/L) [20-250]	415 (758)	381 (303)	306 (199)	342 (411)	31 (-)
HS diagnostics					
EMA (%)	87 (13)	75 (9)	72 (8)	74 (8)	88 (-)

Mean values are presented (± standard deviation). Mean values in hemoglobin ($p < 0.05$), absolute reticulocyte count ($p < 0.01$) and EMA ($p < 0.05$) differed significantly between the categories displayed bright and light grey.

EMA eosin-5-maleimide binding test; NA no available data.

Mutations in the spectrin-binding domains of ANK1, SPTA1 and SPTB are associated with a more severe HS phenotype

Next, we investigated the correlation between clinical phenotype and genetic mutations using protein structure information. Pathogenic mutations were mapped along the protein structures of ankyrin-1, α-spectrin, β-spectrin and band 3. (overview depicted in Figure 2A-D) All mutations in the *ANK1* gene followed an AD inheritance pattern. Six of the identified mutations were positioned in the spectrin-binding domain of *ANK1*: 5/6 of these mutations resulted in a moderate or severe phenotype. Remarkably, mutations closely positioned to each other (e.g. *ANK1* c.841C > T p.(Arg281*) and c.856C > T p.(Arg286*), as well as *ANK1* c.341C > T p.(Pro114Leu) and c.344T > C p.(Leu115Pro)) resulted in completely different HS phenotypes. Intriguingly, three novel pathogenic mutations in *SPTA1* were detected that apparently were inherited in an AD fashion. Disease severity varied in these patients from mild to severe. In our cohort, the majority of patients with autosomal recessive *SPTA1* mutations had a mild phenotype (11/19). Notably, two mutations in repeat 21 of *SPTA1*, the binding domain for β-spectrin, and a mutation in the α-spectrin-binding domain of *SPTB* resulted in a (more) severe phenotype. Interestingly, a large deletion, including the whole *SPTB* gene (*SPTB* c.1-?_6414 + ?del), resulted in a mild phenotype, while the majority of other pathogenic mutations in the *SPTB* gene were associated with a more severe disease phenotype, suggesting that incorporation of a truncated protein might be occurring and be more harmful than an absolute decrease of otherwise normal protein. Pathogenic *SCL4A1* mutations resulted in

a mild phenotype in 9/11 patients. None of the pathogenic mutations involved one of the known binding sites of band 3 with other cytoskeleton proteins. In summary, our data showed that patients with a mutation in the domains for spectrin dimer-tetramer association (*SPTA1* and *SPTB*) and ankyrin-spectrin-binding (*ANK1*) show a more severe disease phenotype compared to patients with other pathogenic HS mutations. No other clear patterns between mutations in specific domains and phenotypes could be identified.

Phenotypic variability of HS exists within families

In order to further study genotype-phenotype correlation, we evaluated the phenotypic expression of HS within families harboring the same HS-causing mutation. Our cohort included nine such families, of which clinical data was available from all family members in seven families. Phenotypic expression of HS was roughly similar among members of three families (family A, F, H in Table 1). However, more broad phenotypic variability was observed in four other families, despite identical genotypes among individual family members (family B, D, G, I). This phenotypic heterogeneity was particularly evident in family G and I. (Figure 3)

In family G, dizygotic twins shared the same pathogenic *ANK1* mutation. In patient I the mutation resulted in a moderate phenotype with mild anemia, reticulocytosis and splenomegaly. In patient II the mutation resulted in a severe phenotype requiring splenectomy at the age of 13 years. Phenotypical variation could not be explained by other (modifying) mutations in the analyzed genes.

In family I, both the father and his three children shared the same pathogenic *SPTB* mutation. All three were also homozygous for the α-LELY mutation (data not shown). Father, sibling I and III had a comparable severe phenotype, including regular transfusion requirements before splenectomy. Surprisingly, sibling II however had a milder phenotype and has thus far never been transfused. Notably, her Osmoscan curve was more right-shifted (increased Ohyper) compared to the other family members carrying the identical pathogenic *SPTB* mutation.
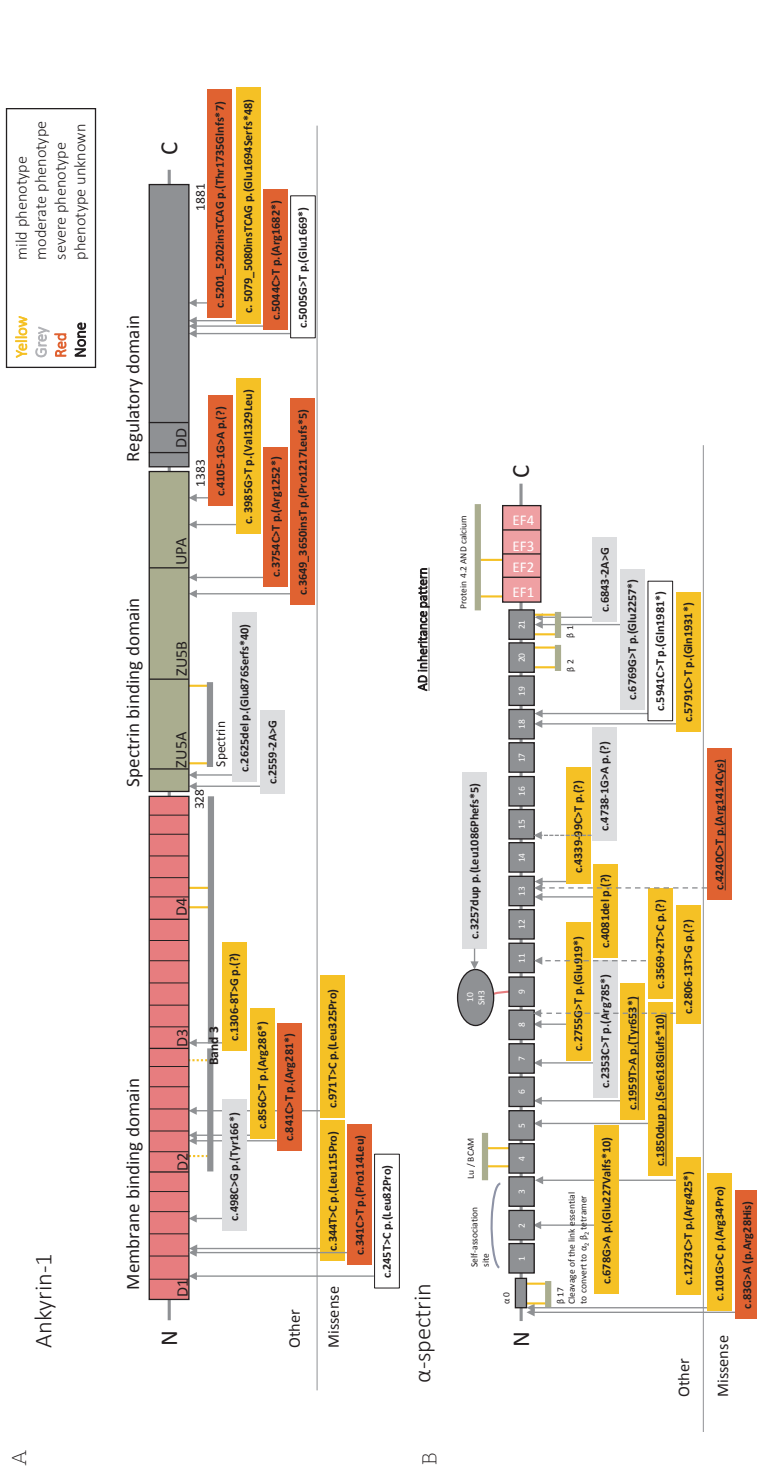
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Figure 2. All pathogenic mutations were mapped along the protein structures of ankyrin-1, α-spectrin β-spectrin and band 3. Mutations were colored by phenotype. All mutations in *ANK1*, *SCL4A1* and *SPTB* were inherited following an AD pattern. Of the novel mutations in the *SPTA1* gene, three mutations were inherited in an AD fashion. Information on coinheritance of the modifying α^{LELY} mutation is provided in *Table 2*.

(A) Ankyrin.[35,55,56] The membrane domain consists of 24 ankyrin repeats grouped per six repeats. The membrane domain harbors two binding sites for Band 3. The spectrin domain consists of three subdomains, ZU5A contains the binding site for spectrin. The regulatory domain might modulate binding properties of the other domains. The function of the death domain (DD) is currently unknown.
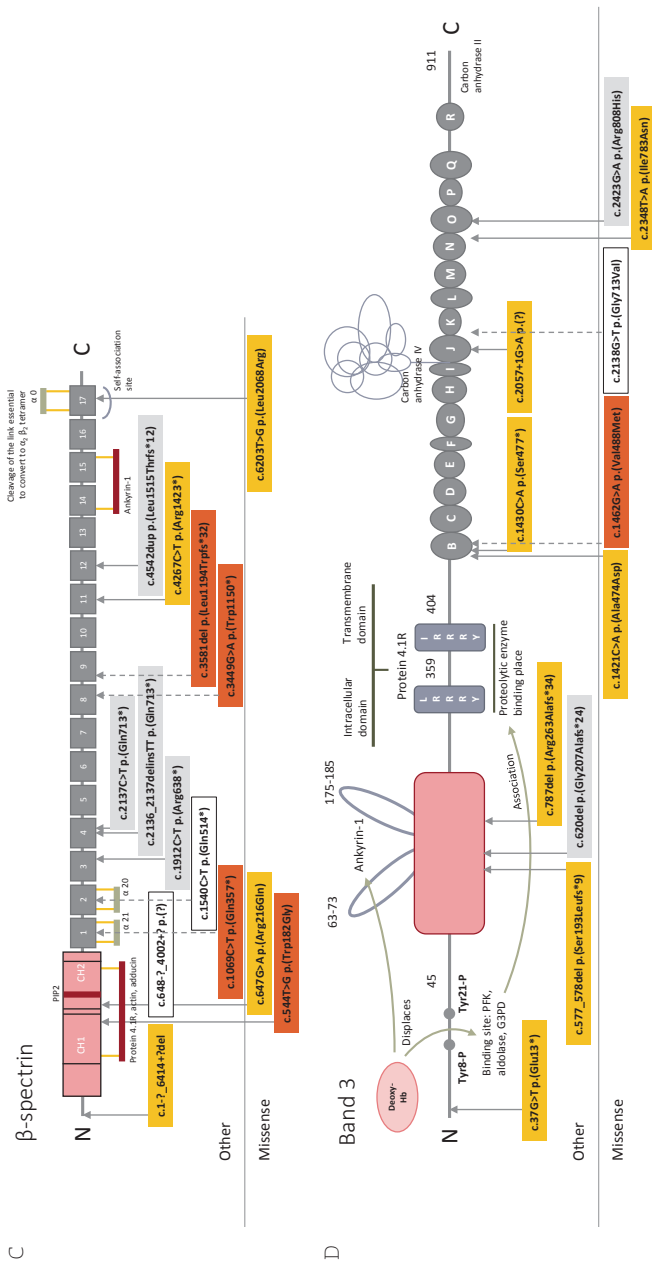
(B & C). A- and β-spectrin.[3,55,57] The membrane protein spectrin is composed of two chains, α- and β-spectrin. Both spectrin chains contain a number of spectrin-type repeats with specialized domains for self-association, spectrin, ankyrin-1 (head), actin, protein 4.1R and other proteins (tail) binding.

(D). Band 3.[3,55,58-60] The protein consists of an intracellular (*N-terminus*) and a transmembrane domain (*C-terminus*). The intracellular domain forms the attachment site for the components of the membrane cytoskeleton, glycolytic enzymes. Deoxyhemoglobin or phosphorylation of Tyr21 or Tyr8 displaces, and thereby activates, PFK, aldolase and G3PD. Deoxyhemoglobin might also displace ankyrin-1. Ankyrin-1 and protein 4.1R inhibit each other's binding to band 3. The transmembrane domain forms an anion-exchange channel.

7

ANK1: c.5201_5202insTCAG p.(Thr1735Glnfs*7) (5-P)

Moderate HS.
Mild anemia, reticulocytosis.
Splenomegaly, no splenectomy.

Severe HS. Splenectomy age 13 y.
Pre-splenectomy: anemia, reticulocytosis.
Post-splenectomy: no anemia, mild reticulocytosis.

SPTB: c.3449G>A p.(Trp1150*) (5-P)
α-LELY

Post-splenectomy:
no anemia,
persistent reticulocytosis.
Ektacytometry: classical HS.

No anemia,
no reticulocytosis.
Ektacytometry: normal.

9 y.
Pre-splenectomy: severe anemia
and reticulocytosis. Transfusion
dependent. Splenectomy age 6 y.
Post-splenectomy: no anemia.
Ektacytometry: classical HS.

7 y.
Mild anemia. No red cell
transfusions. Ektacytometry:
HS, right-shifted O_{hyper}
pattern compared to other
family members.

2 y.
Severe anemia, reticulocytosis.
Transfusion dependent.
Ektacytometry: classical HS.

Figure 3. Overview of genetic mutations and phenotypes in two families with HS (family G and I). (A) Pathogenic *ANK1* mutation, c.5201_5202insTCAG p.(Thr1735Glnfs*7) (5-P) in dizygotic twins. Patient I had a moderate phenotype with mild anemia (Hb 10.5-12 g/dL), reticulocytosis (15-20%) and mild splenomegaly. Patient II had a severe phenotype (Hb 8.0-10.0 g/dL; reticulocytosis ±20%), ultimately leading to splenectomy at the age of 13y. (B) Pathogenic *SPTB* mutation, c.3449G>A p.(Trp1150*) (5-P) in father and three siblings. Father, and sibling I and III had a severe phenotype with transfusion-dependency and the need for splenectomy. Sibling II had a milder phenotype with a remarkably different pattern of the Osmoscan curve with a right-shifted Ohyper.

Osmoscan parameters significantly correlate with clinical severity of HS

To further understand pathophysiological mechanisms underlying HS phenotypes and to identify alternative predictors of clinical severity we analyzed RBC osmotic deformability profiles. RBC Osmoscan profiles and information on disease severity were available for 53 patients. Nine Osmoscan profiles were obtained post-splenectomy and therefore excluded from the analysis. (Figure 4A-D) The analysis included 28 patients with phenotypically mild, 12 patients with moderate and four patients with severe spherocytosis. Each of the four patients with severe spherocytosis harbored a unique mutation in *SPTB* (three patients) or *ANK1* (one patient). The Area Under the Curve (AUC) was below the reference limit in all patients diagnosed with HS; 43/44 patients had an EImax value (maximum Elongation Index) below the reference range; 25/44 patients had an Ohyper value (reflecting cellular hydration status) below the reference limit; and 23/44 had an Omin value (osmotic value where EI is minimal) above the reference range.

In all patients, independent of disease severity, there was a strong correlation between EImax and AUC (r = 0.88, p < 0.01). EImax and AUC were both clearly, negatively associated with Omin values (respectively r = -0.49, r < 0.01, and r = -0.40, p < 0.01), in the absence of a clear correlation with Ohyper values. Interestingly, even in this small subset of our cohort, both EImax and AUC were clearly negatively associated with disease severity (respectively r = -0.46, p < 0.01, and r = -0.39, p = 0.01). Additionally, there was a positive correlation between Omin and disease severity (r = 0.31, p = 0.04). Furthermore, in this subgroup EMA results were also associated with disease severity (r = -0.36, p = 0.03).

EImax values were significantly higher in mild (0.55) compared to moderate phenotypes (0.51) (mean difference 0.04, 95% CI [0.01, 0.07]). The mean AUC was significantly lower in patients with mild phenotypes (129) compared to patients with severe phenotypes (116) (mean difference 13, 95% CI [4, 20]). Mean Omin values were minimal, although significantly, lower in patients with mild (173) compared to patients with severe HS (189) (mean difference 16, 95% CI [1, 33]). No statistical differences in Ohyper values were observed between patients with distinct HS severity.

In summary, our data shows that clinical disease severity in HS is clearly associated with three read out parameters of the Osmoscan profile: EImax, AUC and Omin.
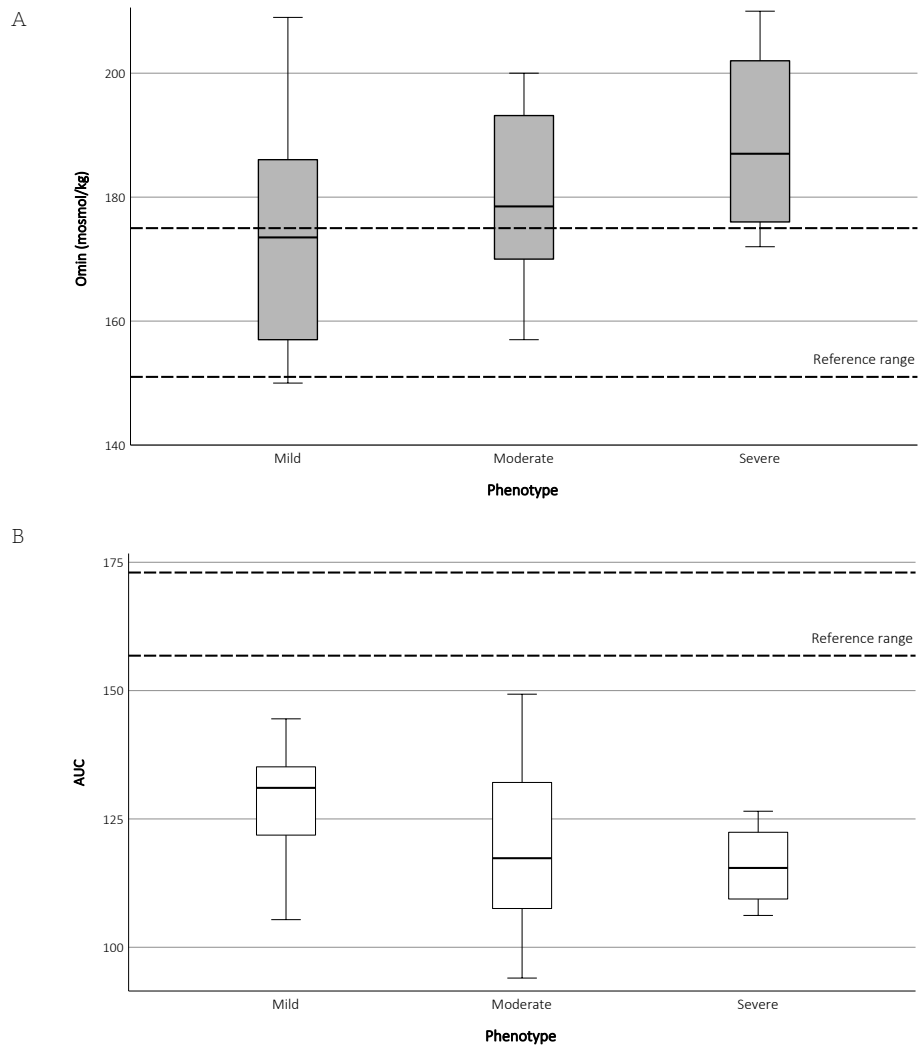
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A

B

Figure 4. Omin, Ohyper, EImax and Area Under the Curve (LoRRca, Maxsis) and their relation to HS phenotype. The graphs show box plots with median value, first and third quartile and minimum and maximum values. Omin (A), AUC (B), EImax (C) and Ohyper (D) values are visualized for a group of 44 HS patients (mild N = 28, moderate N = 12, severe N = 4). The reference range is depicted by two dashed lines (upper and lower limit). Values are organized by HS phenotype (mild, moderate or severe). The AUC was below the reference limit in all patients; EImax was below the reference range in 43/44 patients; Ohyper was below the reference range in 24/44 patients; and 23/44 had an Omin value above the reference range.

C

D
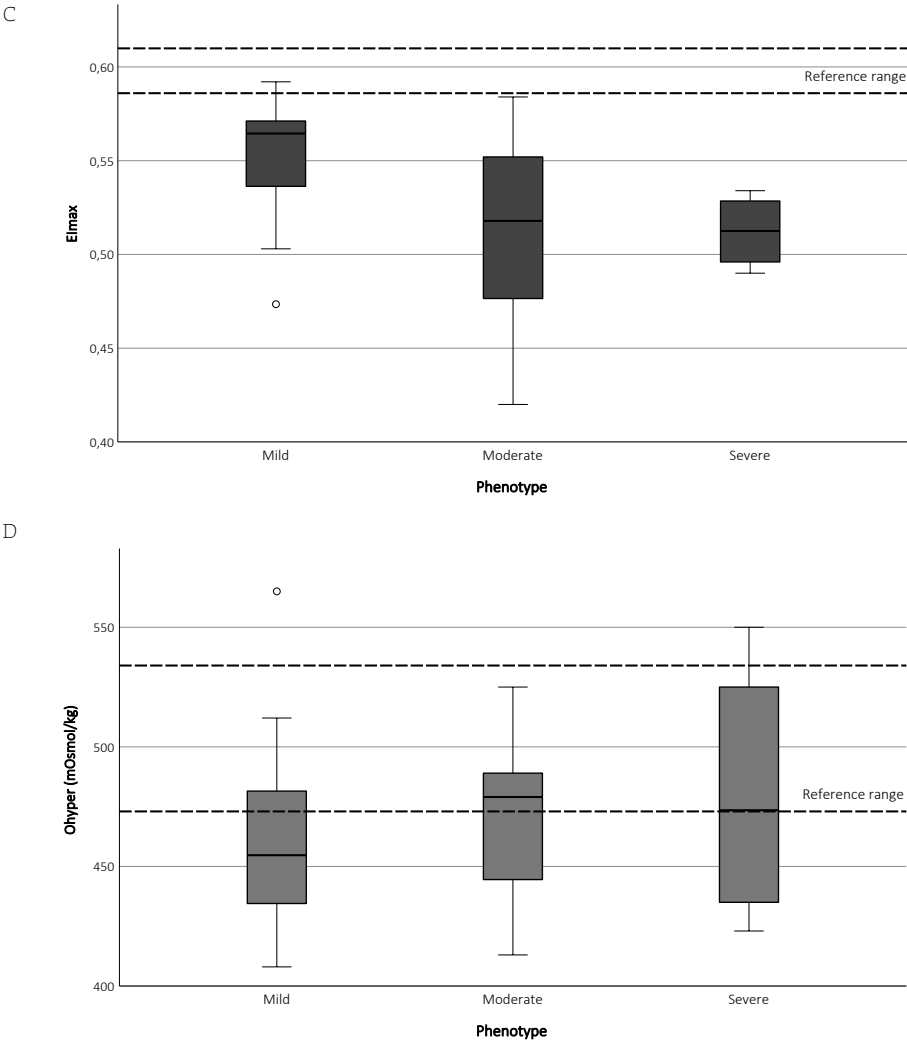
Figure 4. Continued.

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DISCUSSION

We studied the genotype-phenotype correlation in a unique, large cohort of HS patients utilizing targeted-NGS-based gene analysis of *SPTA1, SPTB, ANK1, EPB41, EPB42, SLC4A1* and *RHAG.* Pathogenic mutations were identified in 85/95 patients. As a result, 56 novel mutations were added to the currently known HS mutations. Intriguingly, we identified three novel pathogenic *SPTA1* mutations that were apparently inherited in an AD fashion. We showed that overall patients with *ANK1* and *SPTB* mutations, and especially patients with *ANK1, SPTB* and *SPTA1* mutations in the spectrin-binding domains, were more severely affected. Furthermore, we demonstrated that EImax, AUC and Omin, the diagnostically most important parameters of RBC deformability in the Osmoscan, can be used as markers of disease severity.

While mutations in *SPTB* and *ANK1* were associated with more severe phenotypes in our cohort, we conclude that categorization in genetic subgroups (*ANK1, SPTA1, SPTB, SCL4A1 or EPB42*) is insufficient to precisely predict HS phenotype in our cohort: there was a broad phenotypic variability among patients in each genetic subgroup. To further increase our understanding of genotype-phenotype correlations in HS, insight in the direct effects of mutations on the assembly of the cytoskeleton and its dynamic interactions is required.[3] Previously, it has been demonstrated that some of the interactions in the ankyrin complex of the cytoskeleton are critical: disruptions of these interactions by specific mutations result in more severe disruption of cytoskeleton assembly or functioning and thereby lead to a more severe phenotype.[29] In line with this, we observed in our cohort more severe phenotypes in patients with mutations in the domains for spectrin dimer-tetramer association and ankyrin-spectrin-binding domains of ankyrin-1, α-spectrin and β-spectrin. Earlier research in mice with severe HS due to different homozygous *SPTA1* mutations, showed that the expression of various proteins of the ankyrin complex varied. Interestingly, a missense mutation in the highly conserved cysteine residue at the C-terminus of *SPTA1*, resulted in near-normal amounts of spectrin, band 3 and β-adducin, but still in a severe HS phenotype, due to disruption of a critical interaction domain for membrane stability.[30] Based on these findings, we suggest that determining expression of the distinct membrane proteins may increase our insights in RBC static skeletal conformation.[24]

At the same time, RBC deformability measurements reflect dynamic properties of the assembled membrane.[18,19] To further investigate the genotype-phenotype correlation, we therefore evaluated functional RBC parameters and cellular dynamics. We previously demonstrated in 21 HS patients that, regardless of the genotype, RBC density, intercellular heterogeneity and deformability were strong markers of clinical disease severity.[31] In line with these latter observations, we here demonstrate that various parameters displaying RBC deformability in osmotic gradient conditions, EImax, AUC and Omin, were significantly

associated with disease severity. Omin correspondents to the 50% lysis point determined by the classical osmotic fragility test.[32,33] The lack of an association between disease severity and Ohyper values might be explained by the observation of two distinct Osmoscan profiles in HS patients: a classical profile with low Ohyper values, and a right-shifted one with relatively high Ohyper values.[34] The cause and relevance of this right-shifted profile remains to be established.

In contrast with our findings, Zaninoni *et al.*[34] recently reported the absence of an association between the severity of anemia and Osmoscan parameters in their cohort of 116 HS patients. There were important differences in the assembly of both cohorts: the cohort of Zaninoni *et al.*[34] consisted of splenectomized and non-splenectomized patients, which likely have influenced hemoglobin concentrations. Here we conclude that functional RBC deformability parameters (EImax and AUC) are clearly associated with HS phenotype.

We detected various mutation types in our HS cohort that we categorized as missense and non-missense mutations. It was previously suggested that non-missense mutations would mainly result in the introduction of premature stopcodons, thereby leading to either expression of truncated protein or to nonsense-mediated-decay of the resulting mRNA and a lack of expression from the concerning allele, rather than affecting protein function.[3] While this seems to suggest that non-missense mutations induce largely comparable phenotypes, our data does not support this suggestion. In addition, it has previously been shown that non-missense mutations leading to a premature stop codon in the spectrin-binding domain of ankyrin resulted in a more severe phenotype than similar mutations in one of the other functional domains of the ankyrin protein.[29] In our cohort, we observed that β-spectrin deficiency due to deletion of one *SPTB* allele, resulted in phenotypically milder HS compared to most of the other *SPTB* mutations, missense and non-missense. Thereby, our data is suggestive of incorporation of truncated protein which disrupts cytoskeleton function and is thereby more harmful than reduction in the amount of normally formed protein.

Phenotypic heterogeneity in patients with identical pathogenic HS mutations can be the result of the effects of concomitant mutations in modifier genes, including the low-expression alleles of *SPTA1* α[LEPRA] and α[LELY].[35-37] In our study, we were not able to explain phenotypic heterogeneity in family studies based on known genetic modifiers. Yet-unknown genetic factors might play a role in the observed phenotypic variability. It could even involve non-genetic factors, that might influence the amount of protein, or might disrupt its interactions in the RBC cytoskeleton. With regard to the genetic factors, current research in our laboratory focuses on 88 additional genes hypothetically involved in RBC (membrane) disorders based on their functional role and/or the association with hemolytic anemia in animal models. Over the last years unbiased genetic testing, including whole exome sequencing,[38-40] identified

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new genes involved in hereditary anemias; thereby expanding the targeted-NGS panels for congenital RBC disorders from a few genes to large panels.[23,41-46] Expanding these panels with, for example, genes associated with hyporegenerative anemias, defective erythropoiesis, and metabolic defects allows identification of cases in which phenotypic variability could be explained by coinheritance of multiple RBC diseases.[23,41,42]

We are the first to report three novel *SPTA1* mutations with an apparent AD inheritance pattern. Theoretically this can be explained by distinct underlying pathophysiological mechanisms. First, there might have been co-inheritance of yet-unknown modifying mutations that have influenced α-spectrin expression or its interactions. Second, a *SPTA1* null-allele will become clinically relevant, as α-spectrin becomes the rate-determining component of α/β-heterodimer assembly. Under physiologic conditions there is an overproduction of α- compared to β-spectrin (ratio 3:1).[35,47] Changes in the α-/β-spectrin ratio towards a 1:1 ratio were observed in band 3 deficient membranes,[48] and after erythropoietin stimulation under anemic conditions.[49] Third, we assume that current high rate of screening laboratory assessments plays a role, leading to the diagnosis of very mild, asymptomatic forms of HS resulting from an heterozygous *SPTA1* mutation. Future studies will be necessary to provide insight in those factors resulting in clinically relevant HS in case of one mutated *SPTA1* allele.

In conclusion, our data underline that the genotype-phenotype correlation in HS is highly complex due to the complexity of the interactions in the RBC cytoskeleton. The pathogenic mutation, amount and quality of incorporated protein, effects of truncated protein or its absence on the interactions in the cytoskeleton determine clinical disease severity. Thereby, presence of modifying genetic and even non-genetic factors influence phenotypic variability. Based on our findings, we conclude that knowledge of underlying molecular defects as well as functional analysis of RBC deformability are required to understand phenotypic variability in HS.

MATERIALS AND METHODS

Data source and study population

We retrospectively included patients diagnosed with HS based on analyses performed in the period from January 2014 through January 2018. Patients diagnosed with HS were selected from all patients referred to the tertiary expertise center for rare anemia in the Netherlands (University Medical Center Utrecht, Utrecht, The Netherlands), based on a clinical suspicion of hemolytic anemia due to HS. According to the ICSH (International Council for Standardization in Hematology) guidelines for laboratory diagnosis of nonimmune hereditary RBC membrane disorders,[50] diagnosis of HS was based on a composite of currently available tests including EMA, osmotic fragility test and osmotic gradient ektacytometry (Osmoscan), combined with hematologic and laboratory markers of hemolysis, clinical data, family history and morphological analysis of peripheral blood samples.

Next-generation sequencing

Sequence analysis was conducted in the ISO15189 accredited genome diagnostics laboratory of the UMC Utrecht. In short: Genomic DNA was isolated from peripheral blood samples of the patients and enriched for, among others, seven genes associated with HS (*SPTA1, SPTB, ANK1, SLC4A1, EPB41, EPB42, RHAG*) using a custom designed Agilent SureSelect[XT] capture library (ELID#:0497291) or the SureSelect[XT] Clinical Research Exome V2 (ELID#:30409818). NGS samples were sequenced to a minimum average depth of 100X on either a Life-Technologies/Applied Biosystems SOLiD™ 5500XL Sequencer (50 bp single reads), an Illumina HiSeq2500 Sequencer (2x100 bp paired end reads) or an Illumina Novaseq6000 Sequencer (2x 150 bp paired end reads). Horizontal coverage (the average number of reads that align to the known reference bases)of the seven HS-associated genes analyzed in the gene panel hereditary spherocytosis was at least 99% with > 15 unique reads per base for all sequencers. Raw sequence reads were mapped to the hg19 human genome reference, and variations were called using a in house developed bioinformatics pipeline. Variants were subsequently annotated and classified in the Alissa Interpret software suite (Agilent Technologies) using a custom build, ISO15189 validated, variant classification tree, adhering to the American College of Medical Genetics and Genomics Standards and guidelines for the interpretation of sequence variants.[28] Clinically relevant mutations were confirmed by Sanger sequencing.

Laser Assisted Optical Rotational Cell Analyzer

Osmotic gradient ektacytometry using the Osmoscan module on the LoRRca MaxSis (RR Mechatronics, Zwaag, The Netherlands) measures RBC deformability, expressed as Elongation Index (EI), during constant shear stress as a function of continuously changing osmotic conditions. Deformability depends on the total membrane surface area, surface area to volume ratio, and cellular hydration status. In RBC membrane disorders these features

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are generally altered.[18,19] For osmotic gradient ektacytometry measurements of RBCs from HS patients 250μL of whole blood was standardized to a hemoglobin concentration of 12.9 g/dL and injected in 5mL isotonic polyvinylpyrrolidone (PVP), and osmotic gradient ektacytometry was further carried out as previously described.

Four parameters of the Osmoscan curve are diagnostically relevant: EImax, Omin, Ohyper, and the AUC. These reflect mean surface area (EImax), surface to volume ratio (Omin), and cellular hydration status (Ohyper). The typical Osmoscan curve in HS patients is characterized by a decreased EImax, an elevated Omin value (shift to the right) and decreased Ohyper value (shift to the left), and consequently a decrease in AUC.[34]

Study conduct and data analysis

The study was conducted according to Good Clinical Practice guidelines, defined by the International Conference on Harmonisation (ICH). Mutations were categorized based on affected gene, type of mutation (missense versus other, including nonsense, frameshift, indels or splicesite mutation) and co-inheritance of low expression polymorphisms (e.g. Spα[LELY] or Spα[LEPRA]). Hematologic parameters were provided by the referring institutes. Per patient the mean values of the hematologic and hemolysis parameters from samples obtained during the year before and after genetic analysis were included. If only one assessment was available during this time frame, a second assessment obtained less than five years from genetic analysis was included. In those cases in which splenectomy was performed mean values obtained two years before and/or after splenectomy were reported. In infants, hematologic parameters obtained before the age of one year were not included in the final analyses based on distinct reference values in the first year of life. Due to the existence of inter-institution variance in absolute reticulocyte counts, only reticulocyte counts measured in our own institute were included. Information about the inheritance patterns was provided by the referring hematologist.

Statistical analysis

To explore phenotypic variability patients were categorized based on the underlying genetic defect. Differences between groups were tested with one-way Analysis of Variance (ANOVA), followed by Tukey's HSD post-hoc test. Correlation analyses of hematologic parameters and genetic subgroups were reported using Pearson's correlation coefficient. To correct for potential bias *bootstrapping* was performed to confirm significance.[51,52] The association between Osmoscan curve parameters and disease severity was tested using Spearman's rank correlation test. Statistical significance was set at a two-sided $p < 0.05$. All calculations were performed using IBM SPSS Statistics v. 25.

SUPPLEMENTAL FILES

Table S1. Classification HS based on disease severity.

Classification	**Mild**	**Moderate**	**Severe**
Hemoglobin (g/dL)	11-15	8-12	< 8
Reticulocyte count (%)	3.1-6	> 6	> 10
Bilirubin (µmol/L)	17-34	> 34	> 51
Splenectomy	Not indicated	Not indicated	Indicated after age of 6 years

The original classification was developed by Eber *et al.*[27] The criterion splenectomy is based on current recommendations regarding splenectomy.[26]

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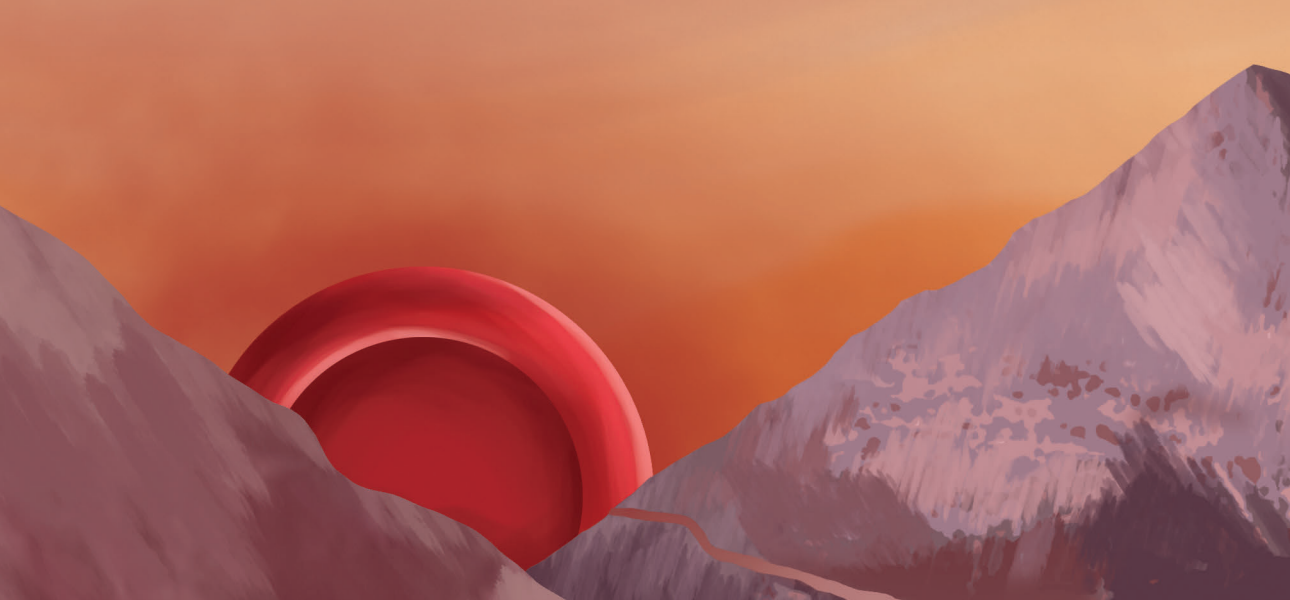
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PART 3

About iron

CHAPTER 8

A comprehensive analysis of the erythropoietin-erythroferrone-hepcidin pathway in hereditary hemolytic anemias

A.J. van Vuren[1], A. Sharfo[2], S.T. Grootendorst[3], S. van Straaten[3], A.M. Punt[3,4], J.B. Petersen[2], D. El Fassi[2,5], W.W. van Solinge[3], M. Bartels[6], R. van Wijk[3], A. Glenthøj[2,5], E.J. van Beers[1].

[1]   Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;
[2]   Department of Hematology, Rigshospitalet, Copenhagen University Hospital, University of Copenhagen, Copenhagen, Denmark;
[3]   Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[4]   Department of Clinical Pharmacology, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[5]   Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark;
[6]   Pediatric Hematology, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

Regulation of systemic iron metabolism and erythropoiesis are closely connected. Iron overload is an important complication in both transfusion-dependent and transfusion-independent patients with hereditary hemolytic anemias. We examined the levels of individual components of the erythropoietin (EPO)-erythroferrone (ERFE)-hepcidin pathway, and levels of soluble transferrin receptor (sTFR) and growth differentiation factor 15 (GDF15) in 115 patients with hereditary hemolytic anemias (β-thalassemia, pyruvate kinase deficiency, hereditary spherocytosis, hereditary xerocytosis and sickle cell disease) and twenty healthy controls.

In general, EPO-values and ERFE-values in patients were increased when compared with controls (25.2 vs 3.4 IU/L, and 14.5 vs 0.2 ng/mL respectively, both $p < 0.001$). And, hepcidin:ferritin ratios were considerably lower in patients when compared to controls (20.8 vs 114.6, $p < 0.001$). Median GDF15 values in β-thalassemia patients (2761 pg/mL) were extremely high compared to the other diseases (all $p < 0.001$).

Furthermore, we report for the first time a strong correlation between all components of the EPO-ERFE-hepcidin pathway. The hepcidin:ferritin ratio was negatively correlated with EPO, ERFE, sTFR and GDF15 (all independent correlations $p < 0.05$). EPO, ERFE, sTFR and GDF15 were, as expected, clearly positively correlated (EPO and ERFE $r = 0.86$, EPO and sTFR $r = 0.80$, and ERFE and sTFR $r = 0.80$; all $p < 0.05$).

Altogether, we confirm the presence of a common regulation of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias. However, variability among disease entities and among individuals illustrates the complexity of the communication between iron and erythropoiesis, which is partly explained by disease severity, spleen-status, iron load and transfusion history.

INTRODUCTION

Iron overload is an important complication in both transfusion-dependent and transfusion-independent patients with hereditary hemolytic anemias. Hereditary hemolytic anemias encompass a heterogeneous group of anemias with varying levels of hemolysis and ineffective erythropoiesis, also varying within disease entities. The increased erythropoietic response observed in hemolytic anemias is known as stress erythropoiesis, which is characterized by expansion of erythroid progenitors in an attempt to preserve the peripheral red blood cell pool.[1] Ineffective erythropoiesis is a term traditionally used to describe the destruction of developing early stage erythroid cells in the bone marrow. It is an example where stress erythropoiesis is not able to replenish the peripheral number of erythrocytes.[1]

Generally, anemia induces production of erythropoietin (EPO), which activates early erythroid progenitors and their production of erythroferrone (ERFE).[2] ERFE is known as a negative regulator of hepcidin, the latter is regarded as the master regulator of systemic iron availability.[3] Hepcidin determines systemic iron availability by binding to the iron transporter ferroportin on enterocytes, macrophages and hepatocytes, which initiates its ubiquitination and degradation, and blocks its iron transporting function.[4,5] Hepatic hepcidin expression is upregulated by iron levels; and ERFE inhibits hepcidin expression.[6] Effectively, this EPO-ERFE-hepcidin pathway secures iron availability for erythropoiesis. One of the clinical features of ineffective erythropoiesis is a dysregulation of the erythropoietin (EPO), erythroferrone (ERFE) and hepcidin pathway. Inappropriately low hepcidin levels induce secondary hemochromatosis in patients with hereditary hemolytic anemias and/or anemias with ineffective erythropoiesis even in the complete absence of regular red cell transfusions.[1,7,8]

In addition, soluble transferrin receptor (sTfR) and growth differentiation factor 15 (GDF15) may play a role in the interplay between erythropoiesis and iron as well. Serum sTfR is derived from cleavage of the membrane-bound transferrin receptor. Higher sTFR values represent an increased erythroid precursor mass, and thereby increased iron demand.[9] GDF15 is expressed and secreted during erythroblast maturation and has been associated positively with EPO. Uncertainty remains on the role of GDF-15 in hepcidin regulation in human, in whom it does not seem to be a physiologic regulator of hepcidin. Plasma levels are high in non-transfusion-dependent β -thalassemia and have been suggested to suppress hepcidin expression,[10] however studies in GDF15-knockout mice indicate otherwise.[11]

In hereditary hemolytic anemias, the bone marrow is dominated by an expanded pool of erythroid progenitors displaying varying degrees of stress erythropoiesis and inefficient erythropoiesis.[1] Currently, no studies are available that report on a combination of the values of the individual components of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias other than

hemoglobinopathies. In order to characterize the interplay between erythropoiesis and iron levels, the parameters of the EPO-ERFE-hepcidin pathway were measured and assessed in patients with various forms of hereditary hemolytic anemias, with varying levels of disease severity and iron burden.

We here report the presence of a correlation of the individual components of the EPO-ERFE-hepcidin pathway as a general feature in hereditary hemolytic anemias. Additionally, we explored the presence of a unique signature of the EPO-ERFE-hepcidin pathway in distinct disease entities and individual patients.

STUDY DESIGN

Data source and study population
Parameters of the EPO-ERFE-hepcidin pathway were measured in two different cohorts. The first (Dutch) cohort consisted of 82 patients and 10 healthy controls from the ZEbRA trial, a cross-sectional observational study on clinical sequelae and pathophysiology of rare congenital hemolytic diseases, which was conducted at the University Medical Center Utrecht (Netherlands Trial Register [NL5189]). The second (Danish) cohort included 33 patients and 10 healthy controls included from the Copenhagen General Population Study [H-KF-01–144/01]. The studies were conducted according to Good Clinical Practice guidelines, defined by the International Conference on Harmonization (ICH).

Regular laboratory parameters related to erythropoiesis (including EPO), hemolysis and iron metabolism were measured using routine laboratory procedures. Blood samples from transfusion-dependent patients were drawn immediately before transfusion.

Laboratory assays
Plasma levels of ERFE and sTFR were quantified by Intrinsic ERFE IE™ ELISA Kit (Intrinsic Lifescience, CA, USA) and human sTfR ELISA kit (RD194011100, Biovendor, Czech Republic), respectively. A custom-built Luminex Screening assay (R&D Systems, MN, USA) was used in combination with the Bio-Plex Multiplex system (Bio-Rad, CA, USA) to perform the analysis of plasma GDF15. Hepcidin in plasma was quantified by liquid chromatography tandem mass spectrometry. Sample clean-up was performed by protein precipitation with use of an isotope labelled internal standard (Peptide Institute, Osaka, Japan). The assay was fully validated and used for routine practice.

Data analysis

Calculations were performed using IBM SPSS Statistics version 25.0.0.2 (SPSS Inc., Chicago, IL). Non-parametric data was reported as median values with interquartile range (IQR). Mann-Whitney U test was used for comparisons between groups. EPO-ERFE-hepcidin pathway and GDF15 and sTFR correlation analyses were performed using Spearman's rho correlation coefficient. Linear regression modelling was applied to assess the predictability of transferrin saturation values by the components of the EPO-ERFE-hepcidin pathway (EPO, ERFE, sTFR, GDF15 and hepcidin). To correct for potential bias, bootstrapping was performed to confirm significance. Quantile normalized, log2-transformed data was used as input for the heatmaps, generated to visualize disease-specific patterns and individual variation. For optimal visualization and clustering only patients with complete data were included (N = 78). Hierarchical clustering of the columns was performed by the complete linkage method.

Hereditary xerocytosis (HX) patients, and sickle cell disease (SCD) patients were excluded from the correlation analysis and prediction model, based on known extrinsic factors influencing hepcidin regulation independent of the EPO-ERFE-hepcidin pathway. Data on EPO and sTFR were not available for the Danish cohort.

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RESULTS

Overview of clinical and laboratory parameters of our cohort

In total 115 patients and 20 healthy volunteers were included. The cohort consisted of patients diagnosed with hereditary spherocytosis (HS; N = 33), pyruvate kinase deficiency (PKD; N = 30), β-thalassemia (N = 29), hereditary xerocytosis (HX due to *PIEZO1* gain-of-function mutations; N = 8) and sickle cell disease (SCD; N = 15). As such, this cohort encompasses a heterogeneous population of patients with various degrees of hemolysis and various degrees of ineffective erythropoiesis. Moreover, this cohort was also heterogeneous with regard to transfusion requirements, degree of anemia, iron load, iron reducing treatments and spleen status. An overview of the main clinical characteristics of the patients (per diagnosis) and individual components of the EPO-ERFE-hepcidin pathway is provided in Table 1.

In general, median EPO-values and ERFE-values were increased in patients when compared to healthy controls (EPO: 25.2 IU/L; IQR 15.3, 40.0 vs 3.4 IU/L; IQR 2.8, 4.4, and ERFE: 14.5 ng/mL; IQR 6.1, 33.3 vs 0.2 ng/mL; IQR 0.0, 1.5, resp. p < 0.001 and p < 0.001). Additionally, levels of sTFR in patients were significantly higher than in controls (8.9 ug/mL; IQR 5.9, 13.3 vs 1.3 ug/mL; IQR 1.1, 1.7, p < 0.001).

Hepcidin:ferritin ratios (x 1000; in order to correct hepcidin values for iron load)[12] were considerably lower in patients when compared to healthy volunteers (20.8; IQR 8.6, 46.9 vs 114.6; IQR 90.3, 195.8, p < 0.001), statistical significance persisted when each disease was compared to the control population (all comparisons p < 0.001). Notably, hepcidin:ferritin ratios were moderately higher in non-transfusion-dependent patients than in transfusion-dependent patients (resp. 32.9; IQR 15.4, 60.1 vs 8.5; IQR 3.9, 13.2; p < 0.001). GDF15 levels were extremely high in β-thalassemia patients compared to all other diagnoses (all inter-disease comparisons p < 0.001). Notably, median GDF15 values in non-transfusion-dependent thalassemia and transfusion-dependent thalassemia patients were comparable (resp. 2534 pg/mL; IQR 979, 4588, and 3145 pg/mL; IQR 2281, 3449; p = 0.56). The SCD population also showed moderately increased amounts of GDF15 with a median value of 682 pg/mL (IQR 274, 836).

Robust correlation in the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias

To explore the relation of the individual components of the EPO-ERFE-hepcidin pathway, correlation analyses were performed between EPO, ERFE, hepcidin:ferritin ratio, sTFR and GDF15; results are reported in Table 2 and visualized in Figure 1. The hepcidin:ferritin ratio was negatively correlated with EPO, ERFE, sTFR and GDF15. EPO, ERFE, sTFR and GDF15 were clearly positively correlated: in particular worth mentioning is the close and strong relationship between EPO and ERFE (r = 0.86; IQR 0.74, 0.91), EPO and sTFR (r = 0.80; IQR 0.65, 0.88), and ERFE and sTFR (r = 0.80; IQR 0.64, 0.89).

As a sensitivity analysis we performed the correlation analyses in all subgroups (HS, PKD and β-thalassemia), the presence of a positive relation between EPO, ERFE and sTFR, and a negative relation between the hepcidin:ferritin ratio and either EPO, ERFE or sTFR was confirmed in all subgroups. The presence of a positive relation between GDF15 and either EPO, ERFE or sTFR, and a negative relation between GDF15 and the hepcidin:ferritin ratio was not consistently present in all disease entities: in contrast to the other disease entities, in HS patients there was no (negative) relation between GDF15 and hepcidin:ferritin ratio, r = 0.04; IQR -0.31, 0.39).

We used a prediction model for transferrin saturation (TSAT) to illustrate the interconnectivity of the EPO-ERFE-hepcidin pathway components. A model constructed of only two variables (GDF15 and ERFE) provided a good prediction of TSAT in individual patients (r = 0.72; ANOVA p < 0.001). Notably, patients included in the model were highly heterogeneous in terms of iron load, use of chelation therapy or phlebotomy and transfusion requirements. Hence, we confirm the presence of a strong correlation between all components of the EPO-ERFE-hepcidin pathway in various hereditary hemolytic anemias.

The EPO-ERFE-hepcidin pathway shows disease-specific and individual patterns

In order to analyze inter-individual variation in the EPO-ERFE-hepcidin pathway and to identify the presence of disease-specific patterns, we depicted individual patient data in a heatmap. (Figure 2) Hierarchical clustering of the individual patients based on EPO, ERFE, sTFR, GDF15, the hepcidin:ferritin ratio, and reticulocyte count was not able to completely separate disease entities, including transfusion dependency. However, several clusters of patients of one disease entity could be discriminated (e.g. β-thalassemia and SCD patients). The anemias with the most divergent patterns were PKD and HX. The EPO-ERFE-hepcidin pathways of the included SCD patients clustered in three subgroups differing in hepcidin:ferritin ratios and EPO values. The influence of disease severity, spleen-status, iron load, and transfusion requirements on clustering is illustrated in Figure S1A-E. To summarize, this analysis suggests the existence of disease-specific patterns of the EPO-ERFE-hepcidin pathway, however with considerable overlap between diseases and with the presence of inter-individual variation in specific hereditary hemolytic anemia sub forms (e.g. PKD and HX).

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Table 1. Demographic, clinical characteristics, parameters of hemolysis, erythropoiesis and iron metabolism per disease.

		Healthy controls[†]
	Number	20
General characteristics	Median age	NA
	Male (fraction)	NA
	Splenectomy (fraction)	0
	Transfusion dependency (fraction)	0
	Iron chelation[‡] OR phlebotomy[§] (fraction)	0
Hemolysis	LDH (IU/L)	NA
	Total bilirubin (umol/L)	NA
Erythropoiesis	Hb (g/dL)	14.2 (13.7, 15.1)
	Reticulocytes (x10^9)	59 (44, 72)
	EPO (IU/L)[*]	3.4 (2.8, 4.4)
	ERFE (ng/mL)	0.24 (0.24, 1.53)
	sTFR (ug/mL)[*]	1.3 (1.1, 1.7)
	GDF15 (pg/mL)	168 (115, 298)
Iron metabolism	Hepcidin (ug/L)	14.9 (5.6, 21.8)
	TSAT (fraction)	0.26 (0.18, 0.33)
	Ferritin (ug/L)	62[$] (25, 135)
	Hepcidin:ferritin ratio (x 1000)	114.6[$] (90.3, 195.8)

Unless otherwise indicated, data are median values (interquartile range). The Dutch cohort included 82 patients diagnosed with HS (N = 24), PKD (N = 23), β-thalassemia (N = 12), HX (N = 8) and SCD (N = 15); the Danish cohort included 33 patients diagnosed with HS (N = 9), PKD (N = 7) and β-thalassemia (N = 17). * No EPO and no sTFR data available in the Danish cohort. † Not reported values were not available (NA). ‡ Iron chelator use was defined as prescription of an iron chelating agent during the

Table 2. Correlation table EPO-ERFE-hepcidin pathway.

	hepcidin:ferritin ratio (x 1000)	ERFE (ng/mL)
hepcidin:ferritin ratio (x 1000)		- 0.74 (-0.85, -0.57)
ERFE (ng/mL)	- 0.74 (-0.85, -0.57)	
EPO (IU/L)[*]	-0.69 (-0.83, -0.50)	0.86 (0.74, 0.91)
sTFR (ug/mL)[#]	-0.68 (-0.81, -0.52)	0.80 (0.64, 0.89)
GDF15 (pg/mL)	-0.43 (-0.65, -0.16)	0.55 (0.32, 0.72)

Values represent the correlation coefficient (R) and 95% confidence interval. All presented values were statistically significant. * No EPO and no sTFR data available in the Danish cohort. EPO erythropoietin; ERFE erythroferrone; GDF15 growth differentiation factor 15.

HS	PKD	β-thalassemia	HX	SCD
33	30	29	8	15
47 (37, 59)	43 (27, 49)	30 (27,36)	48 (32, 59)	30 (27, 35)
0.52	0.47	0.45	0.5	0.4
0.33	0.7	0.52	0.13	0.07
0	0.23	0.83	0	0.33
0.12	0.33	0.86	0.5	0.33
193 (181, 231)	189 (164, 326)	187 (146, 259)	NA	313 (263, 393)
27 (19, 51)	56 (41, 78)	44 (31, 60)	37 (23, 71)	31 (22, 47)
13.2 (11.6, 15.2)	9.6 (8.5, 12.0)	9.2 (8.5, 9.5)	13.9 (13.3, 14.2)	9.8 (8.7, 12.2)
235 (135, 346)	691 (266, 978)	110 (49, 205)	482 (307, 614)	178 (107, 260)
12.8 (5.7, 30.6)	24.7 (17.0, 31.5)	37.5 (18.7, 63.4)	26.9 (18.8, 31.4)	32.0 (20.9, 67.7)
5.7 (2.1, 24.4)	15.2 (7.8, 31.5)	13.2 (9.2, 16.7)	14.7 (10.8, 19.3)	7.4 (5.1, 11.8)
5.5 (2.5, 8.0)	10.6 (7.4, 15.1)	13.2 (9.3, 16.7)	6.9 (5.1, 11.3)	9.7 (6.4, 14.3)
261 (184, 409)	253 (230, 401)	2761 (1629, 3430)	346 (210, 697)	682 (274, 836)
10.8 (6.3, 21.8)	9.6 (5.1, 12.8)	7.5 (2.6, 12.3)	4.6 (1.0, 17.0)	6.2 (2.0, 16.6)
0.36 (0.27, 0.50)	0.50 (0.33, 0.74)	0.93 (0.86, 1.21)	0.48 (0.19, 0.56)	0.43 (0.25, 0.74)
247 (109, 374)	496 (251, 833)	774 (475, 1325)	142 (61, 362)	562 (62, 744)
50.0 (32.2, 68.0)	15.0 (8.7, 27.0)	8.5 (3.8, 14.0)	36.1 (10.5, 47.0)	18.4 (6.4, 25.2)

last 12 months. § Phlebotomies apply to a regular phlebotomy scheme in the past. $ No ferritin data available in the Danish healthy controls. EPO erythropoietin; ERFE erythroferrone; GDF15 growth differentiation factor 15; Hb hemoglobin; HS hereditary spherocytosis; HX hereditary xerocytosis; LDH lactate dehydrogenase; PKD pyruvate kinase deficiency; SCD sickle cell disease; sTFR soluble transferrin receptor; TSAT transferrin saturation.

EPO (IU/L)*	sTFR (ug/mL)*	GDF15 (pg/mL)
-0.69 (-0.83, -0.50)	-0.68 (-0.81,-0.52)	-0.43 (-0.65, -0.16)
0.86 (0.74, 0.91)	0.80 (0.64, 0.89)	0.55 (0.32, 0.72)
	0.80 (0.65, 0.88)	0.59 (0.35, 0.75)
0.80 (0.65, 0.88)		0.39 (0.12, 0.60)
0.59 (0.35, 0.75)	0.39 (0.12, 0.60)	
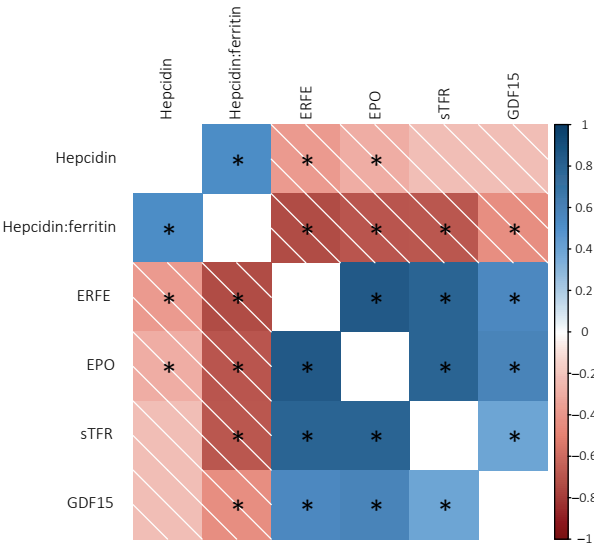
Figure 1. Correlation plot.

Asterix-marked squares represent a statistically significant correlation (p < 0.05).

EPO erythropoietin; ERFE erythroferrone; GDF15 growth differentiation factor 15; sTFR soluble transferrin receptor.
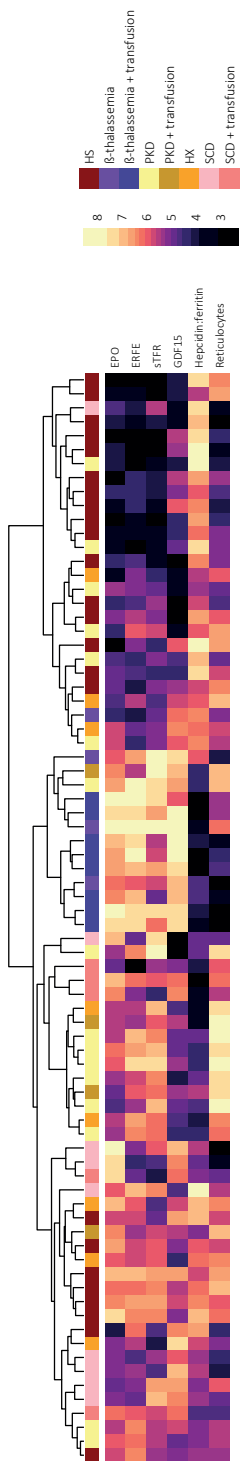
Figure 2. Heatmap of EPO-ERFE-hepcidin pathway in hereditary anemias.

The heatmap is constructed with Z-scores of quantiles normalized, log2 transformed data. Hence, high values represent high quantiles. Hierarchical, unsupervised clustering of the columns was applied by the complete linkage method. The dendrogram branches are colored by diagnosis and transfusion-dependency.

EPO erythropoietin; ERFE erythroferrone; GDF15 growth differentiation factor 15; HS hereditary spherocytosis; HX *PIEZO1*-mutated hereditary xerocytosis; PKD pyruvate kinase deficiency; SCD sickle cell disease; sTFR soluble transferrin receptor.

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DISCUSSION

Here, we studied the activity of the EPO-ERFE-hepcidin pathway in a large group of different hereditary hemolytic anemias. We confirm the presence of a common regulation of the EPO-ERFE-hepcidin pathway in all these hemolytic anemias including hemoglobinopathies, enzymopathies and membranopathies. Although anemia, hemolysis, ineffective erythropoiesis and iron overload are features shared among the hemolytic anemias, marked differences in patterns of EPO, ERFE, sTFR, GDF15 and hepcidin expression exist between diseases as well as between patients diagnosed with one disease entity. Variability is at least partly explained by differences in disease severity, spleen-status, iron load and transfusion requirements.

As expected, hepcidin values were generally suppressed in all patients with hereditary hemolytic anemias compared to healthy controls. Noteworthy is the observation that hepcidin values were comparable in transfusion-dependent and-independent patients. This observation is not in line with the theory that regular transfusions suppress erythropoiesis, reduce erythron iron requirements and thus reduce hepcidin suppression.[13,14] We observed a strong positive correlation between the three parameters involved in hepcidin regulation, all representing the size of the erythron: EPO, ERFE and sTFR. However, our data is limited in size and may represent a biased selection of more severe diseased patients in the transfusion-dependent thalassemia subgroup. Luspatercept is approved for treatment of ineffective erythropoiesis in β-thalassemia. It would be of great interest to investigate its effect on the erythron characterized to an expanded erythroid progenitor pool.

Regarding GDF15, uncertainty remains on the role of (erythron-derived) GDF15 in hepcidin regulation in human,[15] in whom it does not seem to be a physiologic regulator of hepcidin.[16-18] However, in line with our observations in β-thalassemic patients, high GDF15 and suppressed hepcidin levels were previously reported in three forms of hereditary anemias with pronounced ineffective erythropoiesis: β-thalassemia,[10] congenital dyserythropoietic anemia type I and type II.[19,20] In β-thalassemia high GDF15 levels are suggested to stem from increased oxidative stress in erythroblasts.[21] Elevated levels of GDF15 in the included SCD patients may represent cumulative GDF15 secretion by cardiomyocytes, vascular smooth muscle cells and endothelial cells in response to oxidative stress, inflammation and tissue injury, and erythroblasts as a consequence of bone marrow stress.[22]

We have partly excluded patients diagnosed with HX and SCD from our analysis, as factors other than the upstream components of the EPO-ERFE-hepcidin pathway or GDF15 determine hepcidin transcription. *PIEZO1*-mutated HX is a rare hemolytic anemia with delayed reticulocyte maturation.[23] Compared to the other disease entities, hepcidin values

in HX were low in relation to EPO, ERFE and sTFR. Our findings are in line with recent reports of Andolfo *et al.*[24] and Ma *et al.*[25] that showed a direct link between *PIEZO1* gain-of-function mutations and hepcidin suppression. So, low hepcidin in HX represents both the direct effect of the *PIEZO1* mutation on hepcidin gene (*HAMP*) transcription and the size of the erythron.

The EPO-ERFE-hepcidin pathways of the included SCD patients clustered in three subgroups differing in hepcidin:ferritin ratios and EPO values. The pathophysiology of SCD includes chronic inflammation accompanied by leukocyte-platelet-erythrocyte-endothelial adhesive events that trigger vaso-occlusion, acute organ ischemia and reperfusion injury with wide diversity among individuals.[26,27] The inflammatory cytokine interleukin 6 directly promotes hepcidin transcription in hepatocytes.[28,29] Hypoxia augments EPO secretion,[30] but hypoxia also modulates hepcidin expression in an ERFE-independent way via platelet derived growth factor-BB secreted by non-erythroid cells.[31] Altogether, we assume that the variability in EPO-ERFE-hepcidin regulation in the SCD patients represents the complex interplay between iron, inflammation, hypoxia and erythropoiesis.[32]

One limitation of our study is the lack of sTFR and EPO values in the Danish cohort. Exclusion of the Danish patients from the analyses including EPO or sTFR rendered those analyses limited to the Dutch cohort, nevertheless highly significant results were obtained in these analyses underscoring the robustness of the correlations in the EPO-ERFE-hepcidin pathway.

The heterogeneity of our cohort could be regarded as either a strength or a limitation of the study. This first report confirming a uniform regulation of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias raises questions on the individual contribution of factors, such as transfusion requirements, iron load, chelation therapy, or spleen status, that may explain observed differences between disease entities and between patients. In particular worth mentioning is the role of (adequate) iron chelation in β-thalassemia patients as it may reduce the level of ineffective erythropoiesis and thereby may alter all parameters of the EPO-ERFE-hepcidin pathway.[1]

Altogether, this report provides insight in the balance of the EPO-ERFE-hepcidin pathway in hereditary hemolytic anemias. However, variability among disease entities and among individuals illustrates the complexity of the communication between erythropoiesis and iron. For future research it would be of major interest to investigate how the EPO-ERFE-hepcidin pathway responds to (new) treatments (e.g. treatments that increase fetal hemoglobin in β-thalassemia), and to expand the EPO-ERFE-hepcidin pathway analyses to hypoplastic hereditary anemias.
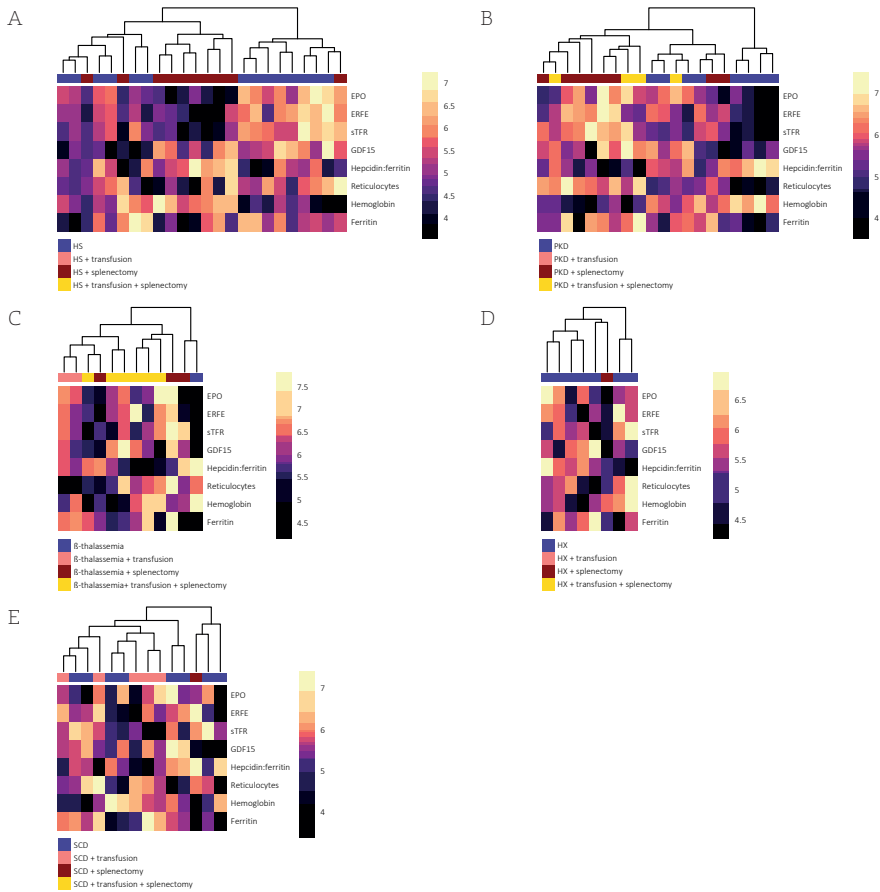
SUPPLEMENTAL FILES

Figure S1. Heatmap of EPO-ERFE-hepcidin pathway hereditary spherocytosis (HS; S1A), pyruvate kinase deficiency (PKD; S1B), β-thalassemia (S1C), *PIEZO1*-mutated hereditary xerocytosis (HX; S1D) and sickle cell disease (SCD; S1E).

The heatmaps are constructed with Z-scores of quantiles normalized, log2 transformed data. Hierarchical, unsupervised clustering of the columns was applied by the complete linkage method. The dendrogram branches are colored by transfusion-dependency and previous splenectomy.

EPO erythropoietin; ERFE erythroferrone; GDF15 growth differentiation factor 15; sTFR soluble transferrin receptor.

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CHAPTER 9

Liver iron retention estimated from utilization of oral and intravenous radioiron in various anemias and hemochromatosis in humans

A.J. van Vuren[1]; R. van Wijk[3]; E.J. van Beers[1], J.J.M. Marx[2].

[1] Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, The Netherlands;
[2] Departments of Haematology and Internal Medicine, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[3] Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.

ABSTRACT

Patients with hereditary hemochromatosis and non-transfusion-dependent hereditary anemia develop predominantly liver iron-overload. We present a unique method allowing quantification of liver iron retention in humans during first-pass of 59Fe-labeled iron through the portal system, using standard ferrokinetic techniques measuring red cell iron uptake after oral and intravenous ^{59}Fe administration. We present data from patients with iron deficiency (ID; N = 47), hereditary hemochromatosis (HH; N = 121) and non-transfusion-dependent hereditary anemia (HA; N = 40). Mean mucosal iron uptake and mucosal iron transfer (±SD) were elevated in patients with HH (59 ± 18%, 80 ± 15% respectively), HA (65 ± 17%, 74 ± 18%) and ID (84 ± 14%, 94 ± 6%) compared to healthy controls (43 ± 19%, 64 ± 18%) ($p < 0.05$) resulting in increased iron retention after 14 days compared to healthy controls in all groups ($p < 0.01$). The fraction of retained iron utilized for red cell production was 0.37 ± 0.17 in untreated HA, 0.55 ± 0.20 in untreated HH and 0.99 ± 0.22 in ID ($p < 0.01$). Interestingly, compared to red blood cell iron utilization after oral iron administration, red blood cell iron utilization was higher after injection of transferrin-bound iron in HA and HH. Liver iron retention was considerably higher in HH and HA compared to ID. We hypothesize that albumin serves as a scavenger of absorbed Fe(II) for delivering albumin-bound Fe(III) to hepatocytes.

INTRODUCTION

Iron absorption and iron utilization studies performed in the 60s and 70s from last century using radiolabeled iron greatly improved our understanding of iron metabolism.[1-3] Over the last 25 years advances in molecular biology and genetics revolutionarily increased the knowledge on normal and abnormal iron biology including the discovery of hepcidin as key player in primary and secondary hemochromatosis.[4,5]

Production of red blood cells (RBCs) requires 20–25 mg iron daily under physiologic conditions. The demand is supplied by import of iron from enterocytes (1–2 mg), iron recycling by macrophages (20–25 mg) and iron from storage tissues like hepatocytes.[4] Production of red blood cells (RBCs) requires 20-25mg iron daily under physiologic conditions. The demand is supplied by import of iron from enterocytes (1-2mg), iron recycling by macrophages (20-25mg) and iron from storage tissues like hepatocytes.[6] Due to the absence of a renal or hepatic iron excretion mechanism, body iron levels are only controlled by intestinal absorption. The tendency to retain iron exposes humans to a substantial risk of iron-overload and related toxicity.[7]

The hepcidin/ferroportin axis regulates iron export from enterocytes, macrophages and iron storage tissue into the circulation. Hepcidin inhibits iron efflux by binding to ferroportin, which leads to degradation of the exporter.[4,6,8-10] Inversely, less hepcidin synthesis or activity will result in increased intestinal iron transfer from enterocytes to the circulation.[11] Duodenal enterocytes, having a life-span of only two days while moving from the crypts of Lieberkühn to tips of the villi, express the divalent metal transporter 1 (DMT1) at the luminal side and intracellular ferritin, incorporating excess iron, which is rapidly lost to the lumen of the gut with exfoliating enterocytes. High expression of ferroportin results in iron depletion of enterocytes, by diffusion of $Fe(II)$ through the basolateral membrane to a compartment where $Fe(II)$ is rapidly oxidized with binding of $Fe(III)$ to a variety of ligands, including transferrin.[6] In patients with hereditary hemochromatosis genetic inactivation of one of the intermediaries of the hepcidin-activation route results in inhibition of BMP-SMAD signaling and, subsequently, reduced hepatic hepcidin expression.[12] The most common forms of hereditary hemochromatosis are caused by mutations of the *Hfe* (Hereditary FE), hemojuvelin (*Hjv*), hepcidin (*Hamp*) or transferrin receptor 2 (*Trf2*) gene. Hereditary hemolytic or dyserythropoietic anemias are characterized by an increased effective or ineffective erythropoietic response.[13] Erythropoietin is the main hormone controlling erythropoiesis. Erythroblasts secrete erythroferrone in response to erythropoietin.[14] Erythroferrone suppresses hepatic hepcidin expression via inhibition of BMP-SMAD signaling.[15] So, both patients with hereditary hemochromatosis and non-transfusion-dependent hereditary anemia are at risk for development of iron-overload in response to low hepcidin.[16-19]

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Iron transport via transferrin seems not to contribute significantly to continuous hepatic iron loading in hemochromatosis, because both transferrin and hepatic transferrin receptor 1 (TfR1) are downregulated under iron-overload conditions. Still, livers of iron-loaded mice take up more iron than non-iron-loaded livers.[20-22] The importance of a non-transferrin-dependent iron-regulating system was demonstrated in hpx (atransferrinemic) mice, characterized by anemia and mainly hepatic iron-overload. Iron taken up by hepatocytes of hpx mice has to be non-transferrin-bound iron (NTBI).[20] Hence, hepatic import of iron reversibly bound to other carrier molecules significantly contributes to continuous hepatic iron-overloading.

Here, we present data of radiolabeled iron absorption and iron utilization studies performed in a large cohort of patients with iron deficiency, primary and secondary hemochromatosis. The combination of these studies in the same patient provides unique information on iron transport after entrance of iron from ferroportin into the portal venous system and its uptake into hepatocytes during the first passage through the liver.

RESULTS

Our cohort contained 278 analyses of subjects with normal iron stores, patients with iron deficiency, and with primary or secondary iron-overload with and without iron reducing treatment. Analyses in the non-transfusion-dependent hereditary anemia group without treatment were obtained in patients diagnosed with congenital sideroblastic anemia (CSA, N = 13), hereditary spherocytosis (N = 3), congenital dyserythropoietic anemia (CDA, N = 5), non-transfusion-dependent β–thalassemia (NTDT, N = 2), HbE/β–thalassemia (N = 1), pyruvate kinase deficiency (PKD, N = 1) and Hb Adana (N = 4). Analyses in non-transfusion-dependent hereditary anemia patients with iron reducing therapy were obtained in patients with hereditary spherocytosis (N = 3), hexokinase deficiency (HKD, N = 2) and congenital sideroblastic anemia (CSA, N = 6). Of the treated non-transfusion-dependent hereditary anemia patient group, three analyses were obtained in patients on iron chelation therapy (deferoxamine), and eight analyses in patients on a regular phlebotomy scheme. All patients with hereditary haemochromatosis (HH, N = 121) were regarded as homozygotes, based on HLA-typing, and clinical and family history. Ferritin assays were not yet available when normal subjects were investigated. All patients in hereditary hemochromatosis patient group treated with iron reducing therapies were on a regular phlebotomy scheme, and one patient was both phlebotomized and treated with deferoxamine. Patient characteristics and median laboratory parameters are summarized in Table 1.

Body iron retention is significantly increased in patients with non-transfusion-dependent hereditary anemia and hereditary hemochromatosis
Median percentages of mucosal iron uptake, mucosal iron transfer and iron retention are summarized in Table 1. Mean mucosal iron uptake, and mucosal transfer were significantly higher in hereditary hemochromatosis and non-transfusion-dependent hereditary anemia patients without iron reducing treatment, and in iron deficient patients, compared to healthy controls (p < 0.05). Together, this resulted in a significantly increased iron retention after 14 days in all groups, with or without iron reducing treatment, when compared to healthy subjects (p < 0.01).

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Table 1. Patient characteristics and laboratory parameters.

	HA NTD	HA NTD-irt
No. of patients	29	11
Male, %	79	82
Age, years	29 (22, 37)	26 (15, 39)
Laboratory parameters		
Hb, g/dL	10.5 (10.5, 12.4)	12.4 (9.5, 15.5)
Reticulocytes, x 10^9/L	15 (12, 39)	8 (5, 243)
Ferritin, mg/L	407 (215, 1800)	222 (109, 367)
Iron saturation, fraction	0.74 (0.50, 0.87)	0.76 (0.53, 0.97)
Serum iron, mmol/L	33 (27, 41)	35 (30, 38)
AST, U/L	22 (16, 42)	18 (13, 27)
LD, U/L	350 (277, 644)	395 (311, 593)
Bilirubin, mg/dL	18 (8, 44)	9 (7, 28)
Iron absorption studies		
Mucosal iron uptake, %	60 (54, 77)	78 (55, 87)
Mucosal iron transfer, fraction	0.76 (0.63, 0.91)	0.90 (0.80, 0.96)
Iron retention, %	42 (33, 63)	66 (55, 74)

Presented values are medians with interquartile ranges (IQR). Iron uptake, retention and transfer were calculated from the amount of ^{59}Fe and ^{51}Cr in the body 24 h after the oral iron test dose (uptake) and 14 days after the oral iron test dose (retention). Mucosal iron transfer is the fraction of iron retained after uptake.

Disappearance half-life of transferrin-bound iron is shorter in non-transfusion-dependent hereditary anemia than in hereditary hemochromatosis

We tested the velocity of disappearance of a single injection of transferrin-bound iron from the circulation. A graphic overview is provided in Figure 1. Mean disappearance half-life of transferrin-bound iron (± standard deviation) was significantly shorter in patients with iron deficiency (47 ± 37 min), untreated hereditary anemia (51 ± 23 min) and treated hereditary anemia (65 ± 22 min), than in treated (96 ± 29min) and untreated (108 ± 22 min) hereditary hemochromatosis patients (all $p < 0.01$). Half-life of iron in patients with non-transfusion-dependent hereditary anemia was comparable with iron deficient patients ($p > 0.05$). Clearance of radioiron from plasma in healthy controls was previously studied,[23] and varied between 75–105 min (N = 6). Clearance in healthy controls is thereby much faster than in untreated patients with hereditary hemochromatosis, while those patients come close to the normal range during phlebotomy. Patients with hereditary anemia and effective erythropoiesis show a higher demand for iron by erythroblasts, due to hemolysis. These patients have a functional iron deficiency while iron due to early red cell destruction is accumulating in macrophages in spleen and liver.

HH	HH-irt	ID	Normal
79	42	47	70
66	86	26	56
44 (35, 56)	44 (37, 54)	38 (23, 47)	67 (26, 70)
15.5 (14.8, 16.4)	16.0 (14.6, 16.8)	11.1 (10.2, 12.6)	15.5 (14.6, 16.3)
11 (7, 17)	13 (7, 26)	12 (8, 15)	10 (6, 15)
953 (440, 1350)	70 (36, 170)	7 (5, 9)	NA
0.85 (0.78, 0.92)	0.69 (0.44, 0.90)	0.08 (0.05, 0.15)	0.36 (0.29, 0.44)
38 (33, 43)	34 (22, 41)	7 (4, 10)	21 (18, 26)
31 (20, 44)	22 (15, 26)	17 (15, 23)	NA
400 (325, 473)	416 (323, 459)	408 (372, 455)	NA
10 (8, 16)	12 (9, 18)	6 (4, 9)	NA
55 (47, 72)	88 (81, 93)	88 (75, 96)	46 (29, 55)
0.84 (0.71, 0.90)	0.97 (0.94, 0.98)	0.96 (0.91, 0.98)	0.62 (0.53, 0.78)
45 (33, 63)	85 (73, 91)	81 (69, 91)	25 (17, 36)

HA NTD hereditary anemia non-transfusion-dependent; HH hereditary hemochromatosis; ID iron deficiency; irt iron reducing therapy (either phlebotomies or deferoxamine). AST aspartate aminotransferase; Hb hemoglobin; LD lactate dehydrogenase.
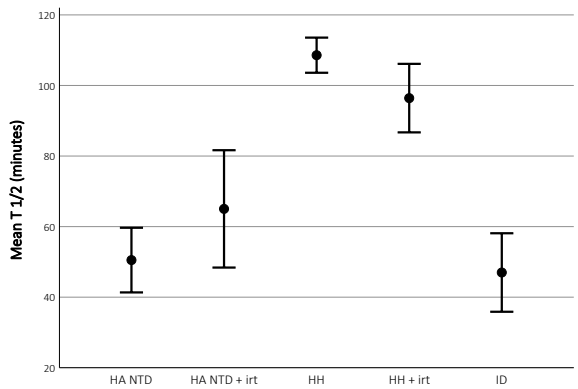
Figure 1. Disappearance half-life of transferrin-bound iron. Disappearance half-life of iron was calculated from blood samples taken at 0, 5, 15, 30, 45, 60, 90 and 120 min after injection. Half-life is expressed in minutes. Plot of the mean half-life categorized per disease group. Error bars represent 95% confidence intervals.

HA NTD hereditary anemia, non-transfusion-dependent; HH hereditary hemochromatosis; irt iron reducing therapy (phlebotomies or deferoxamine); ID iron deficiency; T½ half-life; min minutes.

Of interest was the data of three spherocytosis patients of whom ferrokinetic studies were available before and after splenectomy. We observed striking differences in plasma iron half-life before splenectomy (22, 15 and 23 min), and two years after splenectomy (70, 63 and 67 min respectively). Increase in spleen size was negatively correlated with half-life in the whole group of non-transfusion-dependent hereditary anemia patients (r = −0.50, 95% CI [−0.69, −0.26], p < 0.01). Hemolytic biomarkers were negatively correlated with half-life of iron: absolute reticulocyte count (r = −0.41, 95% CI [−0.51, −0.40], p = 0.02), serum lactate dehydrogenase (r = −0.49, 95% CI [−0.66, −0.27], p < 0.01) and serum bilirubin (r = −0.53, 95% CI [−0.64, −0.42], p < 0.01). In all patients, half-life of iron was positively correlated with serum hemoglobin concentrations (r = 0.61, 95% CI [0.49, 0.72], p < 0.01). Notably, in congenital sideroblastic anemia, a disease characterized by disrupted utilization of iron in erythroblasts, ineffective erythropoiesis and relative low reticulocyte counts,[24] half-life of iron was significantly longer than in other forms of non-transfusion-dependent hereditary anemia (63 ± 21 min versus 45 ± 23 min, 95% CI [4.5, 31.4], p = 0.02).

In summary, disappearance half-life of intravenous transferrin-bound iron was significantly longer in hereditary hemochromatosis patients compared to the patients with various anemias. In non-transfusion-dependent hereditary anemia patients more severe anemia and higher levels of hemolytic parameters were associated with shortened half-life of iron.

Red blood cell iron utilization decreases when iron-overload increases

We measured the amount of ^{59}Fe iron in peripheral blood samples 14 days after the iron test dose to determine the amount of iron utilized for RBC production. Mean percentages (± standard deviation) of RBC iron utilization (RBCIU) after an oral iron test dose were 37 ± 17% in untreated non-transfusion-dependent hereditary anemia, 53 ± 19% in treated non-transfusion-dependent hereditary anemia, 55 ± 20% in untreated hereditary hemochromatosis, 70 ± 22% in treated hereditary hemochromatosis, and 99 ± 22% in iron deficient patients. We previously reported, using the same methodology, a mean RBCIU of 82 ± 13% in ten healthy adults from an oral iron test dose.[25] In iron deficient patients the utilization of oral (99 ± 22%) and intravenous (100 ± 9%) iron were comparable. Surprisingly, RBCIU was significantly higher after intravenous iron than after an oral test dose in patients with hereditary anemia (47 ± 18%) or hereditary hemochromatosis (76 ± 12%), both untreated (p < 0.01). (Figure 2A) In healthy controls, reported previously,[25] RBCIU of intravenous iron was 85 ± 17%, and thereby comparable with the RBCIU after an oral iron test dose.

To understand intergroup differences in iron utilization, we investigated the influence of the pre-existing iron load in all patients and of the degree of anemia and hemolysis in patients with non-transfusion-dependent hereditary anemia. Iron saturation was

negatively correlated with RBCIU after oral iron (r = −0.62, 95% CI [−0.71, −0.51], p < 0.01) and after intravenous iron (r = −0.56, 95% CI [−0.65, −0.47], p < 0.01), (Figure 2B) as was ferritin (respectively r = −0.55, 95% CI [−0.63, −0.45], p < 0.01; and r = 0.42, 95% CI [−0.55, −0.32], p < 0.01). Thus, in patients with a higher iron saturation or ferritin value, representing more severe iron-overload, the amount of iron utilized for RBC production was lower. The treated and untreated hereditary anemia patients were further analyzed to investigate the existence of a correlation between RBCIU and disease severity, based on laboratory parameters of erythropoiesis and hemolysis. The hemoglobin concentration was significantly correlated with RBCIU of intravenous iron (r = 0.55, 95% CI [0.30, 0.75], p < 0.01) and of oral iron (r = 0.61, 95% CI [0.33, 0.78], p < 0.01). RBCIU was not related to reticulocyte count, lactate dehydrogenase or bilirubin. Again, we observed striking differences in the three spherocytosis patients of whom data was available before and after splenectomy. All three patients were anemic before splenectomy and hemoglobin concentrations normalized after splenectomy; patients were phlebotomized after splenectomy. Utilization of oral and intravenous iron for RBC production improved significantly after splenectomy (mean oral RBCIU before splenectomy 41 ± 10%, after splenectomy 73 ± 14%, delta 32%, p = 0.04; mean intravenous RBCIU before splenectomy 50 ± 6%, after splenectomy 85 ± 13%, delta 36%, p = 0.03). In all non-transfusion-dependent hereditary anemia patients increase in spleen size (centimeters under the costal margin) was correlated with lower intravenous RBCIU (r = −0.59, 95% CI [−0.73, −0.45], p < 0.01) and less oral RBCIU (r = -0.50, 95% CI [−0.70, −0.27], p < 0.01).

Summarizing, the amount of iron utilized for RBC production was lower in patients with primary or secondary hemochromatosis compared to iron deficient patients and healthy controls. In non-transfusion-dependent hereditary anemia patients, iron utilization for RBC production was even more suppressed in patients with lower hemoglobin concentrations and splenomegaly.

Liver iron retention is increased in iron-overload
We observed a difference between the utilization of oral and intravenous iron for RBC production in non-transfusion-dependent hereditary anemia and hereditary hemochromatosis patients. (Figure 3A) The difference between oral and intravenous RBCIU was expressed as percentage of intravenous RBCIU and denominated as LIR (liver iron retention). Liver iron retention was close to zero in patients with iron deficiency anemia. Ferritin, serum iron and iron saturation fraction were very low. Free iron binding sites on transferrin could easily accommodate all iron entering the plasma after transport across the basolateral membrane of duodenal cells by ferroportin. The LIR had a mean value (± standard deviation) of 28 ± 26% in untreated hereditary hemochromatosis, 23 ± 24% in untreated hereditary anemia, 16 ± 25% in treated hereditary hemochromatosis patients, all

significantly higher than the LIR of 1 ± 22% measured in iron deficient patients ($p < 0.05$). (Figure 3A) The LIR was strongly correlated to iron saturation ($r = 0.41$, 95% CI [0.26, 0.53], $p < 0.01$), (Figure 3B) and ferritin level ($r = 0.47$, 95% CI [0.30, 0.61], $p < 0.01$). Thus, the fraction of retained iron not utilized for erythropoiesis is considerably increased in patients with primary or secondary hemochromatosis.
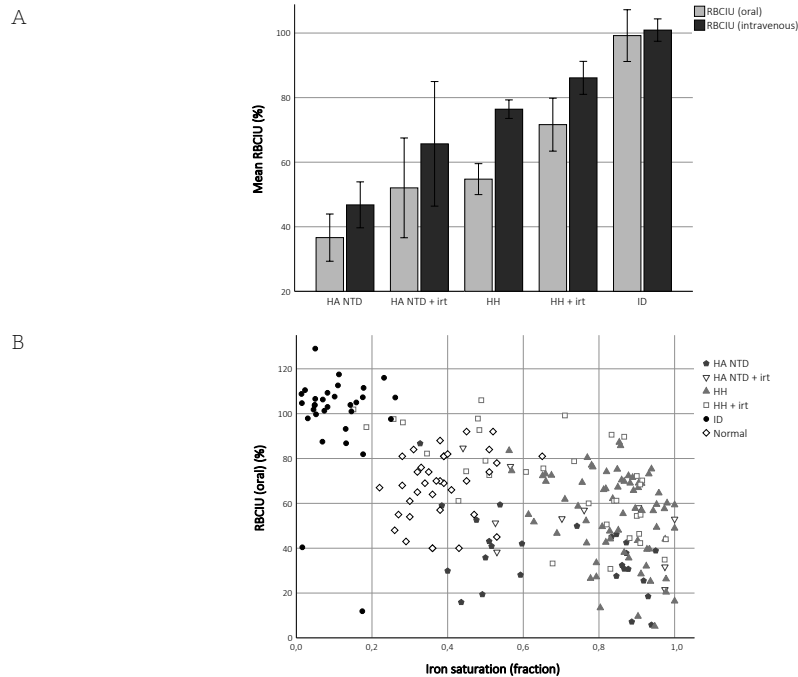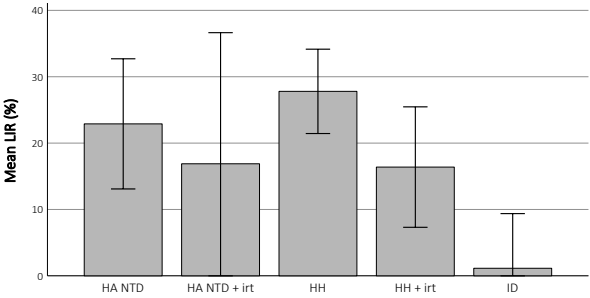

Figure 2. Red blood cell iron utilization (RBCIU). RBCIU was calculated from blood samples obtained 14 days after oral or intravenous iron test dose: RBCIU (oral) (%) = (^{59}Fe in 1 mL blood × blood volume (mL))/^{59}Fe retained in the body after oral iron dose × 100%, and RBCIU (intravenous) (%) = (^{59}Fe in 1 mL blood × blood volume (mL))/^{59}Fe iv dose × 100%. (A) Bar plot with mean percentages of RBCIU. Error bars represent 95% confidence intervals. (B) Scatter plot with iron saturation (*x-axis*) and RBCIU after oral iron (*y-axis*). Every dot represents a single analysis.

HA NTD hereditary anemia non-transfusion-dependent; HH hereditary hemochromatosis; ID iron deficiency; irt iron reducing therapy (phlebotomies or deferoxamine).

A

B
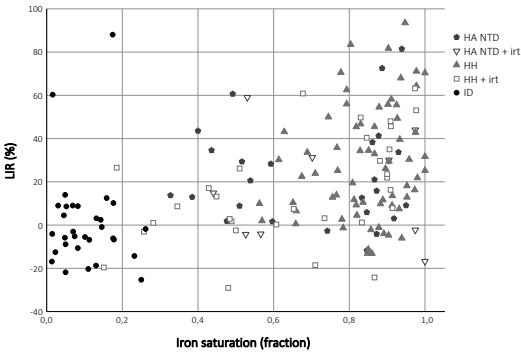
Figure 3. Liver iron retention (LIR). LIR calculated from the difference between red blood cell iron utilization (RBCIU) after intravenous and oral iron test dose and expressed as percentage of intravenous RBCIU: LIR (%) = (RBCIU intravenous–RBCIU oral)/RBCIU intravenous × 100%. (A) Bar plot with mean LIR values. Error bars represent 95% confidence intervals. (B) Scatter plot with iron saturation (*x-axis*) and LIR (*y-axis*). Every dot represents a single analysis.

HA NTD hereditary anemia non-transfusion-dependent; HH hereditary hemochromatosis; ID iron deficiency; irt iron reducing therapy (phlebotomies or deferoxamine).

DISCUSSION

We report here on a unique combination of iron absorption and ferrokinetic data from a large cohort of patients with iron deficiency, primary hemochromatosis, hereditary anemia and secondary hemochromatosis, enabling quantification of liver iron retention.

An older study reported already in 1982 a significantly lower ^{59}Fe-RBCIU in healthy aged subjects 14 days after an oral iron test dose, while RBCIU after an intravenous dose showed no difference.[25] In this small study RBCIU upon intravenous iron could not be measured in the same subjects as RBCIU upon oral iron administration. The authors suggested that the lower RBCIU after an oral dose could probably be a result of increased liver iron retention. In the present study we compared RBCIU after oral with RBCIU after intravenous iron administration in the same subject, demonstrating that both iron mucosal uptake (via DMT1) and mucosal transfer (via ferroportin) were increased in patients with iron deficiency anemia, primary and secondary hemochromatosis. In non-transfusion-dependent hereditary anemia and hereditary hemochromatosis patients a substantial part of absorbed iron was not utilized for erythropoiesis and the amount was highly dependent on the degree of iron-overload. Furthermore, we showed that half-life of transferrin-bound iron in patients with non-transfusion-dependent hereditary anemia is comparable with iron deficient patients but approximately half that in hereditary hemochromatosis patients.

The intravenous test dose of transferrin-bound ^{59}Fe-labeled iron could rapidly interact directly with iron in the peripheral venous system. In our studies oral iron was administered as 7 mg ferrous ammonium sulphate (1 mg [59]Fe(II)), which enters the portal vein via ferroportin. Part of this ^{59}Fe will bind to apotransferrin in plasma, similar to the injected ^{59}Fe, and will be cleared mainly by erythroblasts and other cells expressing transferrin receptors. The remaining ^{59}Fe will bind to other molecules with formation of NTBI and labile plasma iron (LPI), resulting in iron deposition in the liver.

NTBI can be detected in patients with non-transfusion-dependent hereditary anemia and hereditary hemochromatosis with moderately increased transferrin saturation (TSAT) levels.[26-29] Short-lived amounts of NTBI can also be detected after supplemental ingestion of iron, even in patients with very low TSAT values.[30,31] Such values were obtained by venapuncture. Strikingly, we observed that in hereditary and secondary hemochromatosis the amount of retained iron of a ^{59}Fe radiolabeled oral test dose of only 1 mg Fe(II) that was utilized for RBC production was lower when compared to the utilization of injected ^{59}Fe radiolabeled transferrin-bound iron. This iron must have been deposited at a site with high iron storage capacity, most probably in hepatocytes.

We suggest that Fe(II) after entering the plasma in the portal circulation, after oxidation due to hephaestin in the basolateral membrane and plasma ceruloplasmin, will not only bind to free Fe(III) binding sites on transferrin but also to a variety of molecular species with affinity for iron (NTBI). In iron deficiency, with very low transferrin iron saturation, iron absorption will result in a negligible amount of NTBI, whereas in iron-overload, characterized by highly saturated plasma transferrin, iron absorption will result in significant amounts of NTBI in the portal circulation. This NTBI will be available as LPI.[26,32] LPI has a much lower affinity for Fe(III) than apotransferrin with a chemically labile character and a high propensity for redox reactions.[32-34]

The amount of iron entering the portal vein after iron absorption is much smaller than that released from catabolized hemoglobin in macrophages of the spleen and the liver, also into the portal vein. Additional iron transport capacity will be needed for temporary storage of iron in hepatocytes. There are two candidates that may serve as scavengers of iron in the portal system in situations where not enough free iron-binding sites on transferrin are available: citrate and albumin.[35] he plasma concentration of citrate is maintained at about 100–120 μM. Albumin exists in the circulation at an extremely high concentration (35–50 g/L plasma). Albumin has a large number of negative carboxylate sites on its surface, suitable for binding of Fe(III). Albumin is able to bind iron in the presence and in the absence of citrate, and can bind Fe(III) even when transferrin is not fully saturated.[36] Albumin can be considered as a safe iron transporter in plasma, next to transferrin, that is able to donate iron to cells that do not have transferrin receptors, and is able to transport iron into hepatocytes.

In mice, ZIP14, member of the Zrt- and Irt-protein (ZIP) family of metal-ion transporters,[35,37,38] efficiently transports NTBI into the intracellular compartment of hepatocytes.[39,40] In humans, ZIP14 is most abundantly expressed in the liver, and at lower levels in pancreas and heart.[20,39] Hepatic ZIP14 expression is upregulated in iron-loaded rats, which illustrates its regulation by iron itself.[20] Iron accumulation in hepatocytes was observed in a murine model for (juvenile) hemochromatosis resulting from *Hjv* knockout or *Hfe2* knockout with intact ZIP14 expression. However, mice with *Hfe* knockout or *Hjv* knockout and *Slc39a14* (ZIP14) knockout failed to accumulate iron in the hepatocytes.[41,42] Extraction of NTBI by mice liver in a single pass is extremely high (58–76%) and independent of the amount of iron present in the liver.[43-45] So, we hypothesize that the majority of NTBI produced in the portal system (from orally administered iron) is highly efficiently taken up in the liver during the first passage through this organ and that this extraction by hepatocytes depends on ZIP14.[35] Our data quantifies the extent of the hepatic first-pass NTBI extraction in human under various degrees of iron load mediated by ZIP14. (Figure 4)

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This hypothesis corresponds with differences in organ distribution of iron in transfusion-dependent and transfusion-independent iron-overload.[46-49] NTBI released from the reticulo-endothelial system of the spleen might be extracted by the liver, whereas NTBI generated or infused in the systemic circulation will lead to iron influx and accumulation in other organs.[50]
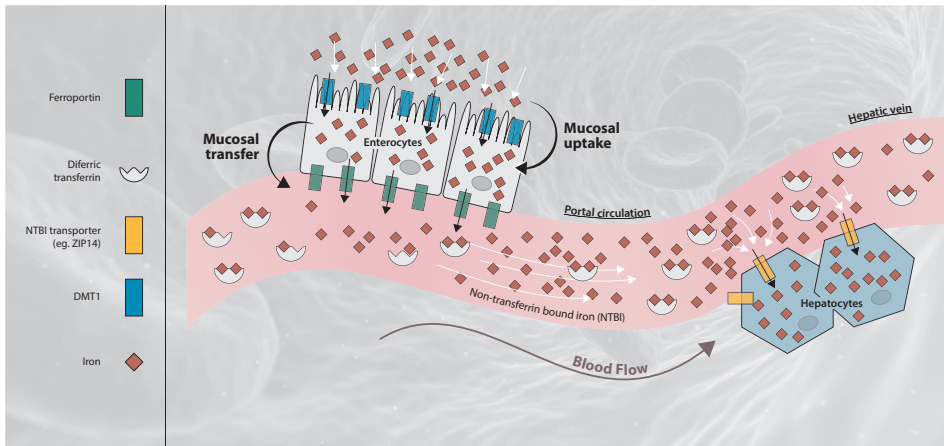

Figure 4. Continuous iron loading in primary or secondary hemochromatosis. Simplified visualization of iron absorption and first passage of the liver. Iron absorption in iron-overload results in generation of NTBI, including LPI. The majority of LPI produced in the portal system is taken up in the liver in the first-pass.

DMT1 divalent metal transporter 1; NTBI non-transferrin bound iron.

We observed that in non-transfusion-dependent hereditary anemia patients less iron from an oral or intravenous iron test dose was utilized for RBC production compared to hereditary hemochromatosis patients, independent from the extent of iron-overload. Iron utilization for red cell production ameliorated in hereditary spherocytosis patients after splenectomy. Presence of ineffective erythropoiesis or extravascular hemolysis results in less RBCs reaching or persisting in the circulation and consequently less iron in RBCs after 14 days. Recombinant transferrin injections in thalassemic mice improved iron availability for erythropoiesis, thereby underlining that despite systemic iron-overload the actual amount of iron available for erythropoiesis does not meet its demands.[51] So, in non-transfusion-dependent hereditary anemia patients increased RBC turnover and pathologic iron trafficking result in low utilization of iron, which is improved in spherocytosis patients after splenectomy.

We showed that anemia and hemolysis are important determinants of time to disappearance of iron from the circulation. In non-transfusion-dependent hereditary anemia and iron deficiency anemia half-life of iron was reduced to half of hereditary hemochromatosis

patients. Iron requirements of an active bone marrow are high. Iron acquisition in precursor RBCs depends on endocytosis of monoferric and diferric transferrin via TfR1.[52,53] (Relative) iron deficiency will result in expansion of erythroid progenitors and increases in the amount of TfR1 per cell.[54]

With exceptions, polymeric iron formulations have a low potential for LPI release; polymeric iron particles remain stable in the circulation until endocytosis and iron processing by macrophages.[55] Disappearance half-life of our test dose of transferrin-bound iron will not be comparable with disappearance half-life of polymeric iron formulations with high potential for LPI release, for example iron saccharate.[55,56] LPI in the circulation will load on transferrin, or enter endothelial cells in seconds,[20] bind to stabilizing anti-oxidant ligands, enter intravascular buffering cells.[57,58]

Determination of iron accumulation is crucial in diagnosing the occurrence and progression of many liver- and iron-related diseases. For interpretation of ferrokinetic data insight in the presence and abundancy of the various forms of iron in the circulation is of major importance. Distinct iron deposition profiles of liver zones in various models with iron homeostasis disorders were studied.[59] Uneven iron distribution was seen in livers of patients with hereditary hemochromatosis and in mice with hemochromatosis (*Hfe* knockout), showing the region with the highest iron concentration near the entrance site of the portal vein and hepatic artery. This is the pattern that can be expected in subjects who have a long term of increased liver iron retention due to iron influx through ferroportin that outnumbers the binding capacity of transferrin in the portal circulation.

In conclusion, insight in the composition of iron in the circulation (transferrin-bound, LPI/NTBI), pre-existing iron load and iron requirements are essential to explain human ferrokinetics. Our data supports that non-transfusion-dependent hereditary anemia patients have increased dietary iron absorption and decreased iron utilization despite fast uptake of transferrin-bound iron. We observed that all these characteristics are dramatically improved after splenectomy in patients with hereditary spherocytosis. Our data points towards the existence of significant hepatic scavenging of NTBI. We hypothesize on an important role for albumin as scavenger of NTBI, not only under iron-overloaded conditions, but as a safeguard for all conditions where the influx of Fe(II) via ferroportin outnumbers the iron binding capacity of plasma transferrin. We suggest that ZIP14 is required to extract NTBI during the first-pass of the liver.

9

MATERIALS AND METHODS

Test subjects
Iron absorption and ferrokinetic studies were performed from 1972 until 1994 as part of routine clinical practice in patients with iron-related health problems by the Iron Expert Clinic at the University Medical Center Utrecht, Utrecht, the Netherlands.

Patient selection
Patients were retrospectively selected based on diagnosis (non-transfusion-dependent hereditary anemia, hereditary hemochromatosis and iron deficiency). Patients receiving iron reducing treatment, either deferoxamine or phlebotomies, were analyzed as separate groups. There was no iron reducing therapy during the two-week periods of examination of iron absorption and ferrokinetics. If more than one iron absorption and ferrokinetic study was performed all analyses were included in the final database. In the hereditary hemochromatosis group one patient was included with three analyses, in the non-transfusion-dependent hereditary anemia group two patients were included with two analyses and in the non-transfusion-dependent hereditary anemia group with iron reducing treatment and iron deficiency group one patient was included with two analyses.

Iron deficient patients and healthy controls were included as reference groups. Patients with iron deficiency due to iron absorption disorders were excluded, defined as iron uptake less than 50% and clinical suspicion of iron uptake disorder. The group of healthy controls was earlier described by Marx.[60,61] Healthy controls did not receive the intravenous iron test dose.

Iron test doses
In all subjects, first iron absorption was studied using a whole-body counter (WBC) as described by Marx.[60-62] In brief, a test dose of 7 mg ferrous ammonium sulphate, containing 1mg Fe(II), labeled with 5 µCi ^{59}Fe and 10mg ascorbic acid (to prevent oxidation of iron in solution) was administered. The dose was ingested in the early morning after ten hours fasting; fasting was continued for two hours after ingestion. ^{51}Chromium was added (as CrCl$_3$, 40 µCi) to the oral test dose as non-absorbable indicator, for estimation of mucosal iron uptake. Red blood cell iron utilization (RBCIU) after the oral iron dose was measured 14 days after ingestion, after the last measurement of total body ^{59}Fe and ^{51}Cr.

The intravenous iron test dose was injected after overnight fasting. The dose contained approximately 10 µCi ^{59}Fe bound to 5 mL autologous serum. Autologous serum was incubated with ^{59}Fe for 30 minutes. Before injection, unbound iron was removed by the

method described by Cavill.[63] In short, an anion exchange column (Amberlite-IRA 400-cl) removed all unbound ferric-citrate. Therefore, all ^{59}Fe in the intravenous test dose was transferrin-bound iron. Before the injection of the intravenous test dose, background 59Fe in RBCs, due to RBCIU from the oral 59Fe administered at least 14 days earlier, was measured.

Measurement of radioactivity

Radioactivity was measured with a whole-body counter, first at the Physical Laboratory, National Institute of Public Health, Bilthoven, The Netherlands, later (after moving the facility) at the University Medical Center Utrecht, Utrecht, The Netherlands. ^{59}Fe activity was measured under the photopeak (1.00-1.40 MeV) and in the Compton area (0.40-1.00 MeV). ^{51}Cr activity was measured under the photopeak (0.28-0.36 MeV) after subtraction of the ^{59}Fe Compton effect. The first measurement was performed one hour after ingestion of the oral test dose; results were considered as 100%. ^{59}Fe radioactivity of peripheral blood samples was measured with an automatic gamma counter and compared to standard solutions containing a preset amount of ^{59}Fe. All measured values were corrected for background radiation and radioactive decay.

Iron absorption studies

The whole process of iron absorption includes mucosal iron uptake, mucosal transfer and retention. Absorption studies were performed according to the protocol described by Marx.[60]

Mucosal iron uptake (the percentage of iron taken up by the mucosal cells from the lumen of the gut) The mucosal iron uptake was calculated from the amount of ^{59}Fe and ^{51}Cr within the body 24 hours after the test dose. If there had been no defecation, counting was postponed for 24 hours. ^{59}Fe and ^{51}Cr measurements at 24 (or 48) hours were expressed as percentage of the amount administered.

Mucosal uptake (%) = 100 x (^{59}Fe - ^{51}Cr)/(100 - ^{51}Cr)

Iron retention (the percentage of iron present in the body 14 days after ingestion)

Iron retention was calculated from the amount of iron in the body 14 days after the test dose as determined with a whole-body counter. ^{59}Fe and ^{51}Cr measurements at 14 days were expressed as percentage of the amount administered.

Iron retention (%) = 100 x (^{59}Fe - ^{51}Cr)/(100 - ^{51}Cr)

Mucosal iron transfer (the fraction of iron taken up by the mucosal cells at day one, that is retained in the body).

Mucosal iron transfer (fraction) = Iron retention (%)/Mucosal iron uptake (%)

Ferrokinetic studies

Ferrokinetic methods used in the present study, based on early work by Finch and coworkers (1970), were described in detail before.[64,65]

Disappearance half-life of iron

The velocity of iron disappearance from the circulation was calculated after injection of intravenous iron, labeled with [59]Fe. Half-life of iron was calculated from blood samples drawn at 0, 5, 15, 30, 45, 60, 90 and 120 minutes after injection based on the slope of the natural logarithm of radioactive decay.

Iron incorporation in RBCs

The method for determining iron incorporation in RBCs was earlier described by Cook and others.[25,61,64,65] Incorporation of oral or intravenous administered iron was calculated from peripheral blood samples collected 14 days after iron administration. RBC iron utilization was expressed as percentage of the total amount of iron retained after an oral test dose or present after an intravenous test dose.

RBCIU (%) = (^{59}Fe in 1 ml blood x blood volume [mL])/total amount of ^{59}Fe in circulation x 100%

An oral test dose was given of 1 mg Fe(II) (as ferrous ammonium sulphate) labelled with 5 μCi [59]Fe. The ^{59}Fe-absorption was measured 14 days after ingestion of the test dose using a whole-body counter. The amount of ^{59}Fe that reached the blood was calculated from the amount of ^{59}Fe in the oral test dose and from the ^{59}Fe absorbed:

$$\text{[59]Fe dose} = \frac{\text{[59]Fe absorption (\%)}}{100} \times \text{[59]Fe administered orally (cpm)}$$

This ^{59}Fe dose is quantitatively comparable with an iron test dose injected directly into the portal vein. RBCIU after ^{59}Fe injection was calculated by:

$$\text{[59]Fe-RBCIU} = \frac{\text{[59]Fe in 1 ml blood (cpm) x blood volume (ml)}}{\text{[59]Fe dose (cpm)}} \times 100\%$$

Liver iron retention

Intestinal absorbed iron enters the portal circulation and passes the liver before reaching the systemic circulation. Intravenous iron is injected directly into the systemic circulation. Liver iron retention (LIR) was defined as the difference in RBC iron incorporation from an oral and from an intravenous test dose, expressed as percentage of the RBC iron incorporation from an intravenous test dose.

LIR (%) = (RBCIU intravenous – RBCIU oral)/RBCIU intravenous x 100%

Laboratory assays

Red cell indices, hemolytic severity parameters and iron indices were measured with commercial assays used in clinical practice by the University Medical Centre Utrecht on the morning of ingestion of the oral iron test dose.

Statistical analysis

Differences in means between different patient groups were tested with a one-way ANOVA, in case of significant differences a Tukey's HSD test was performed testing all possible pairwise comparisons. Correlation analyses were reported using Pearson's correlation coefficient. In order to correct for potential bias bootstrapping was performed to confirm significance.[66] Statistical significance was set at a two-sided $p < 0.05$. All calculations were performed with IBM SPSS Statistics v. 25.

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CHAPTER 10

Proton pump inhibition for secondary hemochromatosis in hereditary anemia, a phase III placebo-controlled randomized cross-over clinical trial

A.J. van Vuren[1], J.L. Kerkhoffs[2], S.E.M. Schols[3], A.W. Rijneveld[4], E. Nur[5,6], D.E.W. Peereboom[7], Y. Gandon[8], P.M.J. Welsing[9], R. van Wijk[10], R.E.G. Schutgens[1], W.W. van Solinge[10], J.J.M. Marx[11], T. Leiner[12,13], B.J. Biemond[5], E.J. van Beers[1].

[1]   Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[2]   Department of Hematology, HagaZiekenhuis, Den Haag, The Netherlands;
[3]   Department of Hematology, Radboud university medical center, Nijmegen, The Netherlands;
[4]   Department of Hematology, Erasmus Medical Center Cancer Institute, Rotterdam, the Netherlands;
[5]   Department of Hematology, Amsterdam University Centers, University of Amsterdam, Amsterdam, The Netherlands;
[6]   Department of Blood Cell Research, Sanquin Research, Amsterdam, The Netherlands;
[7]   Contactgroep Pyruvaatkinasedeficiëntie, Stichting Zeldzame Bloedziekten, Medemblik, The Netherlands;
[8]   Department of Radiology, CHU Rennes, University of Rennes, Rennes, France;
[9]   Division of Internal Medicine and Dermatology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[10]   Central Diagnostic Laboratory - Research, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;
[11]   University Medical Center Utrecht, Utrecht, The Netherlands;
[12]   Department of Radiology, Mayo Clinic, Rochester, MN, United States;
[13]   Department of Radiology, Utrecht University Medical Center, Utrecht, The Netherlands.

ABSTRACT

Importance: Iron overload is a severe general complication of hereditary anemias. Treatment with iron chelators is hampered by important side-effects, high costs and the lack of availability in many countries with high prevalence of hereditary anemias.

Objective: To investigate the safety and efficacy of esomeprazole in treatment of iron overload in patients with non-transfusion-dependent hereditary anemias.

Design: Phase III randomized placebo-controlled cross-over trial.

Setting: Enrollment from March 2018 through April 2019 in five centers of expertise in the Netherlands.

Participants: Thirty adult patients with mild-to-moderate iron overload were enrolled (non-transfusion-dependent thalassemia (N=13), pyruvate kinase deficiency (N=8), congenital sideroblastic anemia (N=3), congenital dyserythropoietic anemia (N=3), sickle cell disease (N=2), and hereditary elliptocytosis (N=1)).

Intervention Esomeprazole 40mg twice daily and placebo for 12 months in a cross-over design.

Main outcome and measure: Change of liver iron content measured by MRI.

Results: Treatment with esomeprazole resulted in a statistically significant reduction in liver iron content that was 0.55mg Fe/g dw larger than after treatment with placebo (95% CI [0.05, 1.06]; p = 0.03). Baseline liver iron content at start of esomeprazole was 4.99mg Fe/g dw (IQR 3.47, 7.21), versus 4.49mg Fe/g dw (IQR 2.96, 6.35) at start of placebo. Mean delta liver iron content after esomeprazole treatment was -0.57mg Fe/g dw (SD 1.20; N=24) versus -0.11 (SD 0.75; N=23) after placebo treatment. Changes in serum ferritin values were non-significant between treatment arms.

Esomeprazole was well tolerated, reported adverse events were mild and none of the patients withdrew from the study due to side effects.

Conclusions and relevance: Esomeprazole 40mg twice daily resulted in a significant reduction in liver iron content when compared to placebo in a heterogeneous group of patients with non-transfusion-dependent hereditary anemias with mild-to-moderate iron overload. From an international perspective this result can have major implications given the fact that proton pump inhibitors may frequently be the only realistic therapy for many patients without access to or not tolerating iron chelators. Longer follow-up is needed to see whether this effect will last for a longer period.

Trial Registration: Netherlands Trial Register NL6659 – PPI in secondary hemochromatosis (www.trialregister.nl).

KEY POINTS

Question: What is the efficacy of proton pump inhibitors in reducing iron overload in patients with non-transfusion-dependent hereditary anemias?

Findings: In this phase III randomized placebo-controlled clinical cross-over trial that included 30 adult patients with various hereditary anemias, one-year esomeprazole treatment resulted in a significantly larger decrease in liver iron content of 0.55mg Fe/g dw when compared to placebo treatment.

Meaning: Proton pump inhibitors are effective in reducing iron overload in patients with non-transfusion-dependent hereditary anemias. Proton pump inhibitors show promise as an effective complementary treatment strategy for iron overload.

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INTRODUCTION

Iron overload is a serious complication in patients with transfusion-dependent congenital anemias.[1] However, iron overload was also detected in 65% of a cohort of previously non-transfused patients with hereditary hemolytic anemia,[2] due to inappropriately low hepcidin levels resulting in excessive intestinal iron absorption in response to ineffective or stress erythropoiesis.[3-5] Upon erythropoietin stimulation, differentiating erythroblasts rapidly increase erythroferrone production, which downregulates hepcidin transcription.[5-9] The resulting elevated levels of total body iron lead to clearance of non-transferrin-bound iron by the liver,[10-13] culminating in iron overload and related pathology.[14,15]

In patients with non-transfusion-dependent thalassemia a liver iron content (LIC) threshold of 3-5mg Fe/g dry weight (dw) was associated with higher prevalence of iron-related morbidity, including vasculopathy, endocrine disturbances and osseous complications.[16] In non-transfusion-dependent thalassemia LIC-values increase approximately 0.5mg Fe/g dw per year already resulting in clinically relevant iron overload in adolescents.[17] Recommendations for monitoring and treatment of iron overload are incorporated in all current guidelines for management of hemoglobinopathies,[18-20] and other anemias.[e.g.21-24] Iron chelation is currently the only treatment modality of iron overload in anemic patients. Treatment-related toxicity (e.g. gastro-intestinal complaints and renal toxicity) is a concern in daily clinical practice, and treatment itself is expensive.

Dietary non-heme iron typically consists of ferric iron which needs to be reduced into ferrous iron for absorption. Ferrireductases present on enterocytes require a proton gradient, additionally protons are needed to solubilize dietary iron salts. Subsequently, proton pump inhibitors (PPIs) might inhibit both processes.[25,26] We hypothesize that treatment with PPIs reduces iron absorption and may limit iron-loading in patients with non-transfusion dependent hereditary anemia. A hint of such an effect was already provided by the Pyruvate Kinase Deficiency Natural History Study.[27,28] Iron overload was better managed in a subgroup with an iron-restricted diet, PPIs and calcium citrate.[27-29] Moreover, PPIs have shown to be effective in reduction of phlebotomy requirements in patients with hereditary hemochromatosis.[30,31]

We investigated the safety and efficacy of PPIs in prevention and treatment of iron overload in non-transfusion-dependent hereditary anemias.

METHODS

Trial design

The PPI Shine Again trial was a phase III double-blind, placebo-controlled, cross-over, multicenter trial designed to assess the efficacy and safety of esomeprazole in the treatment of mild-to-moderate iron overload in patients with hereditary anemia. The study was conducted at five clinical centers in the Netherlands, all were appointed as centers of expertise in hereditary anemia. The study included a one-year inclusion period, and was, upon enrollment, followed by two double-blind treatment phases in a cross-over design of 52 weeks each.

Randomization and study procedures

Patients were randomly assigned to start with one of the two treatments. The randomization sequence was generated with blocks of four. The random allocation sequence was generated by the drug manufacturer and corresponded with the provided sequentially numbered bottles. Patients were allocated by means of a telephone call to the blinded coordinating investigator. Treatment assignment was stratified for use of iron chelation therapy. The participants, care givers and those assessing outcomes were unaware of the group assignments.

Three-monthly laboratory assessments and yearly MRI of liver according to current standard-of-care were implemented in the study design. Extra study laboratory assessments included six-monthly gastrin levels and baseline hepcidin level (measured batch-wise after study close-out). Every three-monthly study visit included review of medication, check of compliance, report of side-effects and airway infections. Iron intake was assessed yearly with the IRONIC-FFQ questionnaire.[32] The full trial protocol is available in the *Supplemental Digital Content.*

Participants

Eligible participants were adult patients (≥ 18 years old), with a confirmed diagnosis of hereditary anemia (hemoglobinopathy, sideroblastic anemia, congenital dyserythropoietic anemia or erythrocyte enzyme deficiency), and had clinically stable mild-to-moderate iron overload defined as a baseline LIC between 3-15mg Fe/g without iron chelation therapy 6 months prior to entering the study, or with documented stable dosage of iron chelation the preceding two months and no expected dose alterations, as no dose alterations were allowed during the trial. Patients who were undergoing regular red-cell transfusion therapy were not eligible. During the trial participants were excluded if they had received > 4 units of blood during one study period. All patients provided written informed consent before trial enrollment.

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Trial intervention

Participants received esomeprazole 40mg and placebo capsules bidaily for 12 months in the randomized order, and were instructed to take one capsule 15-60 minutes before breakfast and dinner. Site investigators were allowed to end treatment for patients experiencing severe side effects. Temporarily cessation of the study drug, or exclusion from the study, was considered in cases of co-administration of medication with potential drug interactions. Drug interactions lowering plasma levels of esomeprazole were not considered as exclusion criterion.

Measurement

A combined MRI of liver and heart was performed at yearly intervals according to current clinical practice. In brief, the protocol consisted of dedicated liver and electrocardiographically triggered cardiac T2*-weighted sequences to assess liver and myocardial iron concentrations. All images were acquired on the same commercially available 1.5 Tesla MR imager using a predefined and previously validated imaging protocol. A detailed description of the MRI protocol, including a thorough description of the R2* method is available in the review paper by Henninger *et al.*[33] and on https://imagemed.univ-rennes1.fr/. Upon completion of the acquisition, images were sent to a dedicated workstation for further analysis using the MRQuantif web application. MRQuantif is a dedicated software package that can be used to quantify LIC from T2* MRI acquisitions.

End points

Primary end point was the difference in change of LIC measured by MRI between esomeprazole and placebo treatment. The LIC was determined by two independent observers (YG/AV). Inter observer variability assessment was calculated by the intraclass correlation coefficient (ICC).[34] Secondary efficacy assessment included changes in serum ferritin values. Safety assessments included incidence of adverse events and abnormal clinical laboratory tests (serum vitamin B12, magnesium and zinc levels). Reported adverse events were graded according to the CTCAE v4.03 criteria.

Trial oversight

The trial was centrally approved by the ethics committee of the University Medical Center Utrecht (Utrecht, NL) and approved by the Board of Directors of the participating centers. The trial was conducted according to Good Clinical Practice guidelines, defined by the International Conference of Harmonisation. All participating centers signed confidentiality agreements with the sponsor regarding the data.

Statistical analysis

We estimated that twenty patients would need to be enrolled to have approximately 80% power (β=0.20) to detect a difference in change in LIC between treatments of 2.0mg Fe/g dw, conform a previous trial with PPIs conducted in patients with hereditary hemochromatosis.[31] In the estimation a yearly expected increase in LIC without intervention of approximately 0.4mg Fe/g dw was incorporated. Estimation of the standard deviation for the sample size calculation was based on the standard deviations reported by Taher *et al.*[17,35] Dropout was estimated to be relatively high due to unscheduled blood transfusions, surgery with blood loss or other complications. Inclusion of a minimum of eight additional patients was proposed to compensate for dropout and lack of compliance.

The main analysis consisted of an intention-to-treat analysis and per protocol analysis. In the per protocol analysis patients who failed to receive their allocated treatment, non-compliant patients or patients who dropped out of the study before completing both treatment periods were excluded. Non-compliance for the placebo period was defined as missing more than 20% of dosages based on pill counts or serum gastrin level within the reference range or less than 50% increase compared to baseline for the esomeprazole period.[36]

For the primary efficacy analysis a linear mixed model was used with change in LIC as dependent variable, a random intercept at patient level and treatment as independent variable. Sex, iron chelator use, (period) baseline LIC and randomized order of treatment were included as covariates. Continuous secondary outcomes were analyzed using the same approach. For safety parameters (serum vitamin B12, magnesium and zinc values) randomized order of treatment and iron chelator use were included as independent variables in the model. Calculations were performed using IBM SPSS Statistics version 26.0.0.1 (SPSS Inc., Chicago, IL).

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RESULTS

Baseline characteristics of the patients

From March 2018 through April 2019, 30 patients were enrolled. (Figure 1) Four treatment periods were excluded based on transfusion requirements (more than four units in one treatment period). The intention-to-treat population included all participants despite those excluded based on set transfusion threshold. A total of six patients dropped out of the study at patient request of whom three completed the first treatment. Data assembled until dropout were included in the intention-to-treat analysis. Baseline characteristics of the randomized population are provided in Table 1. The ICC representing reliability of the LIC-values was 0.996 (95% confidence interval [CI] [0.994, 0.997]). After central re-assessment at the end of the study baseline LIC-values were computed to be < 3mg Fe/g dw in five patients, > 3-7mg Fe/g dw in 18 patients and > 7-15mg Fe/g dw in 7 patients

Primary endpoint

In the intention-to-treat analysis, we observed a significant effect of esomeprazole treatment: the reduction in LIC (delta LIC) was significantly greater after 1-year esomeprazole than after 1-year placebo (mean difference in LIC reduction 0.55mg Fe/g dw; 95% CI [0.05, 1.06]; p = 0.03). (Table 2, Figure 2) Median baseline LIC-value in the esomeprazole period was 4.99mg Fe/g dw (IQR 3.47, 7.21) and in the placebo period 4.49mg Fe/g dw (IQR 2.96, 6.35). Mean LIC reduction in the esomeprazole phase was 0.57mg Fe/g dw (SD 1.20), compared to a reduction of 0.11mg Fe/g dw (SD 0.75) in the placebo phase.

In 20 of the 47 completed treatment periods (43%) pill counts and/or gastrin values indicated therapy non-adherence. Per protocol analysis was performed in the subgroup with adequate adherence (N = 26 treatment periods). Results were in line with the intention-to-treat analysis with a mean difference in reduction of LIC of 0.51mg Fe/g dw (95% CI [0.00, 1.03]; p = 0.05).

A pre-planned modified efficacy analysis including baseline hepcidin/ferritin ratio provided proof for a relevant influence on treatment efficacy. Subsequently, patients were divided in two groups (hepcidin/ferritin ratio above group median, or below group median of 0.021). In patients with a low hepcidin/ferritin ratio treatment with esomeprazole resulted in a significant reduction in LIC of 1.30mg Fe/g dw (95% CI [0.54, 2.06]; p = 0.003) larger than placebo. After esomeprazole there was a mean reduction of LIC of 1.20mg Fe/g dw (SD 1.32) versus an increase of 0.08mg Fe/g dw (SD 0.76) after placebo. In patients with a high hepcidin/ferritin ratio esomeprazole led to a nonsignificant increase in LIC of 0.21mg Fe/g dw (95% CI [-0.39, 0.81]; p = 0.47) greater than placebo. After esomeprazole treatment there was a mean increase in LIC of 0.06mg Fe/g dw (SD 0.63) versus a reduction of 0.28mg Fe/g dw (SD 0.73) after placebo treatment.
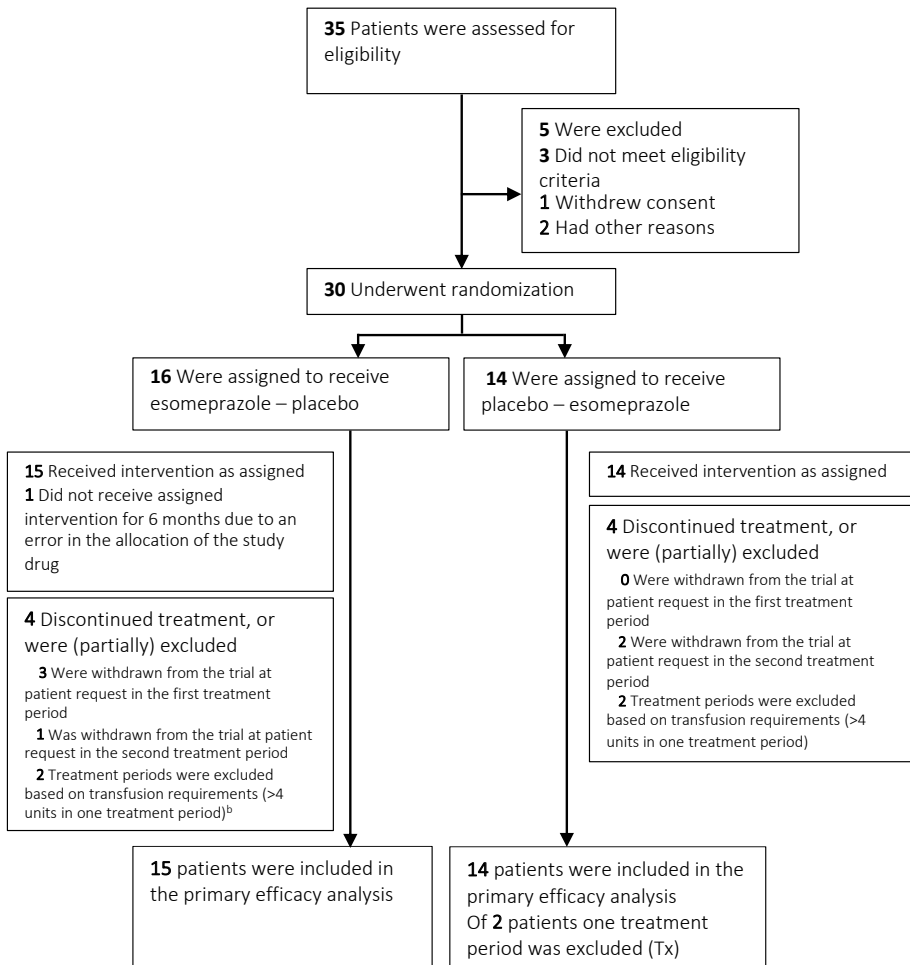
Figure 1. Screening, randomization, and follow-up.

Shown is the disposition of the trial participants. The intention-to-treat population compromised 30 patients who underwent randomization, to receive either esomeprazole followed by placebo, or placebo followed by esomeprazole.

[a]Patients were excluded from the per protocol analysis.

[b]The two treatment periods referred to were the two treatment phases of one patient.

Tx based on transfusion requirements.

Table 1. Baseline demographic and disease characteristics.

Characteristic	Esomeprazole-placebo (N=16)	Placebo-esomeprazole (N=14)
Median age (range) – yr	47 (19, 66)	35 (23, 59)
Female sex – no. (%)	9 (56)	6 (43)
Median body mass index (range)	22.1 (17.8, 28.4)	21.5 (17.4, 30.0)
Diagnosis		
CSA	2	1
CDA	0	3
HE	1	0
NTDT	8	5
PKD	5	3
SCD	0	2
History of splenectomy (%)	5 (31)	2 (14)
History of cholecystectomy (%)	8 (50)	2 (14)
Iron chelation therapy (%)	6 (38)	5 (36)
Relevant other medicaments (%)		
Folic acid	11	10
Bisphosphonate or other therapy osteoporosis	2	2
ACE-inhibitor	0	1
Relevant co-morbidities		
Diabetes	1	0
Hypertension	1	0
Osteoporosis	2	0
Median number of blood transfusions in preceding 12 months (range)	0 (0, 9)	0 (0, 5)
Median iron intake		
Heme iron – mg per day	2.3 (1.8, 3.4)	1.4 (0.7, 2.8)
Non-heme iron – mg per day	5.2 (4.0, 8.5)	6.2 (4.9, 8.9)
Median number of phlebotomies in preceding 12 months (range)	0	0
Median markers of iron metabolism (IQR)		
Serum ferritin – ug/L	483 (302, 705)	603 (346, 807)
Serum transferrin saturation – %	59 (26, 81)	48 (31, 74)
Plasma hepcidin – ug/L	7.4 (4.2, 20.4)	11.3 (4.2, 17.7)
Median hemoglobin value (IQR) – g/dL	9.2 (7.9, 10.4)	9.7 (8.7, 10.2)
Median values of safety parameters (IQR)*		
Vitamin B12 – pmol/L	282 (199, 409)	275 (203, 500)
Magnesium – mmol/L	0.82 (0.81, 0.89)	0.83 (0.79, 0.88)
Median baseline LIC (IQR) – mg Fe/g dry liver weight	4.83 (3.13, 5.40)	5.44 (4.49, 8.37)

Data on all baseline demographics and disease characteristics are shown for all patients who underwent randomization. Characteristics are tabulated against treatment allocation.

* Baseline zinc values are not presented in this table, as reference values (and assays) differed among participating centers. See *results section* for delta zinc values along the trial.

ACE angiotensin converting enzyme; CDA congenital dyserythropoietic anemia; CSA congenital sideroblastic anemia; DFX deferasirox; HE hereditary elliptocytosis; IQR interquartile range; LIC liver iron content; NTDT non-transfusion-dependent thalassemia; PKD pyruvate kinase deficiency; SCD sickle cell disease.

Table 2. Change in levels of liver iron content and iron parameters.

	Esomeprazole Mean (SD)		Placebo Mean (SD)		Effect esomeprazole* Estimate (95% confidence interval)
Primary endpoint					
Δ LIC – mg Fe/g dw	N = 24	-0.57 (1.20)	N = 23	-0.11 (0.75)	-0.55 (-1.06, -0.05)
Secondary endpoints					
Δ Ferritin – ug/L	N = 20	-18 (170)	N = 22	18 (135)	-23 (-121, 76)
Δ Transferrin saturation – %	N = 22	-1.1 (18.4)	N = 23	6.7 (15.9)	-7.7% (-18.8, 3.5)

* Effect estimate of esomeprazole as calculated by linear mixed model analysis with random intercept and treatment as independent variable. Sex, iron chelator use, baseline LIC and order were included as covariates.

Δ delta; LIC liver iron content; SD standard deviation.
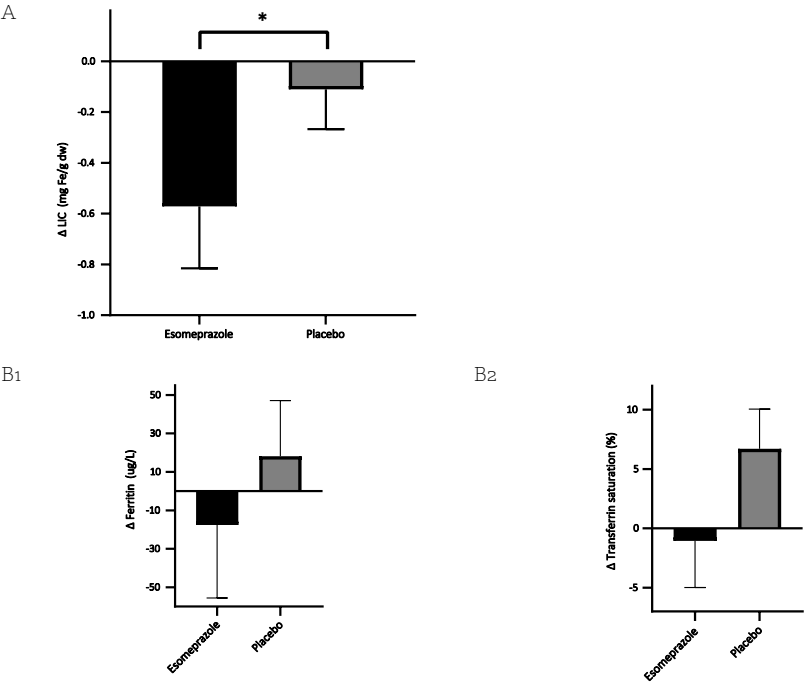
Figure 2. Change in levels of liver iron content and iron parameters.

(A) The change of LIC from baseline to end-of-treatment period for esomeprazole and placebo. Presented values are mean values of those patients included in the intention-to-treat analysis. Error bars represent the standard error of the mean. (B) Mean changes in respectively serum ferritin and transferrin saturation values. The asterix (*) indicates significance of the difference in delta LIC between esomeprazole and placebo assessed by linear mixed model analysis with sex, iron chelator use, baseline LIC and randomized order as covariates.

Iron intake was quantified with the IRONIC-FFQ questionnaire at baseline and after each treatment period. Changes in iron (heme or non-heme) intake were negligible over time (resp. p = 0.81, and p = 0.84). Four patients received red cell transfusions in the esomeprazole period and 2 patients in the placebo period.

Key secondary end points

The change of serum ferritin was included as secondary end point; ad-hoc analysis of change in serum transferrin saturation was performed. (Table 2, Figure 2) The difference in change in serum ferritin and transferrin saturation was nonsignificant when comparing esomeprazole and placebo (respectively -23 ug/L; 95% CI [-121, 76]; p = 0.65, and -7.7%; 95% CI [-18.8, 3.5%]; p = 0.17). (Table 2, Figure 2) Baseline ferritin levels and transferrin saturation levels were respectively 470ug/L (IQR 306, 615) and 78% (IQR 39, 84) in the esomeprazole period and 455ug/L (IQR 288; 694) and 54% (IQR 34, 81) in the placebo period. After esomeprazole treatment there was a decrease of 18ug/L (SD 170) in serum ferritin, and a decrease of -1.1% (SD 18.4) in serum transferrin saturation. After placebo treatment there was an increase in serum ferritin of 18ug/L (SD 135) and an increase of 6.7% (SD 15.9) in serum transferrin saturation.

Safety

Treatment periods of all patients exposed to esomeprazole or placebo were included in the safety analysis (N = 56). Most frequently reported adverse events were gastro-intestinal complaints, in particular in the esomeprazole-treated population. Adverse events occurring in over 10% of patients or graded grade 3 or higher are presented in Table 3. In one patient mild gastro-intestinal complaints (grade 2) led to dose reduction of the study medicament (one capsule once daily), none of the patients discontinued trial participation due to adverse events. The majority of adverse events were graded 1 or 2.

Fourteen serious adverse events occurred, all concerning hospitalization due to various reasons. All serious adverse events were judged by the investigators to be unrelated to esomeprazole or placebo. None of the participants discontinued treatment, and no dose reductions were required.

Respiratory tract infections were reported separately. Three patients were prescribed antibiotics for respiratory tract infections in the esomeprazole period and 2 patients in the placebo period.

None of the participants was prescribed vitamin B12, magnesium or zinc supplementation during trial participation as a consequence of decreasing serum levels during the trial. Delta vitamin B12, magnesium or zinc values did not differ significantly between treatments.

Table 3. Adverse events occurring in at least 10% of patients or graded grade 3 or higher.

Event – no. (%)	**Esomeprazole** (N = 30)	**Placebo** (N = 26)
General disorder or administration-site condition		
Malaise	3 (10)	1 (4)
Fatigue	1 (3)	2 (8)
Gastro-intestinal disorder		
Nausea	1 (3)	3 (12)
Gastric pain or pyrosis	1 (3)	2 (8)
Diarrhea	6[b] (20)	2 (8)
Abdominal pain[a]	3 (10)	0 (0)
Infection or infestation		
Upper respiratory tract infection[c]	17	23
Lower respiratory tract infection[a,c]	3	4
Prosthetic valve endocarditis[a]	1 (3)	1 (4)
Sepsis eci[a]	0 (0)	1 (4)
Cholecystitis[a]	1 (3)	0 (0)
Flue (nos)	2 (7)	1 (4)
(Cardio)vascular disorder		
Vaso-occlusive crisis[a,c]	1	7
Rectus hematoma[a]	1 (3)	0 (0)
Kidney failure	0 (0)	1[b] (4)
Musculoskeletal or connective-tissue disorder		
Backpain	1[b] (3)	0 (0)

Three grade 3 adverse events were reported (rapid decline in kidney function [placebo]; diarrhea in a patient diagnosed with colitis ulcerosa [esomeprazole], and severe backpain [esomeprazole]).

[a] At least one serious adverse event occurred (hospitalization).

[b] One episode was graded grade 3 or higher.

[c] Reported (number) is number of episodes.

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DISCUSSION

In this phase III trial involving patients with non-transfusion-dependent anemias complicated by mild-to-severe iron overload esomeprazole 40mg twice daily resulted in a significantly larger reduction in liver iron load of 0.55mg Fe/g dw as compared to placebo after one year of treatment. We consider this difference clinically relevant given the average iron loading of 0.5mg Fe/g dw in patients with non-transfusion-dependent thalassemia as observed previously.[17] Low individual hepcidin/ferritin ratio was an important predictor for treatment efficacy. Moreover, we established the safety of esomeprazole treatment for this specific indication, esomeprazole was associated with mainly low-grade adverse events.

The cross-over design of our study enabled us to include a heterogeneous group of anemias, which can be considered both a strength and a weakness of the trial. The underlying mechanism of iron loading is similar among these diseases, despite major differences in pathophysiology. Unfortunately, the low number of patients included per disease was too small to allow separate (sensitivity) subgroup analyses. Potency to accumulate iron in the absence of transfusions in SCD is subject of debate, however there is a subgroup of SCD patients with extremely low hepcidin/ferritin ratios who may be at risk of iron overload via this route.[37]

We chose to dose esomeprazole twice daily in order to attain 24-hour gastric acid suppression. Pharmacokinetic data of esomeprazole showed that 40mg once daily attained pH ≥ 4 in 92% and 56% of patients for a minimal duration of respectively 12 and 16 hours per day.[38] It is not known yet whether once daily dosing may also result in a relevant effect, given the fact that food is predominantly ingested during daytime. We strongly advice to address this question in future studies, as therapy adherence was poor for a twice daily regimen.

Five patients met the inclusion criteria (MRI conform standard care LIC ≥ 3mg Fe/g dw), but during the blinded centralized reassessment at the end of the trial LIC-values appeared to be slightly lower. This may have had an effect on the average decrease in LIC, as previous trials with deferasirox reported larger decreases in LIC in patients with higher baseline LIC.[17,35,39] It would be recommendable to specifically study the (added) efficacy of PPI therapy (to iron chelators) in patients with more severe iron load (LIC ≥ 7mg Fe/g dw), a group that was underrepresented in our trial.

In many countries PPIs are currently readily available as over-the-counter drugs, also in low-resource countries. Adverse events are infrequent and generally mild. However, there is a growing concern regarding rare potentially severe side effects including (severe) enteric infections, cardiovascular diseases, pneumonia, hypomagnesemia, acute interstitial nephritis, vitamin B_{12}-deficiency, dementia and osteoporosis, resulting from chronic use of PPIs. For most of these

side effects it is uncertain if the association is based on causality as their incidences are mainly based on observational studies.[40,41] Likewise, long-term use of PPIs was associated with increased risk of gastric cancer.[42,43] Contrasting, extended PPI-use for indications that were not previously related to gastric cancer development PPI-use over 5 years was associated with a decreased risk.[43] Awaiting prospective trials that investigate the absolute risks of long-term PPI treatment on severe complications, these associations should not withhold consideration to use this treatment in patients with a clear indication.

Deferasirox is currently the treatment-of-choice in patients with iron overload. The THALASSA trial reported a LIC reduction of 1.95mg Fe/g dw in the 5mg/kg group after one year from a median baseline LIC-value of 11.7mg Fe/g dw. LIC reductions were larger in patients with higher baseline LIC-values.[17] Similarly, the THETIS trial reported the smallest LIC reduction (1.82mg Fe/g dw) in patients with LIC 5 to ≤ 7mg Fe/g dw with a mean dose of 8.95mg/kg.[39] Compared with these results, deferasirox is likely to be more potent than esomeprazole in reduction of LIC in mild-to-moderate iron overload. However, this does not preclude implementation of PPI-therapy in treatment schedules for iron overload in patients with non-transfusion-dependent hereditary anemias, particularly in those patients with low hepcidin/ferritin ratios. Accounting its favorable safety profile and low costs, PPIs may be considered as an interesting therapeutic option to treat but also to prevent progression of iron overload and thereby the need for treatment with iron chelators in patients with mild iron overload (LIC ≥ 3-5mg Fe/g dw). In case of more severe iron overload (LIC ≥ 5mg Fe/g dw) we suggest to consider addition of PPI-therapy to iron chelators. From an international perspective this positive result may have major implications, as in certain areas of the world the prevalence of hereditary anemia is much higher,[44] but availability of iron chelators much lower. In these areas, PPIs may be the only realistic treatment option for many patients.

In conclusion, high-dose one-year esomeprazole treatment induced a significantly larger reduction in LIC when compared to placebo in patients with mild-to-moderate iron overload due to non-transfusion-dependent hereditary anemias. Longer follow-up is needed to see whether this effect will last for a longer period.

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Summary, discussion
and future perspectives

CHAPTER 11

Summary, discussion
and future perspectives

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Major improvements especially in supportive care for patients with (transfusion-dependent) hereditary anemias color the last decades. The following spectacular increase in life expectancy of these patients shifts the attention of treatment guidelines towards monitoring and treatment of long-term disease complications. This shift in thinking is not limited to transfusion-dependent patients; also milder phenotypic presentations of hereditary anemias harbor risk for long-term complications. The mostly feared, and most thoroughly studied, complication is iron overload. MRI monitoring of heart and liver iron load and chelation therapy have entered current treatment guidelines and 'how I treats' about hereditary anemias.[1-7] In high-income countries these protocols are generally applied in clinical practice.

The fragile balance between iron and erythropoiesis in patients with hereditary anemias is the main topic of this thesis. The history of iron research can nowadays be divided in two eras, parted by the 1990s which have been entitled in later years as the renaissance of iron physiology.[8,9] The first studies on iron physiology stem from as early as the 1930s. In the next decades studies using intestinally absorbed radioiron provided insight in the regulation of iron absorption, the lack of excretion mechanisms and the ways of body iron recycling (e.g. thesis of J.J.M. Marx, Absorption of iron in the aged, 1976; Utrecht).[9,10] In the 1990s studies focusing on patients and animal models with iron disorders led to the identification of genes that encoded iron transporters, the required iron reductases to facilitate iron transport, and iron regulatory molecules.[8] It was not until 2000 that the master regulator of systemic iron homeostasis was described, hepcidin.[11-13] Soon afterwards, researchers showed that indeed this was the long-sought iron-regulatory hormone.[14,15] The discovery of hepcidin led to the description of the so-called iron-loading anemias.[16,17] In later work, in an attempt to understand the exact mechanisms of hepcidin regulation by iron and erythroid activity, erythroferrone (ERFE)[18-20] and growth differentiation factor 15 (GDF15)[21,22] were discovered.

This chapter is too short to remember and honor all major contributors to the unraveling of iron biology. I am proud that with this thesis I will stand in the long tradition of both iron and red cell research in Utrecht. With this thesis we continue iron research in Utrecht in the modern era, this time narrowing the focus towards iron and hereditary anemias.

The backbone of this thesis was to study disease-specific and individual risks of iron overload in hereditary anemias. However, the role of iron was deeper entangled in the pathophysiological aspects of hereditary anemias, thereby adding the aim to describe connections between erythropoiesis, iron and other pathological pathways to the first

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ambition. And finally, an attempt to improve the understanding of the unique features of each anemia, and of each individual, that determines clinical phenotype. This final chapter will provide a summary of the research conducted during my PhD trajectory, and a discussion on the value of the work for this growing and blooming research field.

SUMMARY
PART 1: NEW CROSSROADS.

Chapter 2 focusses on the ununderstood pathophysiological aspects of a single hereditary hemolytic anemia: pyruvate kinase deficiency (PKD). The disease is caused by compound heterozygous or homozygous loss of function mutations in the *PKLR* gene encoding for liver and red blood cell specific pyruvate kinase (PK-R). One remarkable feature of PKD is that post-splenectomy reticulocyte counts tend to be extremely high (up to 70-80%), thereby sharply contrasting the decreased reticulocyte counts post-splenectomy in other hemolytic anemias.[23-25] In this perspective we worked out a model for disturbed red cell maturation in PKD that suggests that the main problem in PKD may not be the PK-deficient erythrocyte, but for it to reach the mature red cell stage. In brief, young PK-deficient reticulocytes fully depend on their reduced PK-R activity to provide the cell with pyruvate to fuel the tricarboxylic acid (TCA) cycle. In order to compensate for the absence of sufficient amounts of pyruvate, glutaminolysis will be the major (additional) source of anaplerotic carbon entrance. As cytosolic glutamine is preferentially used to fuel the TCA cycle, there will be shortage of glutamine for synthesis of glutathione (GSH) and, subsequently, bioavailability of glutathione peroxidase 4 (GPX4) will be impaired. GSH functions as reducing substrate for GPX4, an essential enzyme to protect the cell against oxidative damage.[26]

Reticulocytes, in the presence of heme and iron, are continuously subjected to auto-oxidation, including lipid peroxidation.[27-29] Excessive auto-oxidation, in the presence of a relative GPX4 deficiency, will perturb mitophagy. And, as this is the final step of reticulocyte maturation, this will lead to a failure to fulfill reticulocyte maturation. The latter can be defined as late-stage ineffective erythropoiesis.[30,31] Splenectomy will result in a slower rate of removal of non-maturing reticulocytes in the liver, thereby extending longevity of these immature reticulocytes, which provides an explanation for the profound reticulocytosis in PKD patients after splenectomy.

In **chapter 3** and **4** we explore the relevance of a pathway not previously linked to the pathophysiology of hereditary anemias: the erythropoietin (EPO)-fibroblast growth factor 23 (FGF23) signaling pathway. FGF23 was originally known as a bone-derived hormone, a key player in phosphate and vitamin D metabolism and regulator of bone mineralization. It was shown by others that bone marrow, specifically the early erythroid lineage, does significantly contribute to total circulating FGF23.[32-34] Erythroid progenitor cells do express *FGF23* mRNA under physiologic conditions, and significant increases are observed in response to EPO.[32,33,35] FGF23 is formed as intact, biologically active protein (iFGF23). Proteolytic cleavage results in formation of an assumed inactive C-terminal tail FGF23 (cFGF23). By competitive inhibition, increases in cFGF23 relative to iFGF23 lead to suppression of FGF

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receptor signaling.[36,37] *Fgf23*-knockouts or animals treated with iFGF23-blocking peptides show increased erythropoiesis, reduced erythroid cell apoptosis, and increased *EPO* mRNA and EPO levels.[35,37,38] EPO lowers the i:cFGF23 ratio, thereby overcoming iFGF23-mediated suppression of erythropoiesis.[32,33,35,39-41] Moreover, iron deficiency and acute inflammation also decrease the i:cFGF23 ratio.[42-45] Chapter 3 reviews the importance of the EPO-FGF23 signaling pathways in erythroid cell development and bone mineralization. The information about the amount of FGF23 in hereditary anemia was, at that moment, limited to two studies: one study in a mouse-model for β-thalassemia and one study in sickle cell disease (SCD) patients.[41,46] In this review we speculate on a role of the EPO-FGF23 axis in the pathophysiology of hereditary anemias, in particular the bone mineralization disorders complicating these diseases. In chapter 4 we report the results of the first study that shows a strong connection between EPO and FGF23 signaling in a broad range of hereditary hemolytic anemias, a correlation that remains after adjustment for iron load, inflammation and kidney function. ERFE was included in the study as well, and, as suspected, a close relation between EPO and ERFE was identified. Data argued against a role for ERFE as intermediary in EPO-FGF23 signaling. By establishing a correlation between FGF23 and markers of endothelial dysfunction, we uncovered preliminary evidence for a link between FGF23 and vasculopathic complications (e.g. pulmonary hypertension) in hereditary anemias.

PART 2: THE CHALLENGE CALLED HETEROGENEITY.

Chapter 5 and 6 highlight the unique features of SCD as opposed to the other hereditary hemolytic anemias. In SCD a significant part of hemolysis takes place in the (micro) circulation, whereas other hereditary hemolytic anemias (e.g. PKD, or hereditary spherocytosis) are characterized almost exclusively by extravascular hemolysis, and thus patients are not subjected to large amounts of intravascular cell-free heme. Both cellular free hemoglobin and heme, devoid of anti-oxidant buffering mechanisms normally present in red cells, are recognized as erythroid damage-associated molecular pattern molecules (e-DAMPs),[47-49] and contribute to the development of vasculopathy and platelet activation in SCD.[50-56] Heme-oxygenase 1 mediates heme detoxification, which will lead to carbon monoxide (CO) production. All heme, either metabolized via extravascular hemolysis, as well as cleared from the plasma via scavenger proteins will contribute to endogenous CO production. In contrast to heme, CO has many protective effects as it inhibits polymerization of sickle hemoglobin, and serves as anti-oxidative and anti-inflammatory agent.[57]

It was previously shown that lactate dehydrogenase (LDH) is associated with intravascular heme release. In chapter 5 the results of a study investigating the value of the ratio of LDH to carboxyhemoglobin (HbCO; the transport form of CO) as predictive biomarker are presented. We here show that a LDH/HbCO ratio over 1,200 identifies SCD patients with a high probability to develop pulmonary hypertension and at high risk of early death. Although validation is needed, we denominate the ratio of LDH to HbCO, representative of the intravascular fraction of the total hemolytic rate, as a promising and readily clinically available biomarker in SCD to improve individual risk prediction. Chapter 6 focusses on the capableness of free heme to modulate Toll like receptor 4 (TLR4) signaling in SCD. The aim of this study, in which monocyte transcriptome data of SCD patients were related to those of patients with other hereditary (extravascular) hemolytic anemias, was to identify the main activated pathways in monocytes in response to intravascular hemolysis. Indeed, we identified a characteristic gene expression pattern in SCD patients. Not unexpected, a clear upregulation of *HMOX1* (encoding heme-oxygenase 1) was noted, in line with the current hypothesis that intravascular free heme is an important effector of SCD pathophysiology and gene regulation. Noteworthy are the roles of lipid metabolism and interferon signaling as differentiating pro-immune pathways.

More clinically orientated is **chapter 7** describing a unique, large cohort of hereditary spherocytosis patients. The study could be regarded as our attempt with the growing list of mostly unique spherocytosis mutations to settle the discussion on the existence of a genotype-phenotype correlation in hereditary spherocytosis. The report adds 56 novel mutations to the currently known spherocytosis mutations. Our conclusion is dissatisfying, in a sense that establishing a genotype-phenotype correlation in spherocytosis is highly complex due to the complexity of the interactions in the red cell cytoskeleton. A challenging combination of the pathogenic mutation, amount and quality of (mutant) protein, and effects of truncated protein or its absence on cytoskeleton interactions determines disease severity. Furthermore, modifying genetic and even non-genetic factors seem to influence phenotypic variability.

Summarizing, part 2 shows that there are major pathophysiologic differences between hereditary anemias, as well as between patients diagnosed with one disease entity. The search for ways to understand and define this heterogeneity has just started.

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PART 3: ABOUT IRON.

All chapters of part 3 are centered around one subject: iron-loading. The iron regulatory axis, the EPO-ERFE-hepcidin axis, is the theme of **chapter 8**. A detailed description of the components of the iron regulatory axis is provided in the *General introduction*. A collaboration with Denmark (Rigshospitalet, Copenhagen University Hospital, Denmark) resulted in a large cohort of patients with various hereditary anemias characterized by distinct amounts of hemolysis and ineffective erythropoiesis. Despite heterogeneity of our cohort, including patients with varying disease burden and iron load, we here report the presence of a strong correlation of the individual components of the EPO-ERFE-hepcidin pathway. Notably, significant inter-disease and inter-individual variability in the activity and balance of the pathway existed. Assessment of the EPO-ERFE-hepcidin axis, including GDF15 and soluble transferrin receptor (sTFR), may function as a tool to estimate an individuals' size of the erythron (EPO, ERFE and sTFR; e.g. in response to hemolysis or ineffective erythropoiesis), effectiveness of erythropoiesis (GDF15, reticulocyte count and hemoglobin), and risk of iron-loading (hepcidin).

Chapter 9 offers an interesting step back to the first generation of iron research. Old data are explained with current knowledge about iron physiology. This historic set of not previously published data of combined radiolabeled iron absorption and iron utilization studies was collected in patients diagnosed with iron deficiency, and primary or secondary hemochromatosis due to hereditary anemia. This combination of studies provides unique insights in iron transport directly after entrance of iron out of the enterocytes into the portal venous system and its uptake into hepatocytes during the first passage through the liver. Interestingly, in patients with non-transfusion-dependent hereditary anemias and in patients with hereditary hemochromatosis a substantial part of absorbed iron was not utilized for erythropoiesis and this amount was highly dependent on the degree of iron overload. This points towards the existence of significant hepatic scavenging of nontransferrin-bound iron (NTBI), and we suggest an important role for ZIP14 in this process.[58]

And then, the finale. **Chapter 10** describes the results of the *PPI shine again* study, a phase III randomized, placebo-controlled trial in a cross-over design investigating the efficacy of proton pump inhibitors in the prevention and treatment of iron-loading in patients with non-transfusion-dependent hereditary anemias. Current recommended treatment with iron chelators is potentially toxic and expensive. Dietary non-heme iron typically consists of ferric iron. Enterocytes take up ferrous iron from the intestinal lumen. Dietary ferric iron is reduced to ferrous iron by ferrireductases, a process that requires a proton gradient. Protons are also needed to solubilize dietary iron salts. Proton pump inhibitors will generate a relatively basic environment, thereby inhibiting both processes.[59,60] Theoretically, blockade

of absorption will stop the process of iron-loading in non-transfusion-dependent patients with hereditary anemias. A heterogeneous group of thirty patients diagnosed with hereditary anemia complicated by mild-to-moderate iron overload was enrolled in the trial. The primary study outcome was change in liver iron content (LIC) measured by 1.5 Tesla MRI. It was shown that one-year treatment with esomeprazole 40 mg bidaily resulted in a LIC reduction that was 0.55 mg Fe/g dry weight larger when compared to placebo, a statistically significant finding as well as a clinically relevant finding. A low individual hepcidin/ferritin ratio was an important predictor for treatment efficacy, which is in line with the hypothesis that proton pump inhibitors inhibit intestinal iron-loading. The safety profile of esomeprazole, in line with previous data, was judged to be highly favorable. Chapter 10 includes suggestions for implementation of proton pump inhibitors in treatment strategies for iron overload. Proton pump inhibitors could be a possible candidate to prevent or postpone development of more severe iron overload and thereby the need for treatment with iron chelators in patients with mild iron overload (LIC ≥ 3-5mg Fe/g dw), particularly in those patients with low hepcidin/ferritin ratios. In case of more severe iron overload (LIC ≥ 5mg Fe/g dw) we suggest to consider addition of proton pump inhibitor therapy to iron chelation therapy. From an international perspective this positive result may have major implications, as in certain areas of the world the prevalence of hereditary anemia is much higher, but availability of expensive iron chelators much lower. Proton pump inhibitors may be the only realistic treatment option for those without access to iron chelating agents, or those not tolerating treatment with iron chelators.

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DISCUSSION AND FUTURE PERSPECTIVES

In this paragraph I will discuss two main topics. First, I will recite on the complexity of hereditary anemias, followed by a discussion on the (future) role of iron in red cell research.

The complexity of hereditary anemias

I was hoping to improve the apprehension of the unique disease entities that pertain to the heterogeneous and complex group of hereditary anemias. This hope demands understanding of common and, especially, distinguishing characteristics of diseases and individuals. I tried to translate insights gained in this thesis into a more pragmatic approach to characterize an individual with hereditary anemia. (*section I*) Thereby leaving behind the historic classification system of hereditary anemias (see *General introduction*). This classical division seems to overpass the strong intercommunication between red cell components in all consecutive maturity states that guarantee normal red cell physiology, and thereby the consequences of a single pathogenic mutation for the whole red blood cell (and its environment). The latter we are just beginning to understand. (*section II*)

I. Nowadays diagnostics shift towards upfront genetic testing with targeted hereditary anemia panels.[61] This approach makes it plausible to name one's diagnosis by the mutated gene (e.g. *PKLR*-mutated hereditary anemia). The presence of profound inter-individual variation in disease severity observed in patients with identical (gene-)diagnoses, when considering the mutation (e.g. SCD) or the gene (e.g. PKD), arguments for the addition of a notation that delineates one's clinical disease severity.[62-65] It is dissatisfying to realize that this approach illustrates our own limitation to assemble, as a first step, and interpret all data required to fully understand the consequences of a genetic mutation. An example is provided in *chapter 7* in which we discuss genotype-phenotype correlations in hereditary spherocytosis: it is needless to say that there is a genotype-phenotype correlation as already suggested years ago,[66] but we still fail to predict the consequences of a specific (unique) disease-causing mutation for the amount and quality of protein encoded by the mutated gene and its effect on the interactions in the cytoskeleton, and hence the remaining stability of the red cell membrane in this specific patient.

In the *General introduction* it was described how improvements to restore the anemia led to the current ineluctable shift of attention towards long-term disease complications. This transition calls for early identification of those patients with high risk for long-term disease complications as vasculopathy and iron overload. Information pivotal to estimate one's risks includes degree (and site) of hemolysis and effectiveness of erythropoiesis, and therefore inclusion of this information in the (descriptive) diagnosis is recommendable. This thesis suggests two (future) tools that may help to assemble the required information.

- The LDH/HbCO ratio. In *chapter 5* we illustrated the detrimental consequences of considerable amounts of intravascular hemolysis, which results in the production of free heme and other e-DAMPs. SCD can roughly be divided into two sub-phenotypes with considerable overlap: one side of this gliding scale characterized by chronic intravascular hemolysis, and the other side by vaso-occlusive disease. Both sides of the spectrum are accompanied by their own complications.[67-69] We showed that each SCD patient has its own amount of intravascular hemolysis during steady-state disease relatively stable over time, and which did not depend on the total amount of hemolysis present. This relative contribution of intravascular hemolysis (LDH) to total hemolysis (HbCO; and thereby the amount of the protective gasotransmitter CO produced) is essential, as the balance determines vasculotoxicity. The LDH/HbCO ratio is clearly associated with increased risk of pulmonary hypertension, the main vasculopathic complication of SCD, and all-cause mortality. Incorporation of the LDH/HbCO ratio in clinical practice, may provide a readily available tool to estimate the relative amount of intravascular hemolysis in SCD patients. Furthermore, free heme and iron species are also detected (at lower rates) in other transfusion-dependent hereditary hemolytic anemias, including the more classical extravascular hemolytic diseases as hereditary spherocytosis, and related to vascular dysfunction and immune cell activation.[70] Splenectomy, nowadays still treatment-of-choice in various hemolytic anemias, accelerated intravascular hemolysis and thereby aggravated vascular dysfunction and inflammation.[70,71] Therefore our theory, and the tool (LDH/HbCO ratio), may extent to all hereditary anemias. It is needless to say that this biomarker first needs validation in other patient groups.
- The EPO-ERFE-hepcidin pathway. Knowledge on the activity of one's EPO-ERFE-hepcidin axis provides a tool to estimate an individual's (compensating) size of the erythron in response to hemolysis or ineffective erythropoiesis, which is directly translated into one's risk of iron-loading. *Chapter 8* describes the existence of global disease-specific patterns of the EPO-ERFE-hepcidin pathway including GDF15 and sTFR. Still, a broad inter-individual variation in the set-point of the pathway exists despite identical diagnoses. Assessment of the pathway provides the clinician with a frame to identify patients with severe disease and high-risk of iron-loading, e.g. those with a large erythron (high EPO, ERFE and sTFR), high GDF15 (high degree of ineffectiveness of erythropoiesis), low hemoglobin levels (low output or shortened survival of peripheral erythroid cells), and low hepcidin (high intestinal iron absorption).

Assessing the setpoint of one's pathway at diagnosis provides valuable information on one's specific disease-related risks. Thereby, reassessment is recommendable to investigate how the EPO-ERFE-hepcidin pathway responds to (new) treatments (e.g. fetal hemoglobin inducers in β-thalassemia, or recombinant EPO in X-linked sideroblastic anemia), and, subsequently, how this therapy alters one's risks for (e.g.) iron overload.

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In conclusion, I call for this new approach that provides a meaningful individual naming of diagnosis and include one's disease severity and risk for disease complications (e.g. *ANK1*-mutated hereditary anemia, moderate phenotype, mainly extravascular hemolysis and risk of iron loading). Implementation in clinical practice calls for the availability of, and clinical experience with, hepcidin and ERFE assays, which will hopefully enter clinical practice over the next couple of years, and for further validation of the LDH/HbCO ratio. Despite these restrainers, this concept, the attempt to individualize one's diagnosis and long-term risk prediction, should be a main focus point in our clinical practice.

II. Until recently, the identification of the genes harboring pathogenic mutations in the specific diseases seemed the final step in the understanding of the hereditary anemias. On the contrary: it was at the start of unraveling the pathways linking the truncated or absent protein to hemolysis or ineffective erythropoiesis. One example is our attempt to understand how reduced PK-activity results in failure of reticulocyte maturation. The disease PKD was first described in 1961.[72] PKD is a rare recessive hereditary hemolytic anemia caused by mutations in the *PKLR* gene, discovered in 1988.[73] And still, its pathophysiology is incompletely understood. Our hypothesis is controversial as we suggest the presence of late-stage ineffective erythropoiesis (at the level of the reticulocyte) being the prominent pathophysiological mechanism instead of hemolysis. It turned out that the understanding of newer pathophysiological concepts as ferroptosis, an iron-dependent process of regulated cell death identified in neuron degenerative diseases and various cancers, was required for the understanding of the biochemical interactions underlying this rare red cell disease.[27,74] Disruption of oxidative balance, central in our theory on the pathophysiology of PKD, is not a new concept in the red cell field.[75] As carriers of large amounts of iron (heme) and operating in highly oxygenated tissues, red cells are continuously exposed to limited amounts of superoxide and hydrogen peroxide. To provide protection against reactive oxygen species (ROS) (developing) red cells are equipped with a variety of reductants. So, handling the combination of iron and heme could be regarded as the main challenge of (developing) red cells, consequently I suspect that a mild to severe disruption of the oxidative balance will have a role in the pathophysiology of all hereditary red cell diseases extending beyond SCD and β-thalassemia.[76,77] In the (extravascular) hemolytic disorders this may be of particular importance as (additional) oxidative stress will lead to membrane loss and impaired rheological properties and therefore may accelerate splenic red cell destruction. Overall, this opens a very exciting new field of research with an unperceived number of therapeutic possibilities which we will discuss at the end of this chapter.

Until now I narrowed down the scope of this section to the red cell, however this passes over the gradually unraveling network that connects the erythron, or red cells, with so many other cell types, or even organs. This thesis provides two of such examples. In *chapter 3*

and *4* we discuss the EPO-FGF23 pathway. We report on the intercommunication of EPO and FGF23 in hereditary anemias, still we can only speculate on its consequences on bone marrow functioning and bone metabolism and for example endothelial integrity. The importance of the communication of (components, or signals of) red cells with white cells is illustrated in *chapter 6* where we shift the attention from the sickle cell towards the TLR4-positive cells (monocytes) that play an important role in acute and chronic SCD complications.[51,52,78] Monocyte transcriptomics helped to identify the balance between pro- and anti-inflammatory pathways that seemed to determine one's degree of inflammation. Presence of other factors, as the availability of labile iron (or its absence in the presence of iron chelators) may reprogram this balance. Overall, the (fragile) balance of the complex communication network in and extending from the red cells provides endless opportunities for future research, and each step in each pathway will contribute to the understanding of hereditary anemias.

And the complexity of iron regulation

I started this chapter with a brief overview of the history of iron research. Finally, the connection between iron and erythropoiesis is understood. These days, clinicians are getting familiar with the concept of iron-loading anemias. We are close to the implementation of validated and standardized hepcidin assays in clinical practice,[79] and we expect ERFE assays to follow shortly.[80] The long-sought finesse of iron regulation becomes general knowledge of our medicine students. Hence, the work is done. Does this mean that iron research in hereditary anemias is completed, that we simply know everything? A rhetorical question, sharply contrasting the title of this thesis. Again, we now realize that there is so much more to investigate. However, I expect the scope of iron research to change from the pathways that deliver iron for erythropoiesis (including the concept of iron-loading) towards the influence of the oxidative capacity of iron in (developing) red cells and its contribution in the (disrupted) fragile redox balance (e.g. PKD), or even its effects on other cell types once free heme escapes the redox-regulating mechanisms of the red cell upon hemolysis (e.g. SCD). One pressing research question I would like to put forward is the following: is there a role for ferroptosis in the erythron in hereditary anemias characterized by inefficient erythropoiesis, or even dyserythropoiesis?

Furthermore, we should be prepared for a role of iron in the network of connecting pathways, of which the EPO-FGF23 pathway is just one example. Iron overload was reported to decrease renal expression of α-klotho, the essential cofactor for FGF23 signaling;[81] and iron deficiency is known to decrease the i:cFGF23 ratio.[42,44,45,82] Unfortunately, our dataset (*chapter 4*) was not sufficient to report on the presence or absence of a connection between iron overload and FGF23 signaling. Summarizing, I assume that with this switch in focus iron research in hereditary anemias will bloom over the next years.

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Future therapeutic perspectives

We perform research for our patients with hereditary anemias. So, how does this thesis contribute to the patients' needs? For many years supportive therapy was all we had to offer: we offered red cell transfusions when needed, and extracted iron in return. But with the advances in understanding the individual red cell diseases opportunities arise to modify disease-specific features. One clear example is the application of mitapivat, a small-molecule allosteric activator of PK, which was shown to induce a clinically relevant hemoglobin increase and improvement in hemolytic parameters in PKD patients.[83] I will here discuss four other potential drugs or druggable targets that could be considered for treatment based on the work presented in this thesis.

I. *L-glutamine, vitamin E and N-acetylcysteine in PKD.* In line with our hypothesis on the pathophysiology of PKD, as discussed in *chapter 2*, we suggest three readily available therapies that theoretically may contribute to the correction of the disrupted redox balance underlying the stop in reticulocyte maturation. Supplementation of the amino acid L-glutamine may possess the capacity to correct for the increased fuel demand of the TCA cycle. Additional supply of L-glutamine may restore GSH production, which may accordingly restore excessive auto-oxidation and disruption of mitophagy. Vitamin E interposes in lipid membranes, and acts as a chain breaking antioxidant of lipid peroxidation through its high affinity for unpaired electrons and thereby antagonizes peroxide production. Vitamin E synergizes with GPX4 in antagonizing the action of lipid hydroxyperoxides, and as a consequence may limit perturbation of mitophagy. Repletion of vitamin E deficiency seemed to partly correct lipid peroxidation in the presence of GPX4 deficiency, the role of supra-normal vitamin E doses remains to be explored.[31] N-acetylcysteine is a biosynthetic precursor to GSH. Its supplementation has previously shown to prevent cell death induced by GSH-inhibiting agents.[84] Trials in β-thalassemia and SCD provided prove for its capacity to replenish the amount of GSH and accordingly reduce oxidative stress.[85-87] So, N-acetylcysteine may theoretically correct the GSH deficit in PKD and thereby restore the pathway towards reticulocyte mitophagy. I underline that these drug suggestions are pure theoretical as (pre-)clinical data with one of these drugs in PKD is not yet available.

II. *Statins in SCD patients with a hyperinflammatory phenotype.* In *chapter 6* we discussed the role of cholesterol metabolism, in particular the mevalonate pathway, in the pro-inflammatory response in SCD. HMG-CoA-reductase inhibitors (known as statins) prevent conversion of HMG-CoA into mevalonate, and thereby could be an effective therapy for hyperinflammatory disorders.[88] Concerning the role of the mevalonate pathway in SCD, statins could be an effective therapy by (partly) dampening the hyperinflammatory response in SCD. Previous trials, in humans and mice, seem to

support this hypothesis.[89-93] Statins have a favorable safety profile and its benefits in development of cardiovascular diseases is clear.[e.g.94,95] I therefore suggest that clinicians may consider the prescription of statins in SCD patients, when no contraindications are present, especially in those with a hyper-hemolytic phenotype.

III. *FGF23-pathway modifiers in anemias with ineffective erythropoiesis.* The effects of iFGF23 signaling have been studied by direct infusion of recombinant iFGF23,[35] and by blockage of iFGF23 signaling by knockout,[38] or recombinant cFGF23 injection.[37] *FGF23*-knockouts or animals treated with iFGF23-blocking peptides show increased erythropoiesis, reduced erythroid cell apoptosis, and increased *EPO* mRNA and EPO levels. *In vitro* administration of iFGF23 to *FGF23*-knockout bone marrow–derived erythropoietic cells normalizes erythropoiesis, and administration of cFGF23 in chronic kidney disease mice decreases erythroid cell apoptosis and increases serum EPO levels. Taking into account these effects on erythropoiesis, an interesting question is whether EPO-FGF23 pathway modification forms a potential therapeutic approach to augment (stress) erythropoiesis, or to improve effectivity of erythropoiesis, or even vasculopathic or bone-related disease complications, (partly) bypassing the higher up (side-)effects of hypoxia-inducible factor, EPO and ERFE (and so the connection towards iron-loading). These therapies are not readily available, and more insight in the role of FGF23 signaling in various hereditary anemias is needed.

IV. *Proton pump inhibitors in patients with non-transfusion-dependent hereditary anemias complicated by iron overload.* In *chapter 10* we described the efficacy and safety of proton pump inhibitors in the prevention and treatment of iron overload in patients with non-transfusion-dependent hereditary anemia and mild-to-moderate iron overload. Where iron chelators try to eliminate iron that is present in the body, and which may already have caused damage, proton pump inhibitors inhibit iron absorption. In my opinion the latter is clearly the preferred scenario. Based on our findings we recommend to consider prescription of proton pump inhibitors in mild iron overload to prevent or slow progression towards more severe iron overload. Hepcidin/ferritin ratios could help to select those individuals who benefit most from proton pump inhibitor therapy. This treatment strategy may prevent or postpone treatment with (expensive) iron chelating agents. Furthermore, we suggest to consider addition of proton pump inhibitor therapy to iron chelation therapy. Add-on therapy could reduce dose requirements of iron chelating agents. This concept has been tested in a relatively short-term phase III clinical trial; longer follow-up is needed to see whether this effect will last for a longer period.

Personal future perspectives for red cell research in the University Medical Center Utrecht
A large part of this chapter discusses uncertainties, gaps in knowledge and ununderstood complicating processes. Consequently, this paragraph is not sufficient, and the University Medical Center Utrecht too small, to cover all future research. We, as red cell researchers, face the challenge to reach a detailed understanding of all pathophysiological mechanisms,

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all pathways that intersect with erythropoiesis, red blood cell physiology and red cell clearance in the broad range of hereditary anemias. And despite advances, that is still a lot of work. Achievements in red cell research are built upon a close collaboration between hematologists specialized in red cell disorders and a specialized red cell research laboratory, in the University Medical Center Utrecht represented by the Van Creveldkliniek and the Red Blood Cell Research Group of the department Central Diagnostic Laboratory – Research.

At the end of my PhD trajectory we have worked on two (future) projects, that may be among the next streps of red blood cell research in the University Medical Center Utrecht: the RHiNO registry and biobank, and the DOdO study.

1. The RHiNO study combines a registry and biobank in order to assemble information and blood of all patients diagnosed with hereditary anemias who are treated at the Van Creveldkliniek. Blood samples are taken and data is assembled at steady-state disease, and before and after interventions (e.g. supplements, medicaments, surgery). This will provide red cell researchers with the unique opportunity to study these rare diseases by assembling data on (natural) disease course and collecting samples to study pathophysiologic processes and alterations of red cell biology in response to interventions. The RHiNO may harbor a wealth of data and material to boost red cell research in the near future.

2. The DOdO study is an ambitious project to prove the existence of late-stage ineffective erythropoiesis in PKD, of which the hypothesis is outlined in *chapter 2*. The main objectives of the project are to: characterize the reticulocyte population of PKD patients in terms of metabolic functioning, maturation and ageing, investigate *ex vivo* PK-deficient reticulocyte maturation and establish the influence of PK-activators and redox-modifying agents on the maturation process, and investigate the mechanisms of splenic clearance of PK-deficient reticulocytes.

Concluding remarks

This thesis is an unfinished story. Still, I did not finalize this project discouraged, on the contrary. These years taught me that overseeing and accepting the complexity of hereditary anemias creates many possibilities for future research. With the opportunity to individualize risk prediction of disease-related complications and individualize treatment choices. I hope that this thesis may be a small step in this direction.

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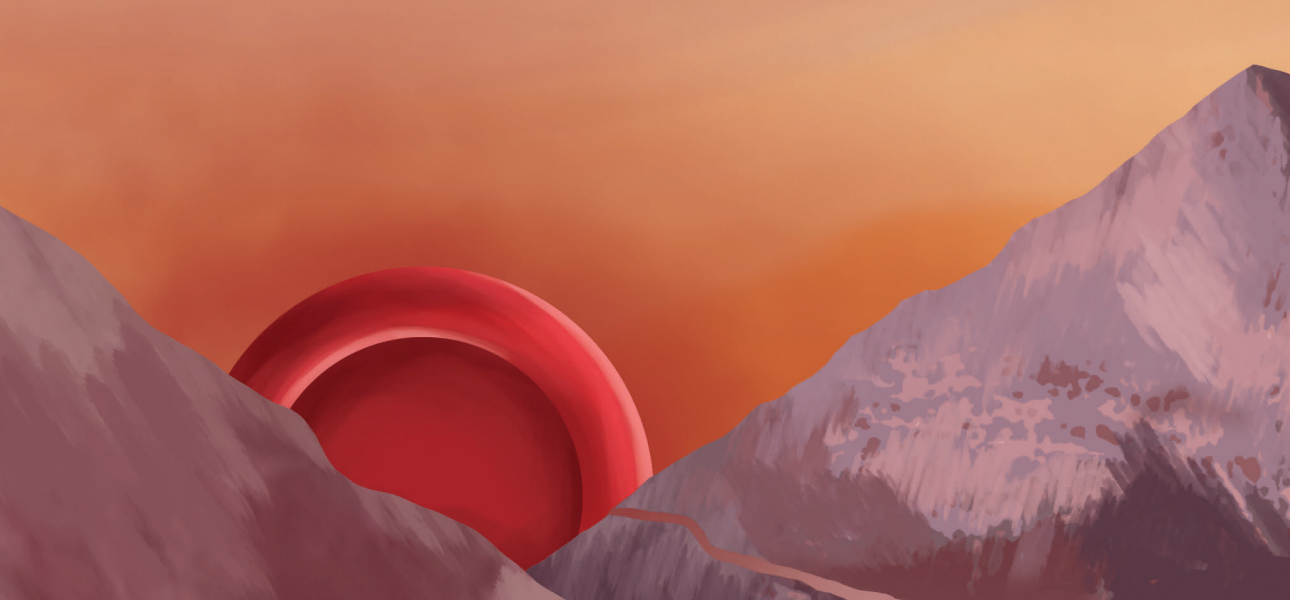
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Appendices

APPENDIX

Nederlandse samenvatting
(Dutch summary)

NEDERLANDSE SAMENVATTING

Dit hoofdstuk bevat een Nederlandse samenvatting van het werk verricht tijdens mijn PhD traject. Voor de (bijbehorende) discussie verwijs ik u naar hoofdstuk 10 (Chapter 10: *Summary, discussion and future perspectives)*. Enkele termen laten zich moeizaam vertalen in de Nederlandse taal, ik heb ervoor gekozen in deze gevallen de Engelse bewoording te gebruiken.

DEEL 1: NIEUW SAMENSPEL.

Hoofdstuk 2 is een hypothese-vormend stuk dat verhaalt over de onbegrepen pathofysiologische aspecten van één enkele erfelijke hemolytische anemie: pyruvaat kinase deficiëntie (PKD). PKD is het gevolg van een compound heterozygote of homozygote loss-of-function mutatie in het *PKLR*-gen dat codeert voor lever en rode bloedcel specifiek pyruvaat kinase (PK-R). Een bijzonder aspect van deze ziekte is dat na een splenectomie reticulocyten aantallen extreem hoog zijn (tot 70-80%), dit in tegenstelling tot de reticulocyten aantallen bij andere hemolytische anemieën postsplenectomie.[1-3] In dit hoofdstuk beschrijven we een model uitgaande van een verstoorde rode cel maturatie in PKD, dat suggereert dat het hoofdprobleem in PKD niet zozeer de PK-deficiënte erythrocyt is, maar het bereiken van dit mature rode bloedcel stadium. In het kort: jonge PK-deficiënte reticulocyten zijn volledig afhankelijk van hun beperkte PK-R activiteit om de cel te voorzien van pyruvaat voor de citroenzuurcyclus (tricarbonzuurcyclus; TCA). In PKD, als noodgreep om te compenseren voor de afwezigheid van voldoende pyruvaat, zal glutaminolyse de belangrijkste (additionele) bron zijn van anaplerose. Als cytosolair glutamine preferentieel wordt gebruikt voor de TCA-cyclus, zal er een tekort zijn aan glutamine voor de synthese van gluthatione (GSH) en, dientengevolge, een verminderde beschikbaarheid van glutathione peroxidase 4 (GPX4). GSH is een reducerend substraat voor GPX4, en GPX4 is een essentieel enzym om de cel te beschermen tegen oxidatieve schade.[4]

Reticulocyten worden door de aanwezigheid van heem en ijzer continu blootgesteld aan auto-oxidatie.[5-7] Excessieve auto-oxidatie, indien beperkt GPX4 aanwezig is, zal het proces van mitofagie verstoren. En, aangezien mitofagie de laatste stap vormt in reticulocyten maturatie, zal dit resulteren in een onvermogen van de rode cel tot het voltooien van maturatie. Dit vastlopen van de maturatie is te definiëren als late ineffectieve erythropoiese.[8,9] Splenectomie zal het gevolg hebben dat de niet-maturerende reticulocyten langzamer worden verwijderd uit de circulatie door de lever, wat de levensduur van de immature reticulocyten verlengt. Dit biedt een verklaring voor de uitgesproken reticulocytose in PKD-patiënten na splenectomie.

In **hoofdstuk 3** en **4** onderzoeken we de relevantie van een signaalroute die niet eerder is gelinkt aan de pathofysiologie van erfelijke anemieën: de erythropoietine (EPO)-fibroblast growth factor 23 (FGF23) signaleringscascade. FGF23 is oorspronkelijk ontdekt als een hormoon van de botten, een centrale speler in het fosfaat en vitamine D metabolisme, en regulator van bot mineralisatie. Eerdere studies hebben laten zien dat beenmerg, in het specifiek de vroege erythroïde lijn, een significante bijdrage heeft in de productie van het totale circulerende FGF23.[10-12] Erythroïde voorlopercellen brengen *FGF23* mRNA tot expressie onder fysiologische omstandigheden, en belangrijke toename in expressie wordt gezien in respons op EPO.[10,11,13] FGF23 wordt gevormd als een intact, biologisch actief eiwit (iFGF23). Proteolyse resulteert in formatie van een vermeend inactief deel van FGF23 afkomstig van de C-terminus (cFGF23). Door competitieve inhibitie leidt een relatieve toename van cFGF23 ten opzichte van iFGF23 tot onderdrukking van FGF receptor signalering.[14,15] *Fgf23*-knockouts of dieren behandeld met iFGF23-blokkende eiwitten laten een toename zien van de erythropoiese, verminderde erythroïde cel apoptose en toegenomen *EPO* mRNA en EPO expressie.[13,15,16] EPO verlaagt de i:cFGF23 ratio, en herstelt daarmee (deels) de iFGF23-gemedieerde suppressie van de erythropoiese.[10,11,13,17-19] Ook ijzer deficiëntie en acute inflammatie verlagen de i:cFGF23 ratio.[20-23] Hoofdstuk 3 reviewt het belang van de EPO-FGF23 signaalroute in de ontwikkeling van de rode cel en botmineralisatie. Er was op dat moment slechts zeer beperkt informatie beschikbaar over de hoeveelheid FGF23 in erfelijke anemieën: één studie in β-thalassemie muizen en één studie in patiënten met sikkelcelziekte (SCD).[19,24] De review eindigt met een speculatie over de rol van de EPO-FGF23 as in de pathofysiologie van erfelijke anemieën, in het bijzonder in de botmineralisatie stoornissen die deze ziektes compliceren.

Hoofdstuk 4 bevestigt het bestaan van een sterke connectie tussen EPO en FGF23 signalering in een brede range aan erfelijke hemolytische anemieën, ook na correctie voor de aanwezige hoeveelheid ijzer, inflammatie en nierfunctie. ERFE-waarden waren eveneens beschikbaar en, als verwacht, lieten een nauwe relatie zien met EPO. De data pleiten tegen een rol voor ERFE als intermediair in de EPO-FGF23 as. Daarnaast beschrijven we een correlatie tussen FGF23 en markers van endotheel dysfunctie, en leveren daarmee vroeg bewijs voor een link tussen FGF23 en vasculpathie (als pulmonale hypertensie) in erfelijke anemieën.

DEEL 2: DE UITDAGING DIE HETEROGENITEIT HEET.

Hoofdstuk 5 en 6 belichten de unieke kenmerken van SCD, en spiegelen het ziektebeeld aan overige erfelijke hemolytische anemieën. Bij SCD vindt een reëel deel van de hemolyse plaats in de (micro)circulatie. Andere congenitale hemolytische anemieën (bijvoorbeeld PKD of hereditaire sferocytose) worden gekenmerkt door vrijwel uitsluitend extravasculaire hemolyse, en daardoor worden patiënten niet blootgesteld aan grote hoeveelheden intravasculair vrij heem. Zowel vrij hemoglobine als vrij heem, vrij van de anti-oxidatieve buffer mechanismen normaal aanwezig in de rode cellen, worden bestempeld als erythroïde damage-associated molecular pattern molecules (e-DAMPs),[25-27] en dragen bij aan de ontwikkeling van vasculopathie en trombocyten activatie in SCD.[28-34] Heem-oxygenase 1 medieert heem detoxificatie, wat leidt tot de productie van koolstofmonoxide (CO). Al het heem, ofwel gemetaboliseerd na extravasculaire hemolyse, danwel verwijderd uit de circulatie via scavenger eiwitten, draagt bij aan de endogene CO-productie. In tegenstelling tot heem heeft CO een beschermende werking: het remt polymerisatie van sikkelcel hemoglobine en functioneert als anti-oxidant en anti-inflammatoir agens.[35]

Eerder is aangetoond dat lactaat dehydrogenase (LDH) geassocieerd is met het intravasculair vrijkomen van heem. In hoofdstuk 5 worden de resultaten besproken van een studie naar de waarde van de ratio van LDH tot carboxyhemoglobine (HbCO; de transport-vorm van CO) als voorspellende biomarker. We laten zien dat een LDH/HbCO ratio boven de 1200 SCD patiënten identificeert met een hoge kans op het ontwikkelen van pulmonale hypertensie en een hoog risico op vroegtijdig overlijden. Hoewel validatie noodzakelijk is, presenteren we de ratio van LDH tot HbCO, welke de intravasculaire fractie van hemolyse ten opzichte van de totale hemolyse representeert, als een veelbelovende en klinisch beschikbare biomarker in SCD om individuele risicopredictie te verbeteren.

Hoofdstuk 6 focust op het vermogen van vrij heem om Toll like receptor 4 (TLR4) signalering te moduleren in SCD. Het doel van deze studie, waarin monocyten transcriptoom data van SCD patiënten werd afgezet tegen data van patiënten met andere vormen van congenitale (extravasculaire) hemolytische anemieën, was om de belangrijkste geactiveerde routes in monocyten te identificeren in respons op intravasculaire hemolyse. Er bleek sprake van een uniek genexpressie patroon in SCD monocyten. Niet onverwacht was er een duidelijke upregulatie zichtbaar van *HMOX1* (coderend voor heem-oxygenase 1), in lijn met de gangbare hypothese dat intravasculair vrij heem een belangrijke effector is in de SCD pathofysiologie en gen regulatie. Belangrijke observaties zijn de rol van het lipiden metabolisme en interferon signalering als differentiërende pro-immuun signaalroutes.

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Hoofdstuk 7 is meer klinisch georiënteerd en beschrijft een in omvang uniek cohort van patiënten met erfelijke sferocytose. Deze studie kan gezien worden als een finale poging om met de groeiende lijst aan veelal unieke sferocytose mutaties de discussie over het bestaan van een genotype-fenotype correlatie te beslechten. In het overeenkomende gepubliceerde artikel worden 56 nieuwe mutaties toegevoegd aan de tot dan toe bekende sferocytose mutaties. De conclusie is echter teleurstellend: de identificatie van een genotype-fenotype correlatie in sferocytose is zeer complex als gevolg van de complexiteit van de interacties in het rode cel cytoskelet. Een uitdagende combinatie van de pathogene mutatie, de hoeveelheid en kwaliteit van ingebouwd eiwit, en het effect van het afwijkende eiwit of zijn afwezigheid op de interacties binnen het cytoskelet bepalen de ernst van de ziekte. Daarbij zijn er modificerende genetische en niet-genetische factoren die fenotypische variabiliteit beïnvloeden.

Deel 2 laat daarmee zien dat de variatie tussen erfelijke (hemolytische) anemieën, maar ook tussen patiënten gediagnosticeerd met eenzelfde ziekte, groot is. De zoektocht naar manieren om die heterogeniteit te begrijpen en inzichtelijk te maken is pas net begonnen.

DEEL 3: OVER IJZER.

Alle hoofdstukken in deel 3 zijn gecentreerd rondom één onderwerp: ijzerstapeling. De ijzer-regulerende as, de EPO-ERFE-hepcidine as, is het thema van **hoofdstuk 8**. Voor een gedetailleerde beschrijving van de componenten van de EPO-ERFE-hepcidine as verwijs ik u naar de *General introduction*. In samenwerking met Denemarken werd een groot cohort samengesteld van patiënten met verscheidene erfelijke anemieën gekarakteriseerd door hemolyse en ineffectieve erythropoiese. Ondanks de heterogeniteit van het cohort, ondermeer in ziektelast en ijzerstapeling, zagen wij een duidelijke sterke relatie tussen de individuele componenten van de EPO-ERFE-hepcidine signaalroute. Voorstaande met de kanttekening dat er een belangrijke variabiliteit is tussen ziekten en tussen individuen in de activiteit en balans van de signaalroute. Het doormeten van de EPO-ERFE-hepcidine as, inclusief GDF15 en soluble transferrin receptor (sTFR) kan dienen als handvat om voor een individuele patiënt een inschatting te maken van de omvang van het erytron (EPO, ERFE en sTFR; bijvoorbeeld in reactie op hemolyse of ineffectieve erythropoiese), de effectiviteit van de erytropoiese (GDF15, reticulocyten getal en hemoglobine), en het risico op ijzerstapeling (hepcidine).

Hoofdstuk 9 biedt een interessante stap terug naar de eerste generatie van het ijzeronderzoek. Oude data worden besproken en verklaard in het licht van de huidige kennis over ijzer fysiologie. Deze niet eerder gepubliceerde historische data omvat gecombineerde radio-gelabelde ijzer absorptie en ijzer utilisatie studies verzameld in patiënten met ijzerdeficiëntie, primaire of secundaire hemochromatose. Deze combinatie van studies geeft een unieke inkijk in het transport van ijzer direct na het vrijkomen van ijzer uit de enterocyten in de portaal veneuze circulatie en de opname van ijzer door de hepatocyten in de eerste passage van de lever. De data levert de interessante bevinding op dat in niet-transfusie afhankelijke erfelijke anemie patiënten en in erfelijke hemochromatose patiënten een substantieel deel van het geabsorbeerde ijzer niet werd gebruikt voor de erytropoiese, en dat deze hoeveelheid sterk afhankelijk was van de mate van ijzerstapeling. Dit wijst op het bestaan van een belangrijke mate van wegvangen van het niet-transferrine gebonden ijzer (NTBI) door de lever, en we suggereren een centrale rol voor de ZIP14 transporter in dit proces.[36]

En dan, de finale. **Hoofdstuk 10** beschrijft de resultaten van de *PPI shine again*, een fase III gerandomiseerde, placebo-gecontroleerde studie in een cross-over design naar het effect van de protonpompremmer in de preventie en behandeling van ijzerstapeling in patiënten met erfelijke niet-transfusie afhankelijke anemieën. De huidige behandeling met ijzerchelatoren kent veel, ook ernstige, bijwerkingen en is duur. Niet-heem ijzer in de voeding bestaat hoofdzakelijk uit driewaardig ijzer. Enterocyten zijn in staat om tweewaardig ijzer op te nemen. Dit maakt dat driewaardig ijzer gereduceerd wordt tot tweewaardig ijzer door ferrireductases,

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een proces afhankelijk van een protonen gradiënt. Bovendien zijn protonen noodzakelijk voor het oplosbaar maken van ijzerzouten. Protonpompremmers remmen beide processen.[37,38] In theorie zal blokkade van de ijzerabsorptie het proces van ijzerstapeling in niet-transfusie afhankelijke patiënten stoppen. Dertig volwassen patiënten met uiteenlopende congenitale anemieën en milde tot matig-ernstige ijzerstapeling werden geïncludeerd in de trial. Het primaire eindpunt was de verandering in het lever ijzergehalte (liver iron content [LIC]) gemeten met 1.5 Tesla MRI. De studie laat zien dat een jaar behandeling met esomeprazol 40 milligram tweemaal daags resulteert in LIC-waarde die 0.55 mg Fe/g droog gewicht lager ligt dan een jaar behandeling met placebo, een statistisch significante en klinisch relevante uitkomst. Een lage hepcidine/ferritine ratio is een belangrijke voorspeller van de mate van effectiviteit van de behandeling, wat passend is bij de hypothese dat de protonpompremmer gastro-intestinale ijzeropname blokkeert. Het veiligheidsprofiel van esomeprazol werd, als bekend, beoordeeld als zeer gunstig.

In het hoofdstuk worden suggesties gedaan voor de implementatie van de protonpompremmer in de behandelstrategie van ijzerstapeling. Bij patiënten met milde ijzerstapeling (LIC ≥3-5mg Fe/g dw) kunnen protonpompremmers een mogelijke kandidaat behandeling zijn voor de preventie van of het vertragen van het ontwikkelen van ernstigere ijzerstapeling, en daarmee de noodzaak tot het behandelen met ijzerchelatoren. Dit geldt in het bijzonder voor patiënten met een lage hepcidine/ferritine ratio. Als er reeds ernstigere ijzerstapeling bestaat (LIC ≥ 5mg Fe/g dw) bevelen we aan om toevoeging van een protonpompremmer aan de behandeling met een ijzerchelator te overwegen. Ook vanuit internationaal perspectief kan het resultaat van dit onderzoek grote implicaties hebben. De beschikbaarheid van ijzerchelatoren is beperkt juist in die gebieden waar erfelijke anemieën frequenter voorkomen. Protonpompremmers kunnen de enige realistische behandeloptie zijn voor patiënten zonder toegang tot ijzerchelatoren, dit geldt ook voor patiënten die behandeling met ijzerchelatoren niet tolereren.

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APPENDIX

Dankwoord
(acknowledgments)

DANKWOORD

Het gevoel dat me overvalt tijdens het schrijven van dit dankwoord is niet anders te verwoorden dan een enorme opluchting. Het boek is vol, het verhaal geschreven. Ondanks mijn sterke voorkeur voor een uitbundige en kleurrijke schrijfstijl heb ik besloten het hier kort te houden. Dit hoofdstuk zou hoe dan ook te kort schieten om voldoende dank te zeggen aan hen die hebben bijgedragen dit boek vol en af te krijgen. Ik hoop dan ook dat ik de kans krijg om jullie binnenkort face-to-face nog eens echt te bedanken.

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COVID-19. Tja, wat valt daar nog over te zeggen. Het meest besproken onderwerp van de afgelopen jaren. COVID heeft mijn promotie in tweeën gehakt. Thuiswerken werd het nieuwe normaal, wandelen de manier om de dag door te komen. Eén van de hoogtepunten van de week was de koffie in het UMCU. Ik dank mijn koffie-genootjes voor het aanhoren van mijn eindeloze (sarcastische) verhalen. Voor het vieren van de (kleine) overwinningen op onderzoeksgebied en persoonlijk vlak. De lijst is lang, maar in willekeurige volgorde verdienen Anne, Marieke, Wobke en Myrthe hier zeker een plekje. Fijn dat jullie er waren. En mocht je hier niet tussen staan, weet dat ik elk pauzemomentje, hoe kort dan ook, met jou gewaardeerd heb.

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APPENDIX

List of publications

THIS THESIS

1. **van Vuren AJ**, Sharfo A, Grootendorst ST, van Straaten S, Punt AM, Petersen JB, El Fassi D, van Solinge WW, Bartels M, van Wijk R, Glenthoj A, van Beers EJ. A Comprehensive Analysis of the Erythropoietin-erythroferrone-hepcidin Pathway in Hereditary Hemolytic Anemias. *Hemasphere*. 2021;5(9):e627.

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APPENDIX

Curriculum vitae

CURRICULUM VITAE

Annelies Johanna van Vuren was born on 24 November 1990 in Moordrecht, the Netherlands. In 2008 she graduated from secondary school at *De Goudse Waarden*, Gouda. She studied medicine at the Utrecht University and obtained her Bachelor's degree *cum laude* in 2011 and her Master's degree *cum laude* in 2014. She obtained her position as resident internal medicine under prof. dr. M.M.E. Schneider (regional training program Utrecht), and started her medical career as resident internal medicine at Gelre ziekenhuizen Apeldoorn (under supervision of dr. C.G. Schaar) for two years, followed by four years of training at the University Medical Center Utrecht (under supervision of prof. dr. H.A.H. Kaasjager). At the start of 2018 she interrupted her residency temporarily for a PhD trajectory at the Van Creveldkliniek, Center for Benign Haematology, Thrombosis and Haemostasis at the University Medical Center Utrecht under direct supervision of prof. dr. R.E.G. Schutgens, prof. dr. W.W. van Solinge, dr. E.J. van Beers and dr. R. van Wijk. Her PhD trajectory focused on hereditary anemias, in particular the interaction of iron and erythropoiesis. During this trajectory she coordinated a multicenter clinical trial known as the *PPI Shine Again*, funded by ZonMw (2018-2021) and Innovatiefonds Zorgverzekeraars. She finished her PhD trajectory at the end of 2021. She is currently working as fellow at the Haematology department at the University Medical Center Utrecht (under supervision of dr. A. van Rhenen). She expects to finish her fellowship at the end of 2023.

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