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# Innovative haematological parameters in clinical practice

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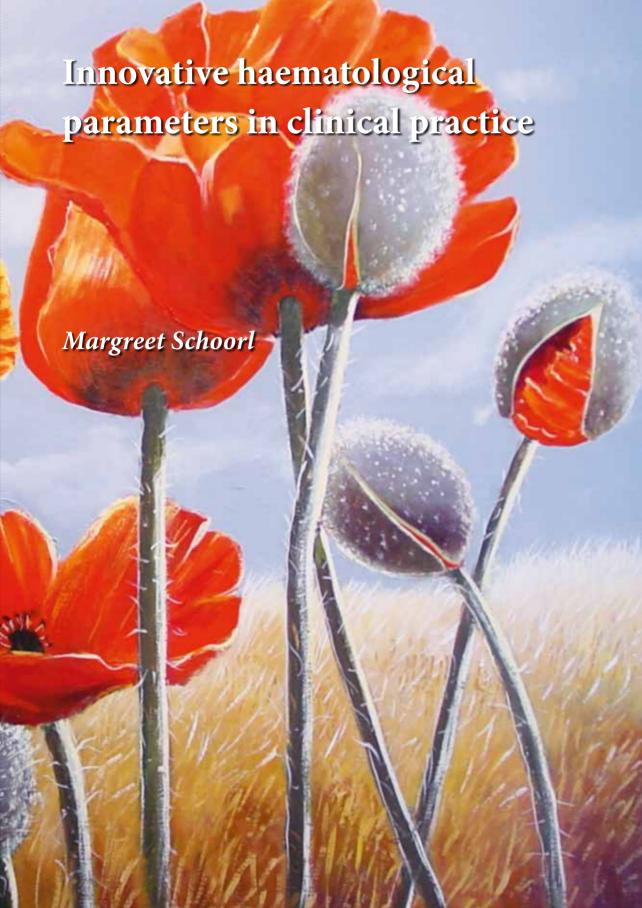
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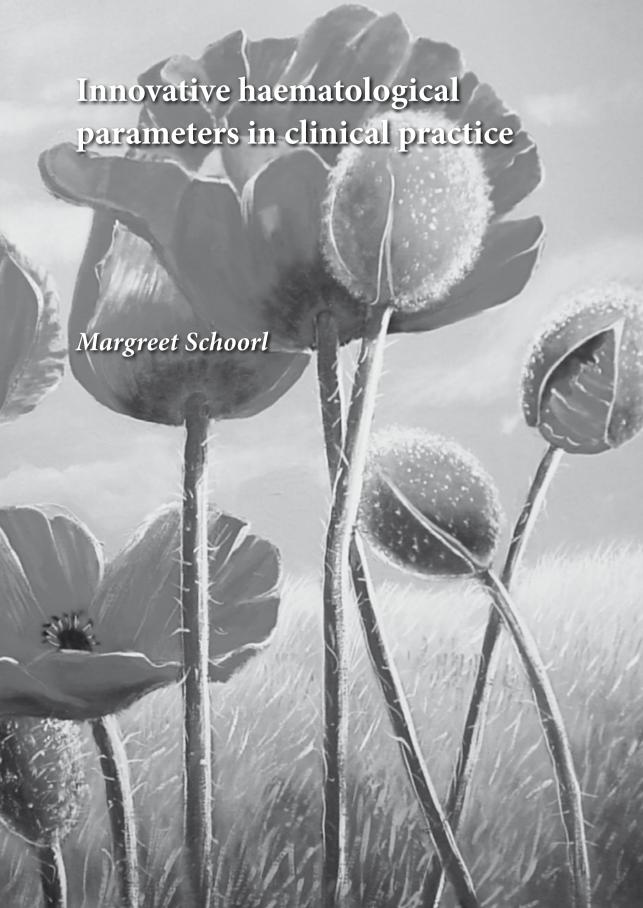
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# INNOVATIVE HAEMATOLOGICAL PARAMETERS IN CLINICAL PRACTICE

# ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D.C. van den Boom

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Aula der Universiteit

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#### 1. INTRODUCTION

Anaemia is a global public health problem affecting populations in both developing and developed countries. According to the World Health Organisation (WHO) anaemia affects 1.62 billion people, which corresponds to 25% of the world population. It is assumed that 50% of the cases of anaemia are due to insufficient iron content in the diet, especially in young children, vegetarians and women in child-bearing age with large menstrual blood loss or during pregnancy. Iron loss in women averages 1 to 3 mg per day, and dietary intake is often inadequate to maintain a positive iron balance. Moreover, pregnancy adds to demands for iron, with requirements of up to 6 mg per day by the end of pregnancy.<sup>2</sup>

Another group at risk for iron deficiency are athletes. Endurance athletes are prone to negative changes in iron status caused by insufficient dietary intake, increases in haematuria, gastrointestinal bleeding, sweating, exercise-induced oxidative stress and haemolysis resulting from 'foot-strike' and/or compression of contracting muscles on capillaries. 'Foot-strike haemolysis', is a disorder that develops from red blood cell destruction in the feet due to frequent contact with hard surfaces. <sup>3,4,5,6</sup> Recent studies have provided evidence that the iron-regulating hormone hepcidin is transiently increased in endurance athletes, and they suggest that this may contribute to iron-deficiency anaemia in athletes.<sup>7</sup>

Further risk factors for iron deficiency are obesity and its surgical treatment. Obese patients are often iron deficient, with increased hepcidin levels being implicated in decreased absorption of iron in the gut. After bariatric surgery, the incidence of iron deficiency might be as high as 50%.<sup>8</sup>

Although the primary cause of anaemia is iron deficiency, it frequently coexists with other causes. The risk of anaemia may be increased by deficiencies in other micro-nutrients, including vitamins A and B12, folic acid, riboflavin, or copper. Furthermore, the impact of haemoglobinopathies on anaemia prevalence needs to be considered within several populations. Consequently, once the diagnosis of anaemia has been established, further investigations are needed in order to identify the underlying cause.

# 2. ERYTHROPOÏESIS

Red blood cells are responsible for oxygen transport in the body. Their diameter is 6.5 to  $8.5 \, \mu m$  and they have a biconcave shape, which ensures a maximum surface-volume ratio for optimized oxygen exchange.

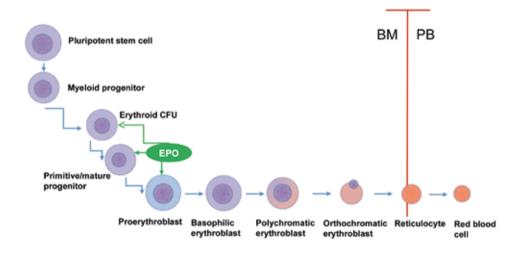
The normal life-span of red blood cells amounts to approximately 100 - 120 days. In healthy adults,  $200 \times 10^9$  red blood cells are replaced each day. Old red cells are trapped in the microcirculation of the spleen, after which they are phagocytosed and degraded by the reticular cells of the spleen. To balance the destruction of red blood cells and their production, the process of erythropoiesis is regulated by a feedback mechanism involving erythropoietin (EPO) stimulation.

Erythropoiesis involves the production of red blood cells from myeloid progenitor cells in the bone marrow. The earliest progenitor cells committed to red blood cell maturation

have been identified as erythroid burst-forming units (BFU-E), which progress to the erythroid colony forming units (CFU-E).

The CFU-E are rapidly dividing cells that are sensitive to low concentrations of erythropoietin. The progenitors CFU-E differentiate into erythroid precursors, identified as proerythroblast, basophilic, polychromatic and orthochromatic erythroblasts, and finally into nucleated reticulocytes. Reticulocytes migrate from the bone marrow to the peripheral blood, where they mature to red blood cells in 1 to 3 days (Figure 1).9

Immature reticulocytes are distinguished from mature reticulocytes by increased RNA content and organelle fragments in the cytoplasm. Immature reticulocytes are released during periods of erythropoietic stimulation, such as in response to iron or erythroid stimulating agents therapy. The immature reticulocyte fraction (IRF) in peripheral blood already increases several days earlier than the number of reticulocytes. 10,11



**Figure 1.** Maturation of red blood cells.

Abbreviations: BM = bone marrow; PB = peripheral blood; EPO = erythropoietin; CFU = colony forming unit

Erythropoietin is the main hormone involved in the regulation of the production of new red blood cells. The hormone is a growth factor for erythroid progenitor cells in bone marrow and induces erythroid proliferation and differentiation, resulting in the increased production of the red blood cells through the various stages described above.

Since erythropoietin is produced in the kidneys, renal failure is frequently associated with anaemia due to decreased erythropoietin production. On the other hand, prolonged stay at high altitude or pulmonary conditions that lead to a chronic hypoxic state, result in increased red blood cell production. In addition to stimulation due to growth factor certain nutrients are required for optimal performance of erythropoiesis.

#### 3. STRUCTURE AND SYNTHESIS OF HAEMOGLOBIN

Normal adult haemoglobin is composed of four polypeptide chains: two  $\alpha$ -chains and two  $\beta$ -chains, which together form a tetramer ( $\alpha_2\beta_2$ ). Each chain is folded around a haem group, consisting of a porphyrin ring and an atom of iron (Figure 2). Iron is an essential component of haem, the iron-porphyrin complex.

The haemoglobin molecule contains four haem groups and is thus able to transport four molecules of oxygen. In healthy adults the principal haemoglobin is HbA (about 97%), while small amounts of HbA<sub>2</sub> (2-3%) and HbF (0-1%) are also present. HbA<sub>2</sub> is a tetramer of two  $\alpha$ -chains and two  $\delta$ -chains ( $\alpha_2\delta_2$ ). HbF is a tetramer of two  $\alpha$ -chains and two y-chains ( $\alpha_3V_2$ ).

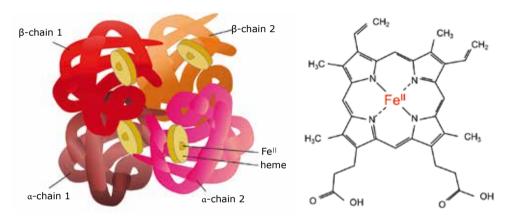


Figure 2. Normal haemoglobin molecule (haemoglobin A), showing two  $\alpha$  and two  $\beta$  chains (left) and normal haemoglobin structure (right).

Modified from the Internet Encyclopedia of Science.

#### 3.1 Disorders of haemoglobin synthesis

Haemoglobinopathy encompasses a group of genetic disorders which involve an abnormal structure of one of the globin chains of the haemoglobin molecule. In contrast, thalassaemias are disorders of haemoglobin synthesis that usually result in decreased production of normal globin proteins, often due to mutations in regulatory genes. 13,14

#### 3.1.1 α-thalassaemia

 $\alpha$ -thalassaemias are the most prevalent disorders of haemoglobin synthesis. <sup>13</sup> The  $\alpha$ -globin genes  $\alpha_1$  and  $\alpha_2$  are situated in a linked cluster on the short arm of chromosome 16. Deletions or mutations in one to four of the  $\alpha$  genes result in reduced or even absent globin chain production. The resulting aberrations involve various degrees of imbalance between  $\alpha$ - and  $\beta$ -chain synthesis. Subjects can be classified according to haematological, biochemical and molecular criteria. Six genotypes are distinguished:

- 1 Normal genotype, with four  $\alpha$ -globin genes:  $\alpha\alpha/\alpha\alpha$
- 2  $\alpha$ -thalassaemia 2 heterogosity, with three functional  $\alpha$ -globin genes:  $\alpha\alpha/-\alpha$
- 3  $\alpha$ -thalassaemia 1 heterogosity, with two functional  $\alpha$ -globin genes:  $\alpha\alpha/-$
- 4  $\alpha$ -thalassaemia 2 homozygosity, with two functional  $\alpha$ -globin genes:  $-\alpha/-\alpha$
- 5  $\alpha$ -thalassaemia 1 and  $\alpha$ -thalassaemia 2 double heterogosity, or Hb H-disease, with one functional  $\alpha$ -globin gene: --/- $\alpha$
- 6  $\,$   $\alpha$ -thalassaemia 1 and homozygosity or Hb Bart's hydrops, with no functional  $\,$   $\alpha$ -globin genes.

The latter condition is not compatible with life; hydropic fetuses are still-born or die shortly after birth.

The conditions listed in sub 2, 3 and 4, the so-called thalassaemia minor variants, are clinically silent. Subjects with  $\alpha$ -thalassaemia 2 heterozygosity, (also called  $\alpha$ -thalassaemia carrier state) are characterised by normal to moderately decreased mean cell volume (MCV) values with normal or slightly decreased haemoglobin concentrations. <sup>15,16</sup> Subjects with homozygous  $\alpha$ -thalassaemia 2 and heterozygous  $\alpha$ -thalassaemia 1 (both indicated as  $\alpha$ -thalassaemia trait), are diagnostically very similar and the genotype of the individuals cannot be distinguished by blood count markers. Erythropoiesis with reduced MCV, frequently associated with mild anaemia, is frequently observed.

Subjects with Hb H-disease (sub 5) demonstrate microcytic anaemia of widely varying degree, dominated by haemolysis due to instability of the HbH molecule.<sup>17</sup>

#### 3.1.2 β-thalassaemia

The  $\beta$ -like globin genes are arranged in a cluster on the short arm of chromosome 11. Mutations causing  $\beta$ -thalassaemia result in a lack of  $\beta$ -globin production, which ranges from minimal (mild  $\beta^+$ -thalassaemia alleles) to a complete absence ( $\beta^0$ - alleles).  $\beta$ -Thalassaemia is an extremely heterogeneous condition. More than 40 different lesions of the  $\beta$ -globin gene have been identified. Most of the lesions are caused by single base substitutions or by DNA-rearrangements, resulting in small deletions or insertions.

With rare exceptions, heterozygotes for  $\beta$ -thalassaemia ( $\beta$ -thalassaemia minor) are clinically asymptomatic. Almost all heterozygous conditions (except the so-called normal HbA $_2$ -thalassaemias, e.g.  $\delta\beta$ -thalassaemia), are characterised by an increased HbA $_2$  content ( $\geq$  3,2 %) and a marked degree of microcytosis, which is frequently associated with mild anaemia. The increased HbA $_2$  content is due to a compensatory increase of  $\delta$ -chain synthesis.

Subjects with homozygous  $\beta$ -thalassaemia suffer from severe microcytic anaemia. The bone marrow demonstrates hyperactive erythropoiesis, with intramedullary destruction of erythroid precursors due to unstable  $\alpha$ -chain tetramers, which precipitate in cells and induce cell death.

Red blood cells have a shortened life-span due to precipitated  $\alpha$ -chain inclusions. Haemo-globin in the red blood cells consists of HbF and a small fraction of HbA<sub>2</sub>, varying from 1-3% in  $\beta^0$ - thalassaemia to 7% in  $\beta^+$ -thalassaemia.<sup>17</sup>

#### 3.1.3 Haemoglobinopathies

Haemoglobinopathies (HbP) include a heterogeneous group of inherited disorders which

affect the structure of the haemoglobin molecule. Four abnormal haemoglobins in particular HbS, HbC, HbE and HbD<sup>Punjab</sup>, are rather common in various parts of the world, such as Africa, the Mediterranean area and Southeast Asia. Heterozygous carriers of HbP have one normal  $\beta^A$  gene and one affected  $\beta$  gene, for instance  $\beta^S$  and  $\beta^C$ . HbS is the most common Hb variant, frequently indicated as HbS/C compound heterozygosity, HbS/βthalassaemia double heterozygosity or HbS/S homozygosity. Although HbP includes this important red blood cell sickling disorder, the vast majority of the HbP are clinically silent diseases in carriers. Subjects with a homozygous type of sickle cell disease generally demonstrate lifelong haemolysis and vaso-occlusive episodes, known as sickle crises. Carriers of HbP, if not associated with α-thalassaemia, usually do not suffer from anaemia, although results of MCV and mean cell haemoglobin (MCH) are situated in the lower part of the reference range. The reticulocyte count may be increased and microscopic evaluation of the red blood cells will demonstrate target cells and occasionally schistocytes. Haemoglobin disorders (thalassaemias and sickle cell disorders) are endemic where falciparum malaria is (or has been) prevalent because carriers are better protected against dying from this infection.<sup>14</sup> Consequently, haemoglobin disorders are common in Southern European countries. However, over the past decades migration from endemic areas has introduced many carriers of haemoglobin disorders to most of the Northern and Western European countries. Modell et al collected data on the prevalence of haemoglobin disorders from countries of the European Union. Results are listed in Table I.<sup>18</sup>

	% of population carrying a signifficant variant								
Region	Country	Residents 2003	AC	AS	AE	AD etc	beta- thal	alfa- thal	total %
Northern Europe	Norway/Sweden/Denmark England & Wales	19.087.200 53.125.596	0,01 0,13	0,08 0,46	0,07 0,06	0,03 0,18	0,22 0,45	0,02 0,07	0,43 1,35
Western Europe	Netherlands Belgium/Luxemburg France Germany Austria/Switzerland	15.810.000 10.720.350 59.768.000 82.087.000 15.093.081	0,10 0,02 0,10 0,01 0,01	0,47 0,18 0,60 0,05 0,08	0,07 0,01 0,15 0,02 0,03	0,03 0,03 0,03 0,03 0,03	0,42 0,36 0,42 0,24 0,24	0,01 0,02 0,02 0,01 0,02	1,10 0,62 1,32 0,36 0,41
Southern Europe	Italy Spain Portugal Greece Cyprus	57.695.000 40.847.371 10.356.586 11.783.586 721.000	0,01 0,03 0,01 0,002	0,22 0,09 0,57 0,53 1,00	0,006 0,002 0,002 0,004	0,03 0,03 0,03 0,03 0,03	3,33 0,25 0,45 6,29 15,00	0,01 0,01 0,003 0,51 1,00	3,61 0,41 1,07 7,34 17,03
Eastern Europe	Bulgaria Romania	7.824.000 21.734.000				0,03 0,03	3,00 1,00		3,03 1,03

**Table 1.** Estimated percentage of residents carrying a significant haemoglobin gene variant. Modified from Modell B. et al. 18

#### 4. NUTRITIONAL ANAEMIA

Nutritional anaemias include a group of disorders in which red blood cell production is reduced by a suboptimal supply of one or more specific nutrients. The deficiency may result from a nutritional deficit when dietary intake fails to meet requirements or from conditions which cause malabsorption. Nutritional deficiencies also result from increased demand or excessive loss of a nutrient. Nutrients with clinical relevance for preventing anaemia are iron, folate and vitamin B12.<sup>19</sup>

#### 4.1 Iron deficiency

In the development of iron deficiency three stages can be defined, *depleted iron stores*, *iron-deficient erythropoiesis*, *and iron-deficiency anaemia (IDA)*. Once iron stores are *depleted*, the size of the iron transport compartment is critically reduced. Plasma iron concentration decreases, total iron binding capacity increases and the iron transferrin saturation decreases.

When iron supply for erythropoiesis is insufficient, this results in the second stage of iron deficiency, *iron-deficient erythropoiesis*.

Once iron in cells is reduced, red blood cell surface transferrin receptors will increase, yielding increased soluble transferrin receptor (sTfR) concentrations. If insufficient iron is available to incorporate ferrous ions into protoporphyrin IX to form haem, zinc protoporphyrin (ZPP) will accumulate resulting in increased ZPP concentrations in red blood cells.<sup>20</sup>

When the iron supply is no longer sufficient to maintain a normal concentration of haemoglobin, the third stage of iron deficiency develops. This stage features an increase in red cell heterogeneity secondary to alterations in red blood cell haemoglobinisation, indicated as increased red blood cell distribution width (RDW). Progress of the iron deficit will gradually result in a decreased MCV, a *microcytic iron-deficiency anaemia*.<sup>17</sup>

In children, the prevalence of depleted iron stores ranges from 2 to 48%, and anaemia ranges from 2 to 4%, depending on age group and country. In adolescents, prevalences are respectively 5-43% and 7-8%.

Adolescents constitute a group with a particularly high risk due to marked iron requirements. Epidemiological surveys in European countries demonstrate that iron depletion occurs in 10 to 30% of menstruating women, and 1.5 to 14% of menstruating women have iron-deficiency anaemia. In pregnant women, the prevalence of iron-deficiency anaemia ranges from 6 to 30%; the highest levels are observed in countries where routine iron supplementation is not usually given during pregnancy.<sup>2</sup> In the Netherlands, the incidence of iron-deficiency anaemia in the general practice is 4.3 per 1000 patients per year.<sup>21</sup>

#### 4.2 Functional iron deficiency

In case of functional iron deficiency, insufficient iron is incorporated into erythroid precursors in spite of adequate body iron stores, as indicated by stainable iron in bone marrow and serum ferritin concentrations within the normal range.<sup>22</sup>

A partial blockade of iron transport to the erythroid marrow occurs in subjects with

inflammatory and malignant diseases, and in chronic kidney disease. Blockade of iron transport is a major cause of the anaemia of chronic disease (ACD). ACD, also called anaemia of inflammation (AI), is characterized by hypoferremia due to iron sequestration which results in iron-restricted erythropoiesis.

The more recent terminology AI better reflects the pathophysiology of this type of anaemia and also includes an acute form of this disorder, anaemia of critical illness, a condition that develops within days of the onset of illness.<sup>23</sup>

While ACD is the main clinical presentation of functional iron deficiency, a second type often occurs when erythroid marrow is stimulated with erythroid-stimulating agents. The understanding of the physiology of anaemia of chronic disease has improved with the discovery of the iron-regulatory peptide hepcidin, a 25-amino acid peptide synthesized in the liver.<sup>23,24</sup>

Hepcidin is up-regulated in the setting of inflammation and cancer, resulting in increased synthesis in the liver due to stimulation by cytokines, the most important of which is interleukin 6. By degrading ferroportin, hepcidin decreases the availability of iron from macrophages. When functional iron deficiency and inflammatory disease coexist, increased hepcidin synthesis will restrict the absorption of oral iron. Suppletion of intravenous iron preparations will overcome this block (Figure 3).<sup>25,26</sup>

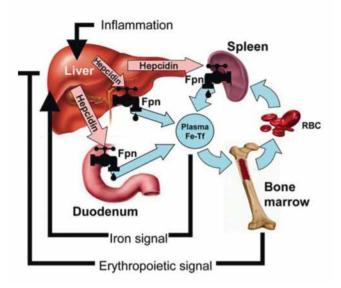


Figure 3. The role of hepcidin in iron metabolism.

Hepcidin - ferroportin interaction deter

Hepcidin - ferroportin interaction determines the flow of iron into plasma. The hepcidin concentration is in turn regulated by iron, erythropoietic activity and inflammation. Abbreviations: RBC = red blood cells; Fpn = ferroportin; Fe-Tf = iron-transferrin From: Nemeth E, Ganz T: Acta Haematol 2009; 122:78-86 (DOI: 10.1159/000243791). (Copyright permission obtained from S. Karger AG, Basel).

In subjects treated with erythroid-stimulating agents (EPO) for anaemia associated with chronic kidney diseases, the response rate improves when intravenous rather than oral iron supplements are given. 11,27,28

Irrespective of the cause, inadequate iron supply results in impaired haemoglobin synthesis and a decrease of mean corpuscular haemoglobin (MCH) and red blood cell haemoglobin content (RBC-He) which become apparent after several weeks of impairment. In contrast, the availability of iron for erythropoiesis as estimated from the haemoglobin content of the reticulocyte (RET-He) is evident within a few days.<sup>11</sup>

#### 4.3 Vitamin B12 / Folate deficiencies

The main cause of megaloblastic anaemia due to vitamin B12 deficiency is autoimmune-mediated atrophic gastritis. Antibodies are directed against parietal cells of the stomach, or to the intrinsic factor (which is required for absorption of vitamin B12). Less common causes of vitamin B12 deficiency are total or subtotal gastrectomy and malabsorption of vitamin B12 due to disorders of the small intestine, such as coeliac disease, Crohn's disease. A vitamin B12 deficiency may also be caused by a diet without food of animal origin (veganism).

Vitamin B12 or folate deficiency frequently results in macrocytic anaemia, which leads to increased MCV and red cell distribution width (RDW). However, due to the occurrence of combinations of iron deficiency, thalassemia trait and inflammation, macrocytosis is less pronounced than expected.

In case of an incomplete response to vitamin B12 or folic acid therapy, iron deficiency might be a complicating underlying factor.<sup>19</sup>

In the Netherlands the incidence of anaemia caused by vitamin B12 or folate deficiency in the general practice is about 1.8 per 1000 patients per year.<sup>21</sup>

# 5. DISCRIMINATION BETWEEN IRON DEFICIENCY AND THALASSAEMIA SYNDROMES

Iron-deficient erythropoiesis and thalassaemia are both associated with mild to moderate microcytic anaemia, which frequently leads to an incorrect diagnosis. It is important to discriminate between iron-deficiency anaemia and thalassaemia, and to avoid unnecessary iron therapy to prevent the development of haemosiderosis, which may result in serious complications like cardiomyopathy, liver fibrosis or endocrine dysfunctions. <sup>13,14</sup>

A wide range of laboratory parameters is available for anaemia screening and assessment of iron status. However, no single marker or combination of tests is optimal for discrimination between iron deficiency, functional iron deficiency and thalassaemia. The available indicators do not provide sufficient information and must be used in combination to obtain reliable information. In addition, iron deficiency often occurs in combination with other diseases which complicates the diagnosis. Diagnosing subjects with combined thalassaemia minor and iron deficiency is even more challenging.

#### 5.1 Bone marrow iron

The gold standard for the evaluation of storage iron includes an examination of iron content in aspirated bone marrow or a bone marrow biopsy. However, a bone marrow puncture is an uncomfortable and painful intervention for the patient, and should only be performed in cases in which other laboratory parameters are not conclusive. Iron within the macrophage compartment of the marrow will stain blue with Prussian-Blue reagent (Perl's reaction). No other single laboratory test demonstrates sufficient sensitivity and specificity for the diagnosis of iron deficiency. In case of iron deficiency the number of sideroblasts is diminished. The absence of sideroblasts indicates impairment of iron availability in the erythroblasts. However, a decreased number of sideroblasts may also be found in a variety of acute and chronic inflammatory conditions.<sup>17</sup>

#### 5.2 Iron status

#### Serum iron and transferrin saturation

A low serum iron concentration and increased transferrin synthesis, resulting in reduced transferrin saturation, yields an indication for depleted iron stores. However, a large number of other diseases have been associated with a reduction in transferrin. Therefore the plasma transferrin level lacks specificity in the diagnosis of iron deficiency.<sup>29,30,31</sup> Normal and increased levels of transferrin saturation are more useful for excluding iron deficiency than decreased values are for identifying it.<sup>32</sup>

#### Ferritin

Serum ferritin is the most widely employed indicator for evaluation of the iron status. It has proven to be the best indicator of iron status in subjects with uncomplicated iron-deficiency anaemia.

The ferritin concentration is proportional to the amount of iron stored in the body in healthy subjects and in subjects with uncomplicated iron deficiency. Results of less than  $12 \,\mu g/L$  are indicative of iron deficiency and of a lack of stainable iron in the bone marrow. Page 17,19,33 Nevertheless, the relationship between serum ferritin and iron stores is affected by acute and chronic infections and inflammatory disorders, liver diseases, and malignancies. Therefore it is recommended to determine an inflammatory marker simultaneously with measuring the serum ferritin concentration, which is routinely determined by immunoassay. C-reactive protein (CRP) is most commonly used, but there is no consensus on the degree of CRP increase and the inadequacy of serum ferritin as an accurate measure of the iron status. A CRP value of less than 30 mg/L might be used to exclude the influence of inflammation.  $^{19,33}$ 

#### Transferrin receptor

Another indicator of iron-deficient erythropoiesis is the soluble transferrin receptor concentration (sTfR). The sTfR concentration is a quantitative measure of iron deficit in uncomplicated iron deficiency. sTfR concentrations are less affected by inflammation or liver disease than serum ferritin. <sup>11,19,34</sup> However, the sTfR concentration is increased by enhanced erythropoiesis in case of haemolytic anaemia and thalassaemia. <sup>19,35,36</sup> The clinical uti-

lity of sTfR measurements is also restricted by the lack of standardization and availability of various antigens being used for the assay.<sup>37</sup> The ratio of sTfR to ferritin (R/F ratio), is used to determine the response to intravenous iron in renal patients with erythroid stimulating agents therapy.<sup>11,38,39</sup>

#### Zinc protoporphyrin

Zinc protoporphyrin (ZPP) is a metabolite which is produced in trace amounts during haem synthesis. When the iron supply is inadequate, ZPP accumulates in red blood cell precursor cells because there is insufficient iron for haem synthesis and zinc is an alternative element for ferrochelatase in the final step in haem synthesis.<sup>20</sup>

Increased red blood cell ZPP concentrations may result from impaired iron metabolism, increased erythropoietic activity and disturbances in the haem synthesis, such as, for example, disturbances caused by inflammation or infections, thalassaemia or lead toxicity. 40,41,42,43

ZPP reflects a *long term* impression in accordance with the lifespan of red blood cells.<sup>44</sup> The assay results reflect the amount of iron that was available to the bone marrow during the preceding 3-4 months.

Measurements of the zinc protoporphyrin in red blood cells are performed on a haematofluorometer (AVIV Biochemical Inc., Lakewood, USA) by application of front surface illumination fluorometry.

#### 5.3 Haemocytometry

#### 5.3.1 Historic overview

Cellular analysis in haematology already has a history of more than three centuries, which is characterized by a high degree of technological developments. The innovations have been accompanied by careful observations, meticulous attention to detail, and the application of various techniques.<sup>45</sup>

#### Microscopy

Van Leeuwenhoek succeeded in producing lenses and the first microscopes, which he used for simple but at the time extremely advanced observations. He identified blood cells in 1675, when he observed that his own blood was composed of 'small red globules, driven through a crystalline humidity of water'. $^{46,47}$ 

In 1877, Paul Ehrlich introduced aniline dyes to stain blood cells. He demonstrated that one group of dyes preferentially stained red blood cells and eosinophil leukocyte granules, whereas the other group stained nuclei and lymphocyte cytoplasm. In 1879 he developed a staining method that could stain red and white blood cells simultaneously.<sup>45</sup>

#### Microscopic blood cell analysis

The addition of quantification by microscopic observation was an important step in the analysis of blood cells. Manual methods for cell counting and cell characterization were highly dependent on the quality of the microscopes.

During the next 60 years several modifications of the manual procedure were introduced, including a variety of haemocytometers, graduated and rectangular chambers into which

diluted blood was injected, and various solutions for dilution. This technique was used for counting red blood cells, white cells and platelets. The Neubauer haemocytometer, which consists of two chambers with finely ruled squares, has become the standard method to perform manual counts. This basic design is still employed for microscopic cell counts today. The quantification of cell size was also initiated by van Leeuwenhoek. However, it was not until 1718 that Jurin accurately established the diameter of red blood cells. The magnified images of cells (flattened in a dried blood film) were compared to a known dimension by calibrating the microscope. More advanced cell counters introduced automated methods for the calculation of haematocrit (HCT), MCV, MCH and mean cell haemoglobin concentration (MCHC).<sup>45</sup>

#### Haemoglobinometry

The first attempts to determine the concentration of haemoglobin in blood included visual matching of dilutions of blood to a liquid colour reference as reported by Gowers (1878), Hoppe-Seyler (1883), Sahli (1895), and Haldane (1901). In 1920, Stahe introduced the determination of haemoglobin as cyanmethaemoglobin or haemiglobincyanide (HiC). This method is still the international reference method (ISLH) for haemoglobin analysis.<sup>45</sup> Current haematology analysers are equipped with modified reagents such as sodium lauryl sulphate or imidazole. Various non-ionic detergents are used to ensure rapid red blood cell lysis and to reduce turbidity caused by cell membranes or plasma lipids.<sup>48</sup>

#### Automated blood cell analysis

The first step towards haematology automation was Walter Coulter's discovery of an aperture impedance method, *the Coulter principle*, for counting and sizing cells. The principle was based on the lower conductivity of red blood cells compared with the dilution fluid. Blood cells suspended in an electrolyte solution were induced to flow through an electric field in a short, small orifice drilled into a thin sapphire. The electric field in and surrounding this orifice was the sensing area, also called *the aperture* (Figure 4). An important feature of the analyser was the aspiration device for aliquoting an accurate volume of blood. In the next decades, several companies have gradually improved and combined various technologies in the haemocytometric analysers, resulting in the current multi-parameter haematology analysers.<sup>45</sup>

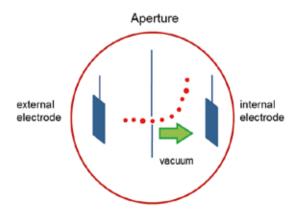


Figure 4. The Coulter principle for counting and sizing cells.

Provided by Sysmex
Europe, Hamburg,
Germany.

#### *5.3.2* 'State of the art' haemocytometry

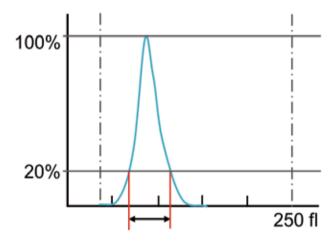
Today's advanced haematology analysers are still based on the Coulter principle. However, the analysers are based on combinations of technologies, such as the application of impedance measurements, fluorescent dyes, flow cytometry and spectrophotometry.

Various haemocytometric parameters are available. Anaemia is usually considered to be present when the haemoglobin concentration has decreased below a cut-off point. Cut-off levels are defined according to sex, age and physiological conditions such as pregnancy. In addition, Hb-content of red blood cells and reticulocytes, and reticulocyte maturity (RBC-He, Ret-He, Ret-He/RBC-He ratio or Delta-He, IRF) can be established. More recently, measurement methods have been developed for the determination of microcytic and hypochromic red blood cells.<sup>11</sup>

#### Red cell distribution width

Red blood cells are usually measured according to the principle of impedance. Cells consecutively pass through an aperture. If a cell passes through this capillary opening, the voltage over the transducer changes, which results in an electrical signal that is proportional to the volume of the cell. Together, all these impulses form a volume distribution curve. The width of the red cell distribution curve reflects the variability of the circulating red blood cells and is a measure of anisocytosis.

Figure 5 shows a red blood cell histogram. Red blood cells can be recorded up to a volume of 250 fL. Normal red blood cells have an average volume of 80 to 100 fL. In the presence of microcytic red blood cells, the whole curve is displaced to the left. With macrocytic red blood cells, the histogram curve is displaced to the right.



**Figure 5.** *Red blood cell histogram.* 

Determination of the RDW-SD is an actual measurement of the width of the red blood cell distribution curve. The measurement is performed at a relative height of 20% above the baseline, indicated by the red lines. The narrower the curve is spread by red blood cells of different sizes, the lower the RDW-SD value will be.

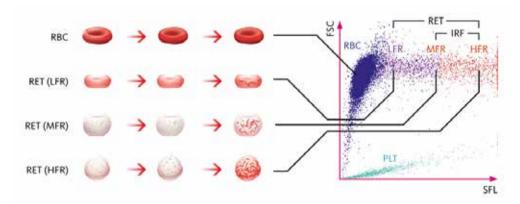
Provided by Sysmex Europe, Hamburg, Germany.

Reticulocyte count and immature reticulocyte fraction

The automation of reticulocyte counting started in 1989, when Toa Medical introduced a benchtop Sysmex R-1000 reticulocyte analyser in particular for counting reticulocytes. A few years later, reticulocyte counting was added to the multi parameter analysers.

The determination of the reticulocyte count gives an impression of the 'quantity' of the erythropoiesis. The increase in precision of the automated reticulocyte count and the possibility to measure the immature reticulocyte count fraction (IRF) provide an opportunity to assess how a changing iron status affects this transient cell population (Figure 6).

The methodology for counting reticulocytes and IRF measurements is based on the application of automated fluorescent flow cytometry, utilizing, for instance, thiazole orange or polymethine dye which binds to cytoplasmic RNA in the reticulocytes. Mature reticulocytes contain only a few colored dots. The IRF is the brightest reticulocyte fraction with the highest RNA-content.



**Figure 6.** Indication of the location of the reticulocyte fractions (LFR, MFR, HFR) in the reticulocyte channel of the Sysmex XE-2100 Haematology analyser. IRF (MFR+HFR) yield higher fluorescent intensity.

Provided by Sysmex Europe, Hamburg, Germany.

Abbreviations: RBC = red blood cells; RET = reticulocytes; LFR = low fluorescent reticulocytes; MFR = mean fluorescent reticulocytes; HFR = high fluorescent reticulocytes; IRF = immature reticulocytes fraction; PLT = platelets; SFL = side fluorescence light intensity; FSC = forward light scatter.

#### Red blood cell and reticulocyte haemoglobin content

The haemoglobin content of reticulocytes (Ret-He) gives an impression of the 'quality' of the erythropoiesis in subjects with iron deficiency, chronic renal failure or ACD. 49,50,51,52,53 Ret-He reflects a *short term* indication of the availability of iron for erythropoiesis and the response to iron therapy. Ret-He reveals a better predictive value for the detection of iron depletion than the MCV, serum ferritin or transferrin saturation. 54

The haemoglobin content of red blood cells and reticulocytes (RBC-He and Ret-He respectively) is determined by means of the flow cytometric reticulocyte count. The mean

forward light scatter intensity in the reticulocyte channel is measured as a parameter for the volume and to establish haemoglobin content of red blood cells and reticulocytes. Results concerning Hb content were initially reported as RBC-Y for red blood cells and RET-Y for reticulocytes. Subsequently, several algorithms were applied to transform the original data into the haemoglobin equivalents (He). Haemoglobin equivalents are expressed in aMol and denoted as RBC-He and RET-He, respectively. The difference between these two parameters is expressed as Delta-He (Figure 7).

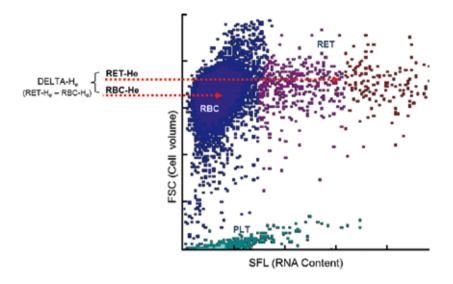


Figure 7. Indication of the location of RET-He and RBC-He in the reticulocyte channel of the Sysmex XE-2100 Haematology analyser. The FSC intensity in the reticulocyte channel reflects the volume and haemoglobin content of red blood cells and reticulocytes.

Provided by Sysmex Europe, Hamburg, Germany.

Abbreviations: RBC = red blood cells; RET = reticulocytes; RET-He = reticulocytes haemoglobin equivalent; RBC-He = red blood cell haemoglobin equivalent; Delta-He = RET-He minus RBC-He; PLT = platelets; SFL = side fluorescence light intensity; FSC = forward light scatter.

#### Hypochromic red cells: %MicroR and %HypoHe

Iron-deficient erythropoiesis is characterized by the production of red blood cells with decreased haemoglobin content, which results in an increased number of microcytic and hypochromic red blood cells. Measurement of the percentage hypochromic and microcytic red blood cells has demonstrated to be useful for detecting rather small changes in the number of red blood cells with inadequate haemoglobinisation. 44,56,57

These specific red blood cells are detected by the current generation of haematology analysers. The percentage hypochromic and microcytic red blood cells are parameters derived from the haemoglobin content of the mature red blood cells, measured in the reticulocyte

channel, and expressed as %HypoHe and %MicroR respectively. %HypoHe and %MicroR are identified by the percentage of hypochromic red blood cells with a Hb content of <17 pg (= 1062 amol) and the percentage microcytic red blood cells with a volume <60 fL respectively.

#### 5.3.3 Discriminating algorithms

For many years, the application of red blood cell indices has been recommended for discriminating between subjects with iron deficiency and subjects with thalassemia.  $^{42,58,59,60}$ . However, application of the England & Fraser formula (MCV-RBC-5xHb-3,4) and Mentzer formula (MCV/RBC) only resulted in appropriate classification of 30-40% of subjects. Additional assessment of ZPP content in red blood cells was recommended for the classification of microcytic red blood cell disorders.  $^{41,42,61,62,63,64,65,66}$  Multivariant discriminant analysis with algorithms including MCV, MCH, RBC and RDW has proven to be useful for the differential diagnosis of  $\alpha$ - or  $\beta$ -thalassaemia and iron deficiency, but for several cases it resulted in an inconclusive diagnosis  $^{42,67}$ 

#### 6. OBJECTIVES OF THE THESIS

As the preceeding paragraphs have shown, several aspects of erythropoiesis and red blood cell haemoglobinisation have a bearing on the diagnosis of impaired iron metabolism in subjects with microcytic anaemia, patients undergoing haemodialysis treatment, subjects with community acquired pneumonia, and women during pregnancy. The laboratory screening for anaemia was improved by the development of new discriminating algorithms for the diagnosis of iron-deficient erythropoiesis and thalassemia.

The aim of the present thesis was to gain insight into the additional value of innovative haemocytometric parameters and to evaluate the applicability of newly derived discriminating algorithms for the screening and diagnosis of haematological abnormalities in several patient groups.

Chapter 2 describes alterations in the degree of haemoglobinisation in reticulocytes in comparison with mature red blood cells in subjects with anaemia resulting from iron deficiency and  $\alpha\text{-}$  or  $\beta\text{-}$ thalassaemia. Further understanding was obtained in the interpretation of the new parameters RET-He and RBC-He. In addition, reference intervals for Ret-He, RBC-He, Ret-He/RBC-He ratio and IRF were established.

**Chapter 3** considers the interdependence between biochemical analytes reflecting iron status and haemocytometric parameters indicating the degree of haemoglobinisation of reticulocytes and red blood cells. In subjects with uraemia, subjects treated with haemodialysis and a reference group of healthy subjects, information with regard to disturbances in erythropoiesis was examined in relation to haemocytometric parameters and concomitant evaluation of serum analytes reflecting the iron status.

**Chapter 4** describes alterations in the degree of haemoglobinisation in reticulocytes and mature red blood cells in pregnant women in the third trimester. A common feature in the

third trimester of pregnancy is the occurrence of decreased haemoglobin concentration (Hb), which is partly due to physiologic haemodilution. As the degree of haemodilution displays considerable inter-individual variation, Hb concentrations show a similar variation. Therefore, establishing reliable cut-off limits for anaemia is a complicated target. Moreover, various diagnostic guidelines used in obstetric practice recommend different cut-off points for anaemia discrimination and as an indication for ensuing iron supplementation. For example, the Hb value used by the Koninklijke Nederlandse Organisatie voor Verloskundigen is 6.3 mmol/L, whereas the World Health Organization advocates a Hb value of 6.8 mmol/L. Therefore, the present study established the additional value of using advanced red blood cell parameters during pregnancy, particularly by assessing immature reticulocyte count and reticulocyte haemoglobin content.

**Chapter 5** describes the effects of iron supplementation on the haemoglobin content of reticulocytes and red blood cells in case of suspected iron-deficient erythropoiesis in the third trimester of pregnancy.

**Chapter 6** presents *short-term* alterations with regard to Ret-He during and after completing antibiotic treatment in subjects with community-acquired pneumonia.

In a longitudinal study design, deviations of Ret-He were investigated in combination with simultaneous monitoring of biomarkers of inflammation. During inflammation proinflammatory cytokines and cells of the reticuloendothelial system induce disturbances in iron homeostasis.

**Chapter 7** presents the results of monitoring inflammation markers and hepcidin-25 concentrations together with alterations in reticulocyte haemoglobinisation (RET-He).

Chapter 8 describes the efficacy of innovative discriminating algorithms for anaemia screening, including new haematological parameters such as the percentage of hypochromic and microcytic red blood cells and parameters for haemoglobinisation of reticulocytes (Ret-He and Delta-He), in order to validate the application of discriminating algorithms for the screening of subjects with iron-deficiency anaemia (IDA) and  $\beta$ -thalassaemia. The study objectives included (1) establishing the sensitivity and specificity of new algorithms in a cohort of subjects with IDA, a group of subjects confirmed to have  $\beta$ -thalassaemia, and a control group of healthy subjects, and (2) comparing the algorithms with currently used formulas for discrimination.

**Chapter 9** presents a minireview of haematological parameters reflecting the haemoglobinisation of red blood cells and reticulocytes which are relevant discriminating between iron-deficient erythropoiesis and thalassaemia. The review demonstrates the applicability of innovative haematological parameters and algorithms in the clinical practice of microcytic erythropoiesis.

#### REFERENCES

- De Benoise B, McLean E, Egli I, Cogswell M. Worldprevalence of anaemia 1993 – 2005; WHO Global Database on anaemia. ISBN 978 92 4 159665 7.
- Hercberg S, Preziosi P, Galan P. Iron deficiency in Europe. Public Health Nutrition 2001;4:537-545.
- 3. Beard J, Tobin D. Iron status and exercise. Am J Clin Nutr 2000;72:594S-597S.
- Burden R, Morton K, Richards T, Whyte GP, Pedlar Cr. Is iron treatment beneficial in, iron-deficient but non-anaemic (IDNA) endurance athletes? A metaanalysis. Br J Sports Med 2014;0:1-10. doi: 10.1136bjsports-2014-093624. (Epub ahead of print)
- 5. Telford RD, Sly GJ, Hahn AG, Cunningham RB, Bryant C, Smith JA. Foot-strike is a major cause of hemolysis during running. J Appl Physiol 2003;94:38-42.
- Chui Y-H, Lai J-I, Wang S-H, How C-K, Li L-H, Kao W-F, Yang C-C, Chen R-J. Early changes of the anemia phenomenon in male 100-km ultramarathoners. J Chin Med Ass 2014;xx:1-6. doi: 10.1016/j. jcma.2014.09.004. (Epub ahead of print)
- Peeling P, Sim M, Badenhorst CE, Dawson B, Govus AD, Abbiss CR, Swinkels DW, Tinder D. Iron status and the acute post-exercise hepcidin response in athletes. PLos One 2014;9:e93002 doi: 10.1371/journal.pone.0093002. (Epub ahead of print)
- DeLoughery TC. Microcytic anemia. N Engl J Med 2014;371:1324-1331.
- Beutler E. Production and destruction of erythrocytes. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Selogsohn U. Williams Hematology, 6th edition, part V. New York: Mc Graw-Hill, 2001:355-368.
- 10. Davies BH. Immature reticulocyte fraction (IRF): by name a useful clinical parameter. Lab Hematol 1996;2:2-8.

- Thomas DW, Hinchliffe RF, Briggs C, Macdougall IC, Littlewood T and Cavill I. Guideline for the laboratory diagnosis of functional iron deficiency. Brit J Haematol 2013;161:639–648.
- Ranney HM, Sharma V. Structure and function of haemoglobin. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Selogsohn U. Williams Hematology, sixth edition, part V. New York: Mc Graw-Hill, 2001:345-353.
- 13. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. The Lancet 2012;379:373-383.
- 14. Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th edition Blackwell Science: Oxford. 2001.
- Maude GH, Higgs DR, Beckford M, Grandison Y, Mason K, Taylor B, Serjeant BE, Serjeant GR. Alpha thalassaemia and the haematology of normal Jamaican children. Clin Lab Haematol 1985;7:289-295.
- Pornpatkul M, Wasi P, Na-Nakorn S. Haematologic parameters in obligatory alpha thalassaemia traits. J Med Assoc Thai 1969;52:801-809.
- 17. E.J. Harthoorn. Erythrocyte zinc protoporphyrin in the diagnosis of iron deficiency. Thesis, Vrije Universiteit Amsterdam, 2000.
- Modell B, Darlison M, Birgens H, Cario H, Faustino P, Giordano PC, Gulbis B, Hopmeier P, Lena-Russo D, Romao L, Theodorsson E. Epidemiology of haemoglobin disorders in Europe; an overview. Scand J Lab Invest 2007;67:39-70.
- Green R. Nutritional Anemia. In: K Kottke- Marchant and B.H. Davis. Laboratory Hematology Practice. Chapter 45. ISBN-13: 978-1-4051-6218-0.
- Labbé RF, Rettmer RL. Zinc protoporphyrin: A product of iron deficient erythropoiesis. Semin Hematol 1989;26:40-46.

- NHG werkgroep Anemie, oktober 2014.
   NHG Richtlijn Anemie. www.nhg.org/standaarden/volledig/nhg-standaard anemie.
- Macdougall IC, Hutton RD, Cavill I, Coles GA, Williams JD. Poor response to treatment of renal anaemia with erythropoietin corrected by iron given intravenously. British Medical Journal 1989;299:157-158.
- 23. Ganz T, Nemeth E. Iron sequestration and anemia of inflammation. Seminars in hematology 2009;46:387-393.
- 24. Goodnough LT, Nemeth E, Ganz T. Detection, evaluation and management of iron-restricted erythropoiesis. Blood 2011;116:4754-4761.
- Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol 2009;122:78-86 (DOI:10.1159/000243791).
- Kroot JJC, Tjalsma H, Fleming RE, Swinkles DW. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-1669.
- Nemeth E, Tuttle MS, Powelson J, Vaughin MB, Donovon A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron reflux binding to ferroportin and inducting its internalisation. Science 2004;306:2190-2095.
- 28. van Wyk DB, Danielson BG, Aronoff GR. Making sense a scientific approach to intravenous iron therapy. J Am Soc Nephrol 2004;15 (Suppl.2):S91-S92.
- 29. Sherwood RA, Pippard MJ, Peters TJ. Iron homeostasis and the assessment of iron status. Ann Clin Biochem 1998;36:693-708.
- Witte DL, Kraemer DF, Johnson GF, Dick FR, Hamilton H. Prediction of bone marrow iron findings from tests performed on peripheral blood. Am J Clin Pathol 1986;85:202-206.
- 31. Baynes RD. Assessment of iron status. Clin Biochem 1996;29:209-215.
- 32. Worwood M. Laboratory determination of iron status. In: Brock JH, Halliday JW, Pippard MJ, Powell LW. Iron metabolism in

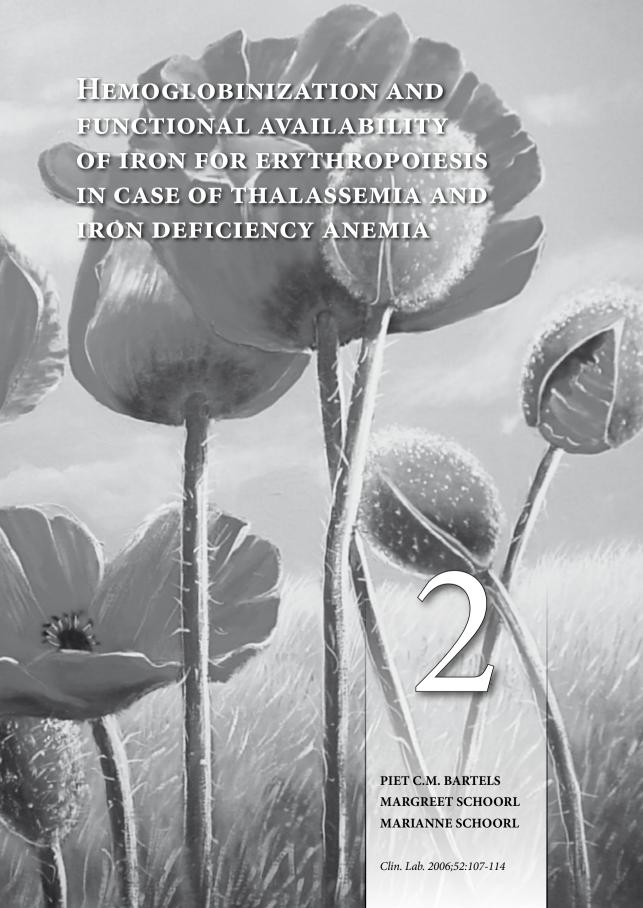
- health and disease. London: WB Saunders, 1994:449-447. ISBN 0-7020-1732-9
- 33. Cook JD, Lipschitz DA, Miles LE, Finch CA. Serum ferritin as a measure of iron stores in normal subjects. Am J Clin Nutr 1974;27:681-687.
- Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: a quantitative measure of iron efficiency. Blood 1990;75:1870-1876.
- 35. Beguin Y. Soluble transferrin receptor for the evaluation of erythropoiesis and iron status. Clin Chim Acta 2003;329:9-22.
- Rees DC, Williams TN, Maitland K, Clegg JB, Weatherall DJ. Alpha thalassemia is associated with increased soluble transferrin receptor levels. Br J Haematol 1998;103:365-369.
- 37. Thorpe SJ, Heath A, Sharp G, Cook J, Ellis R, Worwood M. A WHO Reference Reagent for the Serum Transferrin Receptor (sTfR): international collaborative study to evaluate a recombinant soluble transferrin receptor preparation. Clin Chem Lab Med 2010:48:815–820.
- 38. Punnonen K, Irjala K, Rajamaki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency. Blood 1997;89:1052-1057.
- 39. Chen YC, Hung SC, Tarng DC. Association between transferrin receptor-ferritin index and conventional measures of iron responsiveness in haemodialysis patients. Am J Kidney Dis 2006;47:1036-1044.
- 40. Thomas WJ, Koenig HM, Lightsey AL Jr, Green R. Free erythrocyte porphyrin: haemoglobin ratios, serum ferrtin, and transferring saturation levels during treatment of infants with iron-deficiency anemia. Blood 1977;49:455-462.
- 41. Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM. Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassaemia from iron deficiency anemia. Eur J Hematol 1998;60:245-251.

- Bartels P C M, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocytes count and red cell zinc protoporphyrin for appropriate classification of microcytic anaemia. Sysmex J Int 2003;13:78-82.
- 43. Mijares IA, Lopez P, Rosado JL, Cebrián A, Vera-Aguilar E, Alatore J, Quintanilla-Vega MB, García AE, Stolzfus RJ, Cebrián ME, García-Vargas GG. Delta-aminole-vulinic acid dehydratase genotype and its relationship with blood lead and zinc protoporphyrin levels in lead-exposed children living in a smelter community in Northern Mexico. Toxicol Mech Methods 2006;16:41-44.
- 44. Thomas C, Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.
- Simson E. Historical Perspective on cellular analysis. In: K Kottke- Marchant and B.H. Davis. Laboratory Hematology Practice. Chapter 1. ISBN-13:978-1-4051-6218-0.
- Mohandas N. Gallager PG. Red cell membrane; past, present and future. Blood 2008;112:3939-3948.
- Wintrobe MM. Blood, pure and eloquent: a story of discovery, of people, and of ideas. New York: McGraw-Hill Book Company; 1980.
- 48. Zandecki M, Genevieve F, Gérard J, Godon A. Spurious counts and spurios results on hematology analyzers, white blood cells, red blood cells, hemoglobin red cell indices, and reticulocytes. In: K Kottke- Marchant and B.H. Davis. Laboratory Hematology Practice. Chapter 8. ISBN-13: 978-1-4051-6218-0.
- Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches.
   Clin Chem 2003;49:1573-1578.
- 50. Thomas L, Franck S, Messinger M, Linssen J, Thome M, Thomas C. Reticulocyte

- hemoglobin measurement: comparison of two methods in the diagnosis of iron-restricted erythropoiesis. Clin Chem Lab Med 2005;43:1193-1202.
- 51. Canals C, Remacha AF, Sarda MP, Piazuelo JM, Royo MT, Romero MA. Clinical utility of the new Sysmex XE2100 parameter reticulocyte haemoglobin equivalent in de diagnosis of anemia. Hematologica 2005;90:1133-1134.
- 52. Brugnara C, Schiller D, Moran J. Reticulocyte haemoglobin content (Ret-He) and assessment of iron-deficient states. Clin Lab Haematol 2006;28:303-308.
- 53. Miwa N, Akiba T, Kimata N, Hamagushi Y, Arakawa Y, Tamura T, Nitta K, Tsuchiya K. Usefulness of measuring reticulocyte haemoglobin equivalent in the management of haemodialysis patient with iron deficiency. Int J Lab Hematol 2010;32:248-255.
- Mast AE, Blinder MA, Lu Q, Flax S, Dietzen DJ. Clinical utility of the reticulocyte hemoglobin content in the diagnosis of iron deficiency. Blood 2002;99:1489-1491.
- Franck S, Linssen J, Messinger M, Thomas L. Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. Clin Chem 2004;50:1240-1242.
- Navarro JF, Macia ML. Hypochromic red cells as an indicator of iron deficiency. J Rheumatol 1997;24:804-805.
- Macdougall IC. Merits of hypochromic red cells as a marker of functional iron deficiency. Nephrol Dial Transplant 1998;13:847-849.
- England JM, Fraser PM. Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet 1973;i:449-452.
- 59. Green R, King R. Red cell discriminant function,  $\beta$ -thalassaemia minor versus iron deficiency. Blood cells 1989;15:481-495.
- Mentzer WC. Differentiation of iron deficiency from thalassaemia trait. Lancet 1973;i:882.

- 61. Stockman JA, Weiner LS, Simon GE, Stuart MJ, Oski FA. The measurement of free erythrocyte protoporphyrin (FEP) as a simple means of distinguishing from iron deficiency from beta thalassaemia trait in subjects with microcytosis. J Lab Clin Med 1975;85:113-119.
- 62. Koenig HM, Lightsey AL, Schanberger JE. The micro-measurement of free erythrocyte protoporphyrin as a means of differentiation alpha thalassaemia trait from iron deficiency anemia. J Pediatr 1975; 86:539-541.
- 63. Han P, Fung KP, Teo CP, Tam LP. Alphathalassaemia and beta-thalassaemia traits: biological difference based on red cell indices and zinc protoporphyrin. Clin Lab Haematol 1990:12:169-176.

- 64. Haska J, Lasserre JJ, Schwarzbeck A, Reiter A, Hehlmann R. Laboratory test of iron status: correlation or common sense? Clin Chem 1996;42:718-724.
- 65. Labbé RF, Vreman HJ, Stevenson DK. Zinc protoporphyrin: a metabolite with a mission. Clin Chem 1999;45:2060-2072.
- 66. Akai Y, Kubota F, Nakamoto H, et al. A study of  $\beta$ -thalassaemia screening using an automated haematology analyzer. Sysmex J Int 1998;8:110-114.
- 67. Eldibany MM, Totonchi KF, Joseph NJ, Rhone D. Usefulness of certain red blood cell indices in diagnosing and differentiating thalassaemia trait from iron deficiency. Am J Clin Pathol 1999;111:676-682.



#### **ABSTRACT**

Microcytic erythropoiesis in case of anemia is frequently due to iron deficiency or may be due to  $\alpha$ - and  $\beta$ -thalassemia trait as a result of increased activity of erythropoiesis. The aim of the present study was to evaluate alterations with regard to the degree of hemoglobinization in reticulocytes in comparison with mature erythrocytes. Iron availability in subjects with anemia resulting from iron deficiency and  $\alpha$ - or  $\beta$ -thalassemia was studied by application of conventional as well as hemocytometric parameters that have recently become available. Participants of the study were reference subjects (n=75), subjects with iron deficiency anemia (IDA, n=52) and  $\alpha$ - (n=26) or  $\beta$ -thalassemia trait (n=24). If compared with the reference group obviously increased RBC counts together with decreased values for RDW-sd and MCHC were established in case of  $\alpha$ - and  $\beta$ -thalassemia subjects. Deviations were demonstrated to be more pronounced in case of β-thalassemia. Accelerated erythropoiesis in the case of subjects with IDA and β-thalassemia is manifested by detection of increased results for immature reticulocyte counts. In particular in case of  $\beta$ -thalassemia, elevated reticulocyte counts combined with slightly increased values for ZPP/heme ratio reflect increased activity of erythropoiesis. In the case of subjects with  $\beta$ -thalassemia serum transferrin concentrations revealed slightly decreased results, whereas serum ferritin and iron concentrations demonstrated a tendency towards higher values if compared with the group of reference subjects. At a definite MCV level, the hemoglobin content of reticulocytes is decreased in the case of IDA if compared with the  $\alpha$ - or  $\beta$ -thalassemia trait. For the ratio of hemoglobin content of reticulocytes and erythrocytes, obviously decreased results are demonstrated in the case of subjects with iron deficiency anemia (1.02  $\pm$  0.08, mean  $\pm$ SD) and in the case of  $\beta$ -thalassemia (1.06  $\pm$  0.04) if compared with the group of reference subjects  $(1.11 \pm 0.02)$  and  $\alpha$ -thalassemia  $(1.11 \pm 0.07)$ . Evaluation of the hemoglobinization state should be performed by means of pattern recognition in concordance with characteristic profiles for parameters reflecting the actual iron state. In case of therapy the result of intervention can be appropriately monitored by longitudinal follow-up.

#### INTRODUCTION

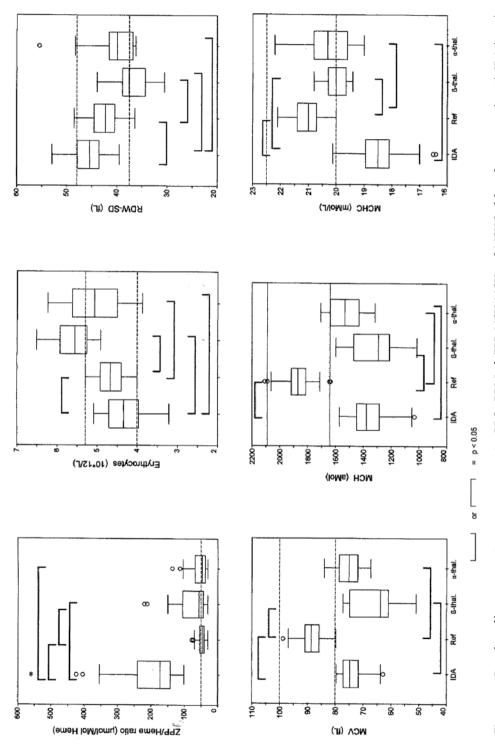
Microcytic anemia is frequently due to iron deficiency or thalassemia. Iron deficiency anemia (IDA) may result from insufficient dietary iron intake, particularly in women of childbearing age. In case of elderly subjects, chronic blood loss in the gastrointestinal tract may occur. Iron deficiency results, amongst others, in impaired activity of several enzymes. As a consequence, iron depletion may give rise to serious health problems such as retarded growth, mental irritability, reduced resistance to infection and impaired intellectual development, particularly in the case of infants in the growth phase (1).

Functional iron deficiency refers to a physiological state of normal or even increased iron content of the body. Nevertheless, iron availability may yet be insufficient for hemoglobinization in erythropoiesis. Functional iron deficiency occurs, for instance, in subjects with chronic inflammation, malignancy or chronic treatment with hemodialysis.

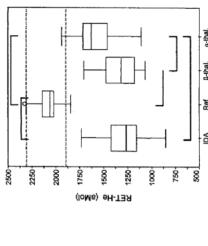
Microcytic anemia in case of thalassemia results from impaired globin chain synthesis and decreased hemoglobinization of RBCs (2,3).

For clinical interpretation and pathophysiological evaluation it should be investigated which parameters demonstrate results that are indicative of functional iron availability. On the basis of results of single hemocytometric parameters, subjects with iron deficiency are inappropriately discriminated from subjects with anemia due to thalassemia or chronic disease (ACD) (1). As a state of iron deficiency proceeds, results of MCV, MCH and RBC count demonstrate a tendency to decline (4,5). With decreased MCV values a tendency towards increased results for RDW-sd and the ZPP/heme ratio is observed (6) Considerations concerning the Hb content of reticulocytes (RET-He) have resulted from recently introduced innovative methodology for reticulocyte counting and qualification of RET-He, amongst others by application of a Sysmex XE-2100 haematology analyzer. RET-He has been demonstrated to be a rather sensitive indicator for detection of functional iron deficiency (7,8).

The aim of the present study was to gain insight with respect to the interpretation of RET-He combined with evaluation of MCV for the detection of functional iron availability in case of IDA and to establish characteristic deviations in the degree of hemoglobinization in subjects with  $\alpha$ - or  $\beta$ -thalassemia.



 $\alpha$ -thalassemia trait (n=26) and  $\beta$ -thalassemia trait (n=24). Box extends from the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to largest and smallest observed results within 1.5 box lengths. Outlying and extreme results corresponding with Box plots of hemocytometric parameters concerning RBCs, RDW-sd, ZPP, MCV, MCH and MCHC of the reference group (n=75), IDA (n=52) values between 1.5-3x box length or > 3x box length, respectively, are designated as (0) and (\*). Figure 1.



Red ĕ

Figure 2.

ters concerning reticulocytes, immature

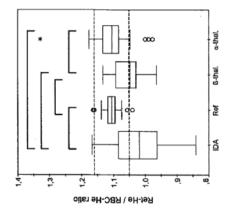
Box plots of hemocytometric parame-

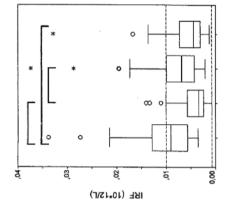
RBC-He and IRF of the reference group (n=75), IDA (n=52) and  $\alpha$ -thalassemia rait (n=26) and  $\beta$ -thalassemia (n=24). Box extends from the 25th to the 75th per-

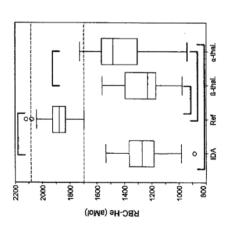
reticulocyte fraction (IRF), RET-He,

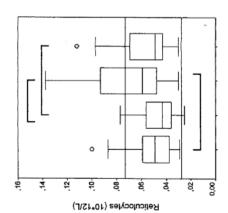
centile. The line inside the box indicates he median value. Whiskers extend to largest and smallest observed values reme results corresponding with values netween 1.5-3x box length or > 3x box length, respectively, are designated as (0)

vithin 1.5 box lengths. Outlying and ex-









= p < 0.05

#### SUBJECTS, MATERIALS AND METHODS

#### Criteria for selecting the groups of subjects

#### Reference subjects

Blood samples of apparently healthy subjects (n=75) were obtained from the Sanquin Blood Transfusion Department Northern Holland, Amsterdam, The Netherlands.

#### Subjects with iron deficient anemia (IDA)

The group consisted of 52 subjects with Hb <7.5 mMol/L and CRP < 5 mg/L, ferritin concentration < 15  $\mu$ g/L, ZPP/heme ratio > 100  $\mu$ Mol/Mol heme.

#### Subjects with $\alpha$ - and $\beta$ -thalassemia trait

The group of thalassemia subjects consisted of subjects with  $\alpha$ -thalassemia trait (n=26) and  $\beta$ -thalassemia trait (n=24), respectively. Detection of abnormal DNA-composition at the  $\alpha$ -chain of hemoglobin was considered to be confirmatory for  $\alpha$ -thalassemia trait, whereas an increased HbA2 content was considered to be confirmatory for  $\beta$ -thalassemia trait. Only subjects with thalassemia trait with serum ferritin concentrations > 15  $\mu$ g/L were included in the study.

#### Preparation of blood samples

Blood samples were drawn into Vacutainer® tubes, anticoagulated with  $K_2$ EDTA (Becton Dickinson, Plymouth, UK) and plain tubes (Vacutainer SST, Becton Dickinson, Plymouth, UK). Blood samples were analyzed within 4 hours after collection. For additional investigations of biochemical parameters reflecting iron state serum samples were frozen in aliquots at -20 °C until analysis.

#### Hemocytometry

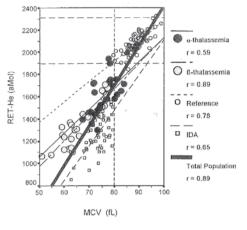
Hemocytometric parameters were measured on a Sysmex XE-2100 Haematology Analyzer (Sysmex Corporation, Kobe, Japan). The methodology of measurement is based on automated fluorescent flow cytometry utilizing a polymethine dye for binding cytoplasmic RNA in reticulocytes. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure for the volume and Hb content of RBCs and reticulocytes respectively. Results concerning Hb content are initially reported as RBC-Y for RBCs and RET-Y for reticulocytes. Subsequently, algorithms

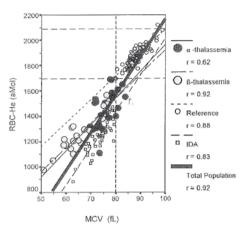
$$y = 400 \text{ x} e^{0.0009 \text{ x Ret-Y}}$$
 and  $y = 400 \text{ x} e^{0.0009 \text{ x RBC-Y}}$ 

are applied in order to transform arbitrarily reported channel numbers of the RET-Y and RBC-Y into hemoglobin equivalents (9). Hemoglobin equivalents are expressed in attomol and denoted as RET-He and RBC-He respectively.

#### Parameters reflecting iron state

Measurement of ZPP/heme ratio in RBC suspensions was performed on a dedicated haematofluorometer (AVIV Biochemical Inc., Lakewood NJ 08107, USA) with application of front surface illumination fluorometry. Serum concentrations of soluble transferrin receptor and transferrin were established by application of a Prospec nephelometer (Dade





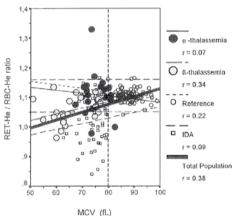


Figure 3. Results for RET-He, RBC-He and RET-He/RBC-He ratio plotted versus MCV values for subjects belonging to the respective groups: reference subjects, IDA,  $\alpha$ -thalassemia trait and  $\beta$ -thalassemia trait.

Behring, Marburg, Germany). Serum iron concentrations were established by application of a Vitros 950 IRC analyzer (Ortho Clinical Diagnostics NV, Beerse, Belgium). Serum ferritin levels were established on an ADVIA Centaur Analyzer (Bayer, Mijdrecht, The Netherlands) by using a two-site sandwich immunoassay.

# Statistical evaluation

The statistical software package SPSS/PC, version 11.5 for Windows, was applied for statistical analysis of results (SPSS, Chicago, IL). Independent Samples T-test was performed in order to detect statistically significant deviations between the groups of subjects.

Median values together with 2.5th and 97.5th percentiles with regard to hemocytometric results and results for analytes reflecting iron state in the groups of reference subjects (n=75) and subjects with IDA (n=52),  $\beta$ -thalassemia (n=24) and  $\alpha$ -thalassemia (n=26), respectively. Table 1.

	Kererence	IDA	β-thalassemia	$\alpha$ -thalassemia	Statistical
rarameters	subjects group (N=75)	(N=52)	(N=24)	(N=26)	signincance between groups
	Median (p2.5-p97.5)	Median (p2.5-p97.5)	Median (p2.5-p97.5)	Median (p2.5 -p97.5)	
RDW-SD (fL)	42.3 (37.5 - 48.1)	45.4 (39.5 - 52.6)	37.6 (30.5 - 43.9)	39.9 (36.2 - 55.5)	1,2,4,5,6
Hb (mMol/L)	8.7 (7.6 - 10.1)	6.0 (4.1 - 7.5)	7.6 (6.0 - 8.7)	7.8 (6.0 - 9.7)	1,2,3,4,5
RBC $(10^{12}/L)$	4.67 (4.05 - 5.28)	4.34 (3.28 - 5.08)	5.55 (4.91 - 6.51)	5.07 (3.87 - 6.23)	1,2,3,4,5,6
MCV (fL)	88.6 (80.2 - 97.1)	74.7 (63.1 - 79.5)	63.8 (50.9 - 77.2)	75.1 (67.2 - 84.0)	1,2,3,4,6
MCH (aMoI)	1868 (1641 - 2089)	1369 (1037 - 1558)	1292 (1015 - 1597)	1534 (1314 - 1705)	1,2,3,4,6
MCHC (mMol/L)	21.0 (20.0 - 22.1)	18.5 (16.4 - 20.0)	20.0 (19.4 - 20.8)	20.3 (19.0 - 22.2)	1,2,3,4,5
Reticulocytes (10 <sup>12</sup> /L)	0.043 (0.028 - 0.073)	0.049 (0.029 - 0.096)	0.059 (0.030 - 0.138)	0.049 (0.030 - 0.112)	2,3,4
RET-He (aMol)	2060 (1897 - 2309)	1265 (879 - 1685)	1318 (1066 - 1708)	1627 (1106 - 1939)	1,2,3,5,6
RBC-He (aMol)	1876 (1698 - 2083)	1272 (916 - 1516)	1230 (975 - 1564)	1484 (940 - 1727)	1,2,3,5,6
IRF $(10^{12}/L)$	0.003 (0.001 - 0.013)	0.009 (0.004 - 0.032)	0.007 (0.002 - 0.037)	0.004 (0.001 - 0.032)	1,2,5
RET-He / RBC-He	1.10 (1.05 - 1.16)	1.02 (0.85 - 1.16)	1.05 (0.96 - 1.13)	1.10(0.98 - 1.33)	1,2,4,5,6
lron (μMol/L)	16.6 (6.9 - 29.5)	3.4(1.0 - 8.8)	21.0 (7.1 - 28.7)	15.9 (8.7 - 27.0)	1,4,5
Transferrin (g/L)	2.53 (1.84 - 3.28)	2.80 (1.42 - 4.16)	2.04 (1.24 - 2.99)	2.66 (1.52 - 3.81)	1,2,4
Ferritin (µg/L)	21 (7 - 140)	4 (1 - 32)	102 (14 - 653)	65 (8 - 458)	1,2,3,4,5
sTfR (mg/L)	1.2 (0.8 - 2.0)	3.5 (1.7 - 8.3)	1.2 (0.8 - 1.8)	1.6 (1.3 - 1.8)	1,4,5
ZPP/Heme (IIMol/Mol Heme)	45 (32 - 73)	173 (103 - 516)	56 (30 - 219)	47 (30 - 136)	1,2,4,5
sTfR/log ferritin	0.9 (0.4 - 2.2)	5.2 (2.6 - 15.4)	0.6 (0.4 - 1.0)	0.7 (0.5 - 0.9)	1,2,4,5

1: statistical significance between reference subjects' group and IDA (p< 0.05) 2: statistical significance between reference subjects' group and B-thalassemia ( $\rho$ <0.05) 3: statistical significance between reference subjects' group and  $\alpha$ -thalassemia ( $\rho$ <0.05) 4: statistical significance between IDA and  $\beta$ -thalassemia 5: statistical significance between IDA and  $\alpha$ -thalassemia (p< 0.05) 6: statistical significance between  $\beta$ -thalassemia and  $\alpha$ -thalassemia (p< 0.05). (p < 0.05)

## **RESULTS**

Box plots representing the results of hemocytometric parameters are depicted in Figures 1 and 2 for the group of reference subjects (n=75), IDA (n=52),  $\alpha$  (n=26) and  $\beta$ -thalassemia trait (n=24), respectively. Statistically significant deviations between the groups are indicated in the Figures.

In Figure 1 is demonstrated that the IDA subjects' group can be discriminated from the reference subjects' group on the basis of parameters such as RBC count, RDW-sd, ZPP/heme ratio, MCV, MCH and MCHC (p = 0.000). If compared to subjects with IDA, subjects with  $\beta$ -thalassemia trait yield markedly reduced results for MCV and RDW-sd (p=0.000). Except for the results of RBC, MCV, MCH and RDW-sd, a considerable overlap was established for hemocytometric parameters from the  $\alpha$ - and  $\beta$ -thalassemia groups. As depicted in Figure 2 the reticulocytes counts in subjects with  $\alpha$ - or  $\beta$ -thalassemia trait are slightly increased if compared with results from the reference subjects' group (p=0.028 and p=0.001 respectively). In case of subjects with  $\beta$ -thalassemia trait the ZPP/heme ratio results as listed in Table I are markedly increased if compared with the reference subjects' group (p = 0.007). Discrimination of subjects with IDA and  $\beta$ -thalassemia trait can be performed by application of the results obtained for RBC, RDW-sd, MCV, MCHC, ZPP/heme ratio (p=0.000) and reticulocyte counts (p=0.005). Discrimination of subjects with IDA and  $\alpha$ -thalassemia trait can be appropriately performed by application of results for RBC, MCHC, MCH, RDW-sd and ZPP/heme ratio (p = 0.000).

Results concerning RET-He, RBC-He and the RET-He/RBC-He ratios are depicted in Figure 2. In the case of subjects with IDA results concerning IRF, RET-He, RBC-He and RET-He/RBC-He ratio were demonstrated to be statistically significantly different (p=0.000) from the results of the reference subjects' group. If compared with the hemocytometric parameters of the reference group the  $\beta$ -thalassemia subjects' group revealed statistically significant deviations for the parameters IRF (p = 0.006), RET-He, RBC-He and RET-He/RBC-He (p = 0.000). The  $\alpha$ -thalassemia subjects' group yielded statistically significant deviations in comparison with the reference subjects' group for RET-He and RBC-He (p = 0.000). Comparison of results for the IDA and the  $\alpha$ -thalassemia subjects' groups revealed statistically significant deviations in the case of RET-He, RBC-He and RET-He/RBC-He (p = 0.000) .

In the  $\beta$ -thalassemia subjects' group the results of RET-He, and RBC-He were not statistically significant from results in the case of IDA. For the parameters just mentioned, both groups demonstrated a considerable overlap, in contrast with results for the RET-He/RBC-He ratio (p = 0.012). If compared with IDA subjects, subjects with  $\alpha$ -thalassemia trait yielded markedly increased results for RET-He (p = 0.000), RBC-He (p = 0.001) and RET-He/RBC-He ratio (p = 0.003).

Median values, together with 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles ranges, for hemocytometric results together with parameters reflecting iron status in the various groups of subjects are listed in Table I. If compared with subjects with  $\alpha$ - and  $\beta$ -thalassemia trait, subjects with IDA yielded markedly reduced levels for serum iron (p = 0.000) and ferritin concentrations (p = 0.013 and p = 0.004), respectively, whereas increased results were established in the case of IDA for the ZPP/heme ratio (p = 0.000). Transferrin concentrations in subjects with IDA were increased if compared with  $\beta$ -thalassemia trait (p=0.000).

In the  $\alpha$ - and  $\beta$ -thalassemia group statistically significant deviations for iron state parameters reflecting iron deficiency could not be detected.

At a definite MCV value, results represented in Figure 3 indicate decreased results for RBC-He as well as for RET-He in the case of subjects with IDA if compared with the thalassemia subjects. A less pronounced decrease of hemoglobin content in RBCs and reticulocytes was established in subjects with  $\alpha$ -thalassemia. Decreased RET-He/RBC-He ratios were established in IDA and  $\beta$ -thalassemia subjects.

# **DISCUSSION**

Reticulocytes are slightly immature RBCs representing output of erythroid proliferation immediately preceding the stage of mature RBCs. Reticulocytes are only for a few days recognizable in peripheral blood before developing into mature RBCs.

In the case of IDA and thalassemia increased IRF results reflect accelerated output of immature reticulocytes if compared with the group of reference subjects. Reticulocyte maturation occurs with progressive reduction in RBC size and Hb content (7). A close relationship is established between RBC-He and RET-He (10) because forward light scatter intensity is related to size and hemoglobin content of RBCs and reticulocytes, respectively. In subjects with depleted iron stores RET-He will increase within a few days if iron supplements are administered. In contrast, RET-He will decrease quickly in subjects with iron deficient erythropoiesis (10). With steadily decreasing RET-He content in this condition, reticulocytes will become gradually smaller if compared with circulating RBCs. If compared with mature RBCs, RET-He is able to yield more appropriate information concerning various states of functional availability of iron for hemoglobinization of erythroid precursors, amongst others in the case of subjects with inflammation, cancer, hemodialysis (11).

The ZPP/heme ratio is concluded to be an appropriate indicator for classification of microcytic erythropoiesis (12). Iron deficient erythropoiesis without complications can be appropriately identified by detection of RBCs with decreased Hb content combined with increased ZPP/heme ratio (13).

However, increased ZPP/heme results are not only due to iron deficiency but may also be indicative of disarrangements of iron utilization in case of chronic inflammatory states and neoplasms (14). In case of normal or only slightly increased ZPP/heme results, IDA can be excluded.

Iron utilization is a dynamic process which cannot be defined by one test parameter only. Therefore, in case of screening procedures for iron deficiency which are based on hemocytometry, application of serum parameters reflecting the actual iron state would be obligatory when results are beyond reference ranges.

Serum iron and transferrin saturation are influenced by acute phase response and strongly affected by the circadian rhythm. In the case of clinical interpretation of ferritin results, conditions such as inflammation, hepatic disease, malignant neoplasms, chronic disease and application of oral contraceptives should be taken into consideration (10). Discrimination between subjects with IDA and anemia due to chronic disease may yield serious

problems (14). Therefore, when considering clinical interpretation of iron state parameters, infection parameters like CRP should also be evaluated before conclusive diagnosis is made.

In case of increased CRP concentrations combined with ferritin and sTfR concentrations within the reference range, reduction of RET-He or RET-He/RBC-He ratio is considered to be a conclusive indicator for the detection of actual iron deficiency.

In the concept of functional iron deficiency it is assumed that demand for utilization of iron in the erythroid and release of iron from stores should be in good balance. Algorithms combining results from simultaneous determinations of serum sTfR and ferritin concentrations have been introduced for evaluation of disturbances in the balance. A frequently applied index is the sTfR/ log ferritin ratio. It has been demonstrated that increased values reliably indicate functional iron deficiency (11).

Thalassemia trait is initially suspected on the basis of evaluation of hemocytometric parameters. Irrespective of the presence of inflammation, reticulocyte hemoglobin content is decreased in the case of IDA.

In agreement with the results of previous studies it is demonstrated in Figures 1 and 2 that discrimination between subjects with thalassemia trait and IDA is mainly supported by detection of decreased RBC production and increased RDW-sd values in the case of IDA (3,15). Classification of deviations in hemocytometric parameters reflecting actual output of erythropoiesis yields additional value for differential diagnosis. In  $\alpha$ - and  $\beta$ -thalassemia reticulocyte counts revealed increased results if compared with IDA. In  $\beta$ -thalassemia, ferrokinetic studies are indicative of detection of ineffective erythropoiesis, whereas a wide range of case-to-case variation has been observed (16).

It has already been demonstrated in previous studies that RET-He adds an appropriate measure for the detection of IDA (4,7,17,18). However, decreased results for RET-He indicating a reduced degree of hemoglobinization in reticulocytes are also observed in case of  $\alpha$ - and  $\beta$ -thalassemia trait. In our study combined evaluation of RET-He and MCV results revealed new insights with regard to discrimination between the groups of subjects. If compared with results from the  $\alpha$ - and  $\beta$ -thalassemia groups the degree of hemoglobinization at a definite MCV value is obviously decreased in the case of IDA (Figure 3).

The results of our study are in agreement with studies that demonstrate a better correlation between RBC-He and MCH (r=0.97) if compared with RBC-He and MCV (r=0.89) (8,9). In the case of subjects with IDA, a shortened lifespan of RBCs may amongst others, be due to decreased RBC deformability (19). Alterations in the geometry of RBCs probably account for exaggerated impedance signals which yield deviations of RBC sizes.

Correlation between RET-He and mean reticulocyte volume was marginally less good. This observation is explained by the fact that the degree of forward scattering applied for RET-He measurement is related not only to cell size but mainly to cell content (10).

After a longer time period of iron deficiency reduced RET-He results are observed in comparison with RBC-He. In order to evaluate whether RET-He/RBC-He ratio would supply additional information, the ratio was established for the respective groups of subjects. For subjects in the reference group the ratio amounted to  $1.10 \pm 0.02$  (mean  $\pm$  SD). In the case of subjects with IDA,  $\alpha$ -thalassemia and  $\beta$ -thalassemia trait the RET-He/RBC-He ratios amounted to  $1.02 - \pm 0.08$ ,  $1.11 \pm 0.07$ , and  $1.06 \pm 0.04$  (mean  $\pm$  SD), respectively. In contrast to a previous study (20), ratios below 1.00 were observed in our study, implicating

that the Hb content of reticulocytes is smaller if compared with the Hb content of RBCs. Decreased RET-He results in comparison with RBC-He are indicative of acute onset of iron deficiency (18, 20). The finding was supported by Brugnara by comparing RET-He and MCH (7).

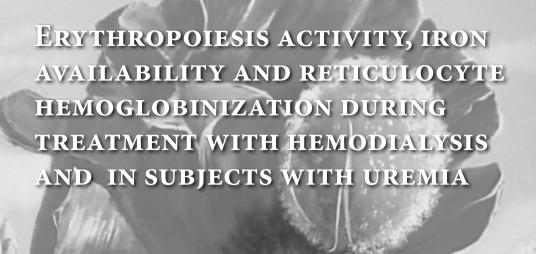
The volume of RBCs, measured by application of aperture impedance technology, is not only determined by cell volume, but also by cell shape, orientation, viscosity and deformability, which are highly dependent on Hb concentration in RBCs (21). In a recent study (22) after transformation of original data a linear relationship has been established between Ret-He (Sysmex XE-2100) and reticulocyte Hb content (Bayer ADVIA 120). A cut off level for RET-He of 1750 aMol has been published as a tool for discrimination of subjects belonging to the reference group from subjects with microcytic anemia (9). In the present study a two-dimensional approach is recommended for appropriate interpretation of results below the cut off level. Interpretation which is based on a combined plot of RET-He or RET-He/RBC-He ratio with regard to MCV results yields a sophisticated method for gaining insight into the degree of hemoglobinization and RBC characteristics in the case of subjects with IDA and thalassemia.

## REFERENCES

- Fairbanks VF, Beutler E. Iron deficiency. In: Beutler E, Lichtman MA, Coller B, Kipps TJ, Seligsohn U (eds) Williams Hematology, 6<sup>th</sup> edition. New York: Mc Graw-Hill, 2001:447-470
- 2. Bunyaratvej A, Fucharoen S, Greenbaum A, Mohandas N. Hydration of red cell in  $\alpha$  and  $\beta$ -thalassemias differs. Am J Clin Pathol 1994;102:217-222
- Eldibany MM, Totonchi KF, Joseph NJ, Rhone D. Usefulness of certain red blood cell indices in diagnosing and differentiating thalassemia trait from iron-deficiency anemia. Am J Clin Pathol 1999;111:676-682
- Briggs C, Rogers R, Thompson B, Machin SJ. New red cell parameters on the Sysmex XE-2100 as potential markers of functional iron deficiency. Sysmex J Int 2001;11:63-68
- Schaefer M, Reinecke T. The analysis of iron status in clinical laboratories. Sysmex J Int 2001;11:59-62
- Baldus M, Walter H, Thies K, Stein M, Hellstern P, Brass H. Transferrin receptor assay and zinc protoporphyrin as markers of iron-deficient erythropoiesis in endstage renal disease patients. Clin Neph 1998;49:186-189
- Brugnara C. Reticulocyte cellular indices: a new approach in the diagnosis of anemias and monitoring of erythropoietic function. Crit Rev Clin Lab Sci 2000;37:93-130
- Noronha JFA, Grotto HZW. Measurement of reticulocyte and red blood cell indices in patients with iron deficiency anemia and β-thalassemia minor. Clin Chem Lab Med 2005;43:195-197
- Franck S, Linssen J, Messinger M, Thomas L. Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. Clin Chem 2004;50:1240-1242
- 10. Buttarello M, Temporin V, Ceravolo R, Farina G, Bulian P. The new reticulocyte

- size parameter (RET-Y) of the Sysmex XE-2100. Am J Clin Pathol 2004:121:489-495
- 11. Hinzmann R. Iron metabolism, iron deficiency and anemia. From diagnosis to treatment and monitoring. Symex J Int 2003;13:65-73
- 12. Bartels PCM, Schoorl M, Schoorl M. Application of hemocytometry in combination with reticulocyte count and red cell zinc protoporphyrin for appropriate classification of microcytic anemia. Symex J Int 2003;13:78-82
- Brugnara C, Colella GM, Cremins J, Langley RC, Schneider TJ, Rutherford CJ, Goldberg MA. Effects of subcutaneous recombinant human erythropoietin in normal subjects: Development of decreased reticulocyte hemoglobin content and irondeficient erythropoiesis. J Lab Clin Med 1994;123:660-667
- 14. Metzgeroth G, Hastka J. Diagnostic work-up of iron deficiency. J Lab Med 2004;28:391-399
- Tillyer M, Tillyer C. Use of red cell distribution width and erythrocyte zinc protoporphyrin in differential diagnosis of α- and β-thalassemia and iron deficiency.
   J Clin Pathol 1994;47:205-208
- 16. Skarmoutsou C, Pappassotiriou I, Traeger-Syndinos J, Stamou H, Ladis V, Metaxotou-Mavrommati A, Stamoulakatou A, Kanavakis E. Erythroid bone marrow activity and red cell hemoglobinization in iron-sufficient  $\beta$ -thalassemia heterozygotes as reflected by soluble transferrin receptor and reticulocyte hemoglobin content. Correlation with genotypes and HbA2 levels. Haematologica 2003;88:631-636
- 17. Brugnara C, Zurakowski D, Di Canio J, Boy T, Platt O. Reticulocyte hemoglobin content to diagnose iron deficiency in children. JAMA 1999;23:2225-2230

- Fishbane S, Galgano C, Langley RC Jr, Canfield W, Maesaka JK. Reticulocyte hemoglobin content in the evaluation of iron status of hemodialysis patients. Kidney Int 1997;52:217-222
- Vaya A, Simo M, Santaolaria M, Todoli J. Red blood cell deformability in iron deficiency anemia. Clinical Hemorheol Microcirc 2005;33:75-80
- d'Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995:85:818-823
- 21. d'Onofrio G, Zini G, Ricerca B.M, Mancini S, Mango G. Automated measurement of red blood cell microcytosis and hypochromia in iron deficiency and β-thalassemia trait. Arch Pathol Lab Med 1992;116:84-89
- Thomas L, Franck S, Messenger M, Linssen J, Thorné M, Thomas C. Reticulocyte hemoglobin measurement comparison of two methods in the diagnosis of iron restricted erythropoiesis. Clin Chem Lab Med 2005;43:1193-1202



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## **ABSTRACT**

In hemodialysis subjects correction of anemia is facilitated by combined supplementation of intravenous iron and recombinant human erythropoietin. Reticulocyte hemoglobin content (RET-He) is considered to be an actual indicator reflecting functional iron availability for erythropoiesis. In the present study, interdependence between biochemical analytes reflecting iron status and hemocytometric parameters indicating the degree of hemoglobinization of reticulocytes and red blood cells, respectively, is established. Participants of the study were reference subjects (n=75), subjects with iron deficiency anemia (n=52), subjects with uremia (n=19) and subjects undergoing hemodialysis treatment (n=43). If compared with the reference subjects the results for RBC counts and MCHC are statistically significantly decreased in case of subjects with hemodialysis and uremia, whereas increased results are established with regard to RDW-sd values. Significantly increased results for absolute reticulocyte counts and immature reticulocyte fractions (IRF) are also observed in case of subjects with hemodialysis and uremia. Slightly increased values for the ZPP/heme ratio in combination with elevated reticulocyte count reflect increased activity of erythropoiesis. At a definite MCV value, decreased levels for the hemoglobin content of reticulocytes (RET-He) and hemoglobin content of red blood cells (RBC-He) are observed in case of subjects treated with hemodialysis and in subjects with uremia if compared with identical MCV values of the group of reference subjects. For the ratio of RET-He and RBC-He obviously decreased results are demonstrated in case of subjects with iron deficiency anemia  $(1.02 \pm 0.08, \text{mean} \pm \text{SD})$ , hemodialysis  $(1.05 \pm 0.05)$  and uremia  $(1.02 \pm 0.10)$  if compared with the group of reference subjects  $(1.11 \pm 0.02)$ . From the combined interpretation of the MCV values within the reference range and decreased values for RET-He and RET-He/ RBC-He ratios, respectively, a decreased degree of hemoglobinization is concluded in the case of subjects with hemodialysis or uremia. The conclusion implicating the presumption of reduced functional availability of iron for hemoglobin synthesis is supported by the detection of increased results for sTfR concentrations and ZPP/heme ratios.

# 3

## INTRODUCTION

Due to HD treatment-related blood loss and poor iron absorption in the gastrointestinal tract depletion of iron stores readily occurs as a result of treatment. Annual blood loss in case of HD and frequent drawing of samples for the purpose of biochemical analyses amounts to approximately 500 mL. Functional iron deficiency may refer to a physiological state of normal or even increased iron content of the body. Nevertheless, iron availability may yet be insufficient for hemoglobinization in erythropoiesis. Moreover, increased demand for iron occurs in case of accelerated erythropoiesis after stimulation by erythropoietin. After administration of erythropoietin increases in reticulocyte count, immature reticulocytes and reticulocyte mean cell volume are detected together with a decrease in RET-He independent of the iron parameters (1).

In case of treatment with HD, indicators concerning functional availability of iron for hemoglobin synthesis should be established in order to optimize actual conditions for application of erythropoietin and iron supplementation dosage. In case of chronic or complicated diseases, results concerning serum ferritin concentration and transferrin iron saturation reveal inappropriate results in order to indicate iron-deficient erythropoiesis (2, 3). In the case of functional iron deficiency serum ferritin concentration may still be within the reference range, whereas insufficient iron is available to meet increased demand for hemoglobinization. Therefore, iron availability should be optimized by supplementation with iron sucrose polymerization complex (Venofer®). Hemocytometric parameters indicating short-term effects of iron supply for hemoglobinization in reticulocytes and RBCs have been recently introduced. Hemoglobin content in reticulocytes (RET-He) is thought to be a sensitive indicator for monitoring short-term deteriorations in functional iron supply for erythropoiesis (4). RET-He has already proved to be a reliable marker for establishment of adequate iron availability for erythropoiesis in case of supplementation with erythropoietin (5).

The aim of the present study was to obtain additional information with regard to disturbances in erythropoiesis together with the establishment of hemocytometric parameters and concomitant evaluation of serum analytes reflecting the iron status. The investigations concerned subjects with uremia and subjects treated with HD.

# **SUBJECTS AND METHODS**

## Criteria for selecting the groups of subjects

## Reference subjects

Blood samples from apparently healthy subjects (n=75) were obtained from the Sanquin Blood Transfusion Department Northern Holland, Amsterdam, The Netherlands.

## Subjects with iron deficiency anemia (IDA)

The group with IDA consisted of 52 subjects (10 men and 42 women) with a mean age of 57 years (range 24- 92 years). Selection criteria for IDA comprised Hb < 7.5 mmol/L, ZPP/heme ratio >  $100 \mu mol/mol$  heme, ferritin concentration <  $15 \mu g/L$ , serum iron concentra-

Median values together with 2.5th and 97.5th percentiles with regard to hemocytometric results and results for analytes reflecting the iron status in the groups of reference subjects (n=75), subjects with IDA (n=52), subjects with HD treatment (n=43) and subjects with uremia (n=19), respectively. Table 1.

Parameters	Reference subjects' group (N=75)	IDA (N=52)	HD (N=43)	Uremia (N=19)	Statistical significance between groups
	Median (p2.5 - p97.5)	Median (p2.5 - p97.5)	Median (p2.5 - p97.5)	Median (p2 p97.5)	
RDW-SD (fL)	42.3 (37.5 - 48.1)	45.4 (39.5 - 52.6)	52.9 (44.7 - 59.1)	50.7(40.5 - 59.7)	1,2,3,4,5
HB (mMol/L)	8.7 (7.6 - 10.1)	6.0 (4.1-7.5)	7.1 (5.1 - 8.6)	7.1 (5.6 - 8.5)	1,2,3,4,5
RBC $(10^{12}/L)$	4.67 (4.05 - 5.28)	4.34 (3.28 - 5.08)	3.89 (2.82 - 5.05)	3.94 (3.38 - 4.51)	1,2,3,4,5
MCV (fL)	88.6 (80.2 - 97.1)	74.7 (63.1 - 79.5)	92.8 (73.1 - 104.2)	92.0 (81.1 - 95.8)	1,2,4,5
MCH (aMol)	1868 (1641 - 2089)	1369 (1037 - 1558)	1866 (1343 - 2107)	1807 (1642 - 1966)	1,3,4
MCHC (mMol/L)	21.0 (20.0 - 22.1)	18.5 (16.4 - 20.0)	20.1 (18.3 - 21.5)	20.1 (18.9 - 21.4)	1,2,3,4,5
Reticulocytes (1012/L)	0.043 (0.028 - 0.073)	0.049 (0.029 - 0.096)	0.054 (0.013 - 0.131)	0.058 (0.033 - 0.093)	2,3
RET-He (aMol)	2060 (1897 - 2309)	1265 (879 - 1685)	1846 (1211 - 2110)	1809 (1300 - 2095)	1,2,3,4,5
RBC-He (aMol)	1876 (1698 - 2083)	1272 (916 - 1516)	1764 (1149 - 2059)	1699 (1549 - 1856)	1,2,3,4,5
IRF $(10^{12}/L)$	0.003 (0.001 - 0.013)	0.009 (0.004 - 0.032)	0.009 (0.001 - 0.028)	0.007 (0.001 - 0.015)	1,2,3,6
RET-He/RBC-He	1.10(1.05 - 1.16)	1.02 (0.85 - 1.16)	1.05 (0.96 - 1.19)	1.03 (0.77 - 1.15)	1,2,3,4
Iron (μMol/L)	16.6 (6.9 - 29.5)	3.4 (1.0 - 8.8)	8.2 (2.2 - 18.8)	8.3 (1.9 - 21.7)	1,2,3,4,5
Ferritin (µg/L)	29 (7 - 140)	4 (1 - 32)	120 (13 - 522)	221 (22 - 731)	1,2,3,4,5
Transferrin (g/L)	2.5 (1.8 - 3.3)	2.8 (1.4 - 4.2)	1.6 (1.1 - 2.5)	1.9 (1.0 - 3.7)	1,2,3,4,5
s-TfR (mg/L)	1.2 (0.8 - 2.0)	3.5 (1.7 - 8.3)	2.3 (1.0 - 4.2)	1.8 (0.8 - 4.0)	1,2,3,4,5
ZPP (μMol/Mol Heme)	45 (32 - 73)	173 (103 - 516)	70 (43 - 151)	65 (30 - 107)	1,2,3,4,5
Iron saturation (%)	25.9 (8.9 - 63.4)	4.3 (2.0 - 16.4)	19.6 (5.7 - 50.1)	19.1 (3.6 - 62.9)	1,2,4,5

1: statistical significance between reference subjects' group and IDA (p<0.05) 2: statistical significance between reference subjects' group and uremia (p<0.05) 4: statistical significance between IDA and HD (p<0.05) 5: statistical significance between IDA and uremia (p< 0.05)  $\,$  6: statistical significance between HD and uremia (p< 0.05)

tion  $< 7 \mu mol/L$  and CRP concentration < 5 mg/L. Pregnant women and subjects with thalassemic trait or chronic disease were excluded from the study.

## Subjects with HD treatment

Samples from subjects with chronic renal failure (n=43) were obtained from the Department of Nephrology, Medical Center Alkmaar, Alkmaar, The Netherlands. The group of HD patients consisted of 15 male and 28 female subjects with a mean age of 60 years (range 26-83 years). In periods of more than one year, patients were treated (standard procedure 3-4 hours of bicarbonate dialysis during 3 times/week) on a high-flux polysulphone F-60 membrane (Fresenius, Bad Homburg, Germany). The dosage schedule for intravenous iron application is based on the guidelines of the Dutch Dialysis Society. Patients subcutaneously received 10-150 μg/week erythropoietin-α (Amgen Europe by, Breda, The Netherlands), and 47% of the subjects (n=20) received intravenously 0-100 mg/2 weeks iron sucrose (Venofer®, American Reagent Inc, Shirley, NY 11967, USA).

# Subjects with uremia

Samples of subjects with serum urea concentrations within a range of 20-25 mmol/L (n=19) were obtained from the Department of Internal Medicine, Medical Center Alkmaar. The group consisted of 11 males and 8 females with a mean age of 75 years (range 34-93 years).

## Preparation of blood samples

Blood samples were drawn into Vacutainer<sup>®</sup> SST plain tubes and tubes anticoagulated with  $K_2$ EDTA, respectively (Becton Dickinson, Plymouth, UK). Blood samples were analyzed within four hours after collection. For additional investigations of biochemical parameters reflecting the iron status serum samples were frozen in aliquots at -20  $^{\circ}$ C until analysis.

## Hemocytometry

Hemocytometric parameters were measured on a Sysmex XE-2100 Hematology Analyzer (Sysmex Corporation, Kobe, Japan). The methodology of measurement is based on automated fluorescent flow cytometry utilizing a polymethine dye for binding cytoplasmic RNA in the reticulocytes. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure for the volume and Hb content of RBCs and reticulocytes, respectively. Results concerning Hb content are initially reported as RBC-Y for RBCs and RET-Y for reticulocytes. Subsequently, algorithms  $y=400 \text{ x} \text{ e}^{0.0009 \text{ x RET-Y}}$  and  $400 \text{ x} \text{ e}^{0.0009 \text{ x REC-Y}}$  are applied in order to transform arbitrarily reported channel numbers of RET-Y and RBC-Y into hemoglobin equivalents. Hemoglobin equivalents are expressed in attomol and denoted as RET-He and RBC-He, respectively (6).

## Parameters reflecting the iron status

Measurement of the ZPP/heme ratio in RBC suspensions was performed on a dedicated hematofluorometer (AVIV Biochemical Inc., Lakewood NJ 08107, USA) with application of front surface illumination fluorometry. Concentrations of serum transferrin and soluble transferrin receptor were established on a ProSpec nephelometer according to the manufacturer's instructions (Dade Behring, Marburg, Germany). Analyses of serum iron

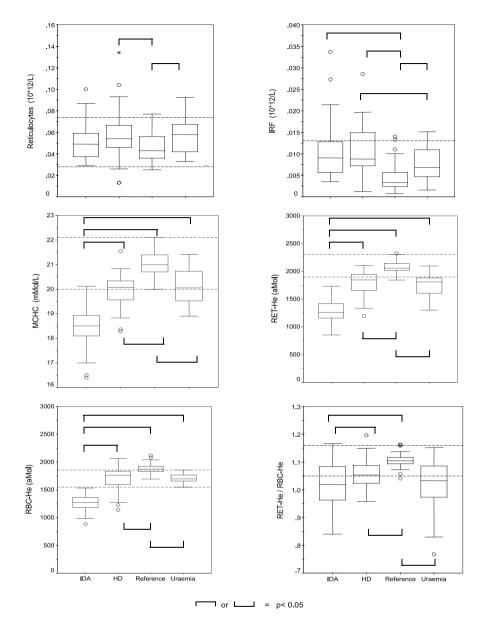
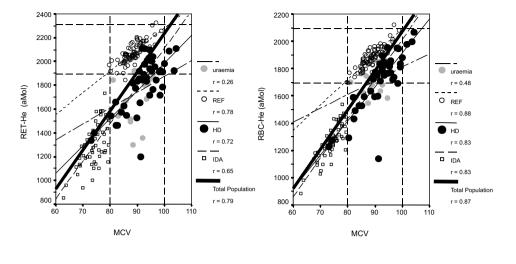


Figure 1. Box plots for evaluation of hemocytometric parameters concerning reticulocytes, IRF, MCHC, RET-He, RBC~He and RET/RBC-He ratio. Results of the groups of subjects with HD treatment, IDA and uremia are compared with results from the group of reference subjects. Box extends from the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to the largest and smallest observed values within 1.5 box lengths. Outlying and extreme values corresponding with values between 1.5-3x box length or >3x box length, respectively, are designated as (0) and (\*). Statistically significant deviations between groups (p<0.05) are indicated by — and ——.



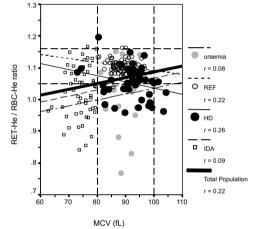


Figure 2. Relationship between MCV values and RET-He, RBC-He and RET-He/RBC-He ratios in the group of reference subjects and the subject groups with uremia, HD treatment and IDA, respectively.

concentrations were performed on a Vitros 950 IRC Analyzer (Ortho Clinical Diagnostics NV, Beerse, Belgium). Serum ferritin concentrations were established by application of a two-site sandwich immunoassay on an ADVIA Centaur Analyzer (Bayer, Mijdrecht, The Netherlands).

## Statistical evaluation of analytical results

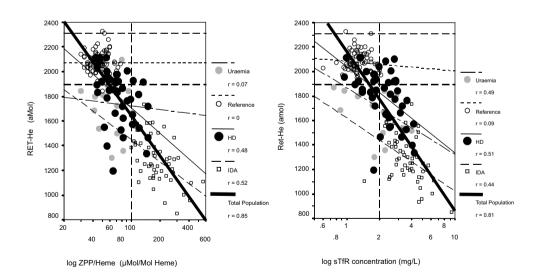
The statistical software package SPSS/PC, version 11.5 for Windows, was applied for statistical analysis of the results (SPSS, Chicago, IL 60606, USA), Independent Samples T-test was performed in order to detect statistically significant deviations between the groups of subjects; p-values less than 0.05 were considered to be statistically significant. Correlation coefficients were calculated by the Pearson method.

## **RESULTS**

Median values together with 2.5th and 97.5th percentiles ranges for hemocytometric results together with parameters reflecting the iron status in the various groups of subjects are listed in Table 1. Decreased results for RBC counts in combination with increased RDW-sd values were observed in the case of uremic subjects and in the case of subjects treated with HD. In several subjects belonging to the HD subjects' group the MCV values exceeded the upper limit of the range established for reference subjects. In the case of HD subjects and uremic subjects the MCHC results were statistically significantly decreased until an intermediate level between the results obtained for the IDA and reference subjects' groups (Figure 1). The intermediate position of box plots reflecting MCHC results in the subjects' group with HD and uremia was considered to be an indication for insufficient availability of iron for hemoglobin synthesis during increased activity of erythropoiesis. As represented in Table I and Figure 1, absolute reticulocyte counts and results concerning immature reticulocyte fractions (IRF) were increased in subjects with HD and uremic subjects if compared with the group of the reference subjects.

In the case of subjects with HD and uremia the results for RET-He and RET-He/RBC-He ratio were statistically significantly decreased until beyond the lower limit of the reference range (Figure 1). At a definite MCV value decreased RET-He and RBC-He results amounting to approximately 20% reduction were observed in the groups of subjects just mentioned (Figure 2).

From evaluation of the ranges, which are noted in Table I, and the results for individual subjects, which are depicted in Figure 3, it is concluded that ZPP/Hb results in subjects



**Figure 3.** Relationship between results concerning log ZPP/heme, log sTfR and RET-He in a group of reference subjects and groups of subjects with uremia, HD treatment and IDA, respectively.

with HD and uremia are obviously increased if compared with the results established for the group of reference subjects. In the case of uremic subjects the results of ZPP/heme and sTfR at a definite RET-He value reveal a tendency towards decreased values if compared with the HD subjects (Figure 3). Evaluation of sTfR concentrations (Table 1) reveals an obvious trend towards increased values beyond the reference range interval in the case of subjects with HD and to a lesser extent in subjects with uremia, whereas for transferrin concentrations obviously decreased results are observed.

Establishment of the interdependence of RET-He results with additional parameters reflecting the iron status (Figure 3) reveals a negative linear relationship with log ZPP/heme (r = -0.87) and log sTfR concentrations (r = -0.86), respectively. For results concerning the RETHe/RBC-He ratio a similar trend is detected (Figure 4).

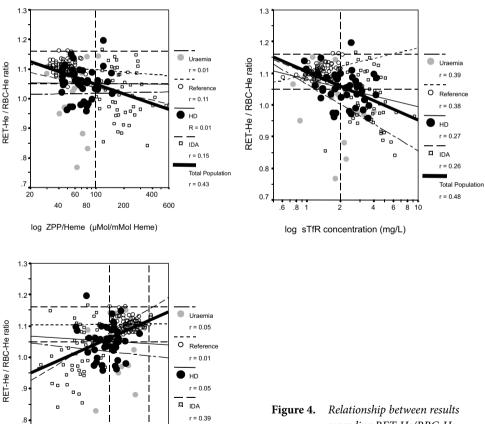
## **DISCUSSION**

Anemia in the case of renal failure may result from a reduced survival time of RBCs, retained inhibitors of erythropoiesis, chronic mucosal blood loss due to uremic coagulopathy, iron or folate deficiency, and aluminium toxicity. Application of rHuEPO facilitates correction of anemia in subjects with end-stage renal disease (7). Reticulocyte maturation occurs with progressive reduction in RBC size and Hb content (8). A close relationship has been established between RBC-He and RET-He because forward light scatter intensity is related to size and hemoglobin content of RBCs and reticulocytes, respecttively (9). In subjects with depleted iron stores RET-He will increase already within a few days after iron supplementation. On the contrary, RET-He results will decrease quickly in the case of subjects with iron deficient erythropoiesis (9). If compared with mature RBCs, RET-He is able to yield additional short-term information concerning the various states of functional availability of iron for hemoglobinization of erythroid precursors, particularly in subjects with inflammation, cancer, hemodialysis (10).

As a result of our study, slightly increased reticulocyte counts were observed in HD and uremia, whereas immature reticulocyte counts were obviously increased in HD subjects (Figure 1). Reduced RBC lifespan has been demonstrated to be a contributing factor to renal anemia in an uremic environment (11). In some subjects, iron availability may be insufficient for additional transfer of storage iron as a response to increased demand for hemoglobinization in erythroid progenitor cells. Discrimination between absolute and functional iron deficiency state is of clinical importance because decreased iron availability in case of accelerated erythropoiesis is a frequent cause of suboptimal erythroid response to rHuEPO. In suboptimal conditions of iron status rHuEPO dosage might be unnecessarily high, resulting in inappropriately expensive costs of treatment. Therefore, iron deficiency should be corrected in an early stage before initiating treatment with rHuEPO (12). Intravenous administration of Venofer® is done according to guidelines corresponding with a strict schedule in order to prevent iron accumulation-associated complications such as anaphylaxis, hemosiderosis, hepatic dysfunction, cardiovascular disease, and infection (12). Early identification of non-responsiveness will reduce inadvertent iron-related toxicity effects. In order to balance optimization of rHuEPO stimulus with the avoidance of reverse effects of iron toxicity, an appropriate monitoring system is required for early detection of beneficial effects of iron administration and establishment of optimal dosage (13).

Application of RET-He for detection of sideropenic erythropoiesis is particularly recommended in case of simultaneous occurrence of complicated diseases (3,13,14). It is a real advantage that beneficial effects of iron supplementation become already apparent within 2-4 weeks after initiation of therapy. For the purpose of periodical monitoring, hemocytometric parameters yielding insight into the degree of hemoglobinization in reticulocytes are indicators of choice.

According to our observations a close correlation (r = 0.96) is established between results of RET-He and RBC-He. In a reference group of apparently healthy subjects, RET-He results exceeded RBC-He amounting to 5-15%. From evaluation of a widely scattered range concerning RET-He/RBC-He ratio results, an obvious trend towards decreased results is established in the case of subjects with HD (range 0.96-1.19) and uremic subjects (range 0.77-1.15). An analogous trend is demonstrated for MCHC results as indicator for



Total Population

r = 0.48

regarding RET-He/RBC-He ratios and log ZPP/heme ratio, log sTfR and MCHC, respectively.

17

MCHC (mMol/L)

insufficient availability of iron during increased activity of erythropoiesis. Survival time of RBCs is reduced in a toxicological environment due to increased urea concentration (11). In various subjects increased percentages of hypochromic RBCs were established together with RET-He within the reference range (14). For clinical interpretation of RET-He results a cut-off value amounting to 1750 aMol has been applied in a previous study (3). In contrast to the study just mentioned, we definitely prefer interpretation of the RET-He results in combination with the corresponding MCV value in a 2-dimensional graph, as depicted in Figure 2, and by subsequent comparison of the results with the group results of the reference subjects. In HD subjects and uremic subjects, RET-He results are significantly decreased if compared with the group of reference subjects. The so-called index of rigidity for RBCs has been found to be increased in HD subjects (15,16). As a consequence of increased RBC rigidity, shape and deformability are changed, which may result in inappropriate RBC volume measurement (17).

In HD subjects the clinical interpretation of results established for transferrin saturation is complicated by frequently observed decreased serum transferrin concentrations (Table I). Even continuous low dosage amounts for iron maintenance supplementation may result in decreased serum transferrin concentrations which are not related to decreased serum albumin concentrations. It should be stressed that hypotransferrinemia is considered to be an independent risk factor for iron toxicity (18). On the basis of combined interpretation of results exclusively concerning transferrin saturation and serum ferritin determinations, iron-deficient subjects are at risk to be incorrectly monitored during treatment (3). In case of inflammation, results for serum ferritin concentration as well as a hypochromic RBC count may yet be within the reference range, whereas iron availability is insufficient. Simultaneous determination of an acute phase reactant, for instance C-reactive protein serum concentration, yields additional information in order to appropriately identify subjects with a clinical condition that complicates diagnosis of the iron status.

If compared with apparently healthy subjects a tendency towards an increased ZPP/heme ratio is observed in the case of subjects treated with HD and in case of uremic subjects. In autologous blood donors receiving rHuEPO, iron-deficient erythropoiesis as indicated by increased zinc protoporphyrin/heme ratios in RBCs is observed, whereas serum iron and ferritin concentration are still within the reference range (19). An analogous discordance between parameters reflecting functional iron availability and analytes reflecting iron storage is noted in uremic subjects (20). Weak correlations between results of RET-He and serum iron parameters are conclusive for the fact that conventional parameters reflecting iron storage are inappropriate measures for detection of iron deficiency in the case of subjects treated with HD (3).

From the combined interpretation of MCV values within the reference range and decreased values for RET-He and RET-He/RBC-He ratios, respectively, a decreased degree of hemoglobinization is concluded in the case of subjects with HD or uremia. The conclusion implicating presumption of reduced availability of iron for hemoglobin synthesis is supported by the detection of increased results for sTfR concentrations and ZPP/heme ratios.

# **CONCLUSION**

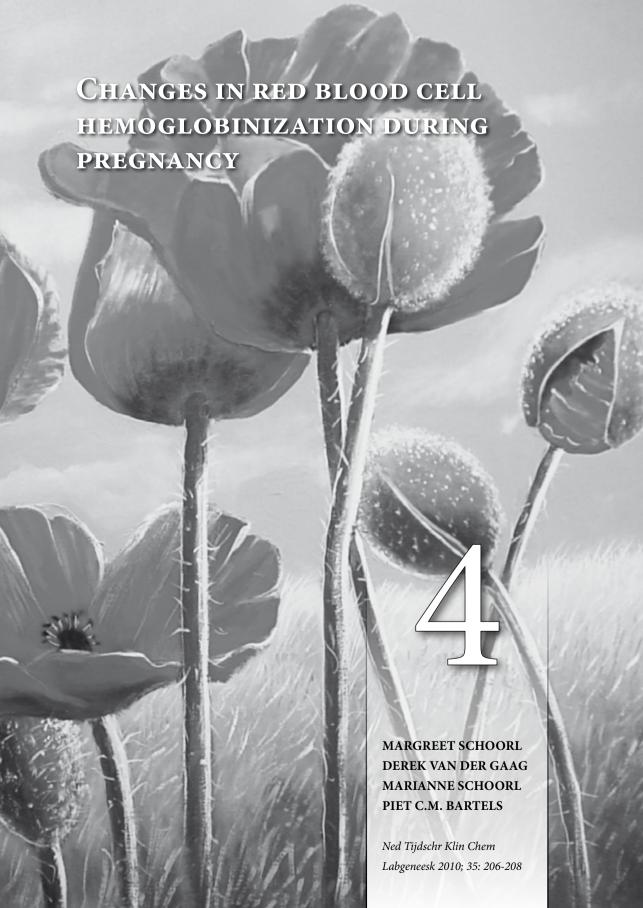
Monitoring of functional iron availability for erythropoiesis requires longitudinal follow-up of RET-He in addition to biochemical parameters reflecting the iron status. The evaluation procedure will support short-term correction of intravenous iron supplementation at an individual level.

## **REFERENCES**

- Sowade O, Sowade B, Brilla K, et al. Kinetics of reticulocyte maturity fractions and indices and iron status during therapy with epoitin beta (recombinant human erythropoietin) in cardiac surgery patients. Am J Hematol 1997;55:89-96
- 2. Fishbane S, Kowalski EA, Imbriano LJ, Malsaka lK. The evaluation of iron status in hemodialysis patients. *J Am Sac Neph-rol* 1996;7:2654-2657
- Mittman N, Sreedhara R, Mushnick R, Chattopathyay J, Zelmanovic D, Vaseghi M, Avram MM. Reticulocyte hemoglobin content predicts functional iron deficiency in hemodialysis patients receiving rHuEPO. Am J Kidney Dis 1997;30:912-922
- Bartels PCM, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-114
- Bhandari S, Turney JH, Brownjohn AM, Norfolk D. Reticulocyte indices in patients with end stage renal disease on hemodialysis. J Nephrol 1998;11:72-82
- 6. Franck S, Linssen J, Messinger M, Thomas L. Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. *Clin Chem* 2004;50:1240-1242
- Marsden JT. Erythropoietin measurement and clinical applications. Ann Clin Biochem 2006;43:97-104
- Brugnara C. Reticulocyte Cellular Indices: a new approach in the diagnosis of anemias and monitoring of erythropoietic function. Crit Rev Clin Lab Sci 2000;37:91-130
- 9. Buttarello M, Temporin V, Ceravolo R, Farina G, Bulain P. The new reticulocyte size parameter (RET-Y) of the Sysmex XE-2100. *Am J Clin Palhol* 2004;121:489-495

- Hinzmann R. Iron metabolism, iron deficiency and anaemia. From diagnosis to treatment and monitoring. Sysmex J Int 2003;13:65-73
- 11. Marticorena LJ, Donnelly S. Red blood cell survival in chronic renal failure. *Am J Kidney Dis* 2004;44:715-719
- 12. Kletzmayr J, Sunder-Plassmann G, Horl WH. High dose intravenous iron: a note of caution. *Nephrol Dial Transplant* 2002; 17:962-965
- Chuang CL, Liu RS, Wei YH, Huang TP, Tarng DC. Early prediction of response to intravenous iron supplementation by reticulocyte hemoglobin content and high fluorescence reticulocyte count in haemodialysis patients. Nephrol Dial Transplant 2003;18:370-377
- 14. Hackeng CM, Beerenhout CM, Hermans M, Van der Kuy H, Van der Dussen DH, Van Dieijen-Visser MP, Hamulyak K, Van der Sande FM, Leunissen KM, Kooman JP. The relationship between reticulocyte hemoglobin content with C-reactive protein and conventional iron parameters in dialysis patients. *J Nephrol* 2004;17:107-111
- 15. Sotirakopoulos N, Tsitsios T, Stambolidou M, Athanasiou G, Peiou M, Kokkinou M, Mavromatidis K. The red blood cell deformability in patients suffering from end stage renal failure on hemodialysis or continuous ambulatory peritoneal dialysis. Ren Fail 2004;26:179-83
- 16. Ibrahim FF, Ghanram MM, Ali FM. Effect of dialysis on erythrocyte membrane of chronically hemodialyzed patients. *Ren Fail* 2002;24:779-90
- Bartels PCM, Schoorl M. Mean corpuscular volume-dependent deviations in red blood cell distribution width between Sysmex NE-8000 and SF-3000. Eur J Clin Chem Clin Biochem 1997;35:549-550

- Canavese C, Bergamo D, Ciccone G, Burdese M, Maddalena E, Barbieri S, Thea A, Fop F. Low-dose continuous iron therapy leads to a positive iron balance and decreased serum transferrin levels in chronic haemodialysis patients. Nephrol Dial Transplant 2004;19:1564-1570
- Brugnara C, Chambers LA, Malynn E, Goldberg MA, Kruskall MS. Red cell regeneration induced by subcutaneous recombinant human erythropoietin: iron-deficient erythropoiesis in ironreplete subjects. *Blood* 1993;81:956-964
- 20. Schaefer RM, Schaefer L. Hypochromic red blood cells and reticulocytes. *Kidney Internat* 1999;55 (suppl 69):544-548



## INTRODUCTION

Decreased hemoglobin concentration (Hb) is a common feature in the third trimester of pregnancy, partly due to physiologic hemodilution. As the degree of haemodilution display considerable inter-individual variation, Hb concentrations show similar variation. Therefore it is complicated to establish reliable cut-off limits for anaemia. Several diagnostic guidelines are used in obstetric practice. Koninklijke Nederlandse Organisatie voor Verloskundigen (KNOV) and World Health Organization (WHO) practise Hb values of 6.3 and 6.8 mmol/l respectively for anaemia discrimination and providing an indication for subsequent iron supplementation (1-4).

The aim of the study was to gain insight into the additional value of advanced red blood cell parameters, particularly immature reticulocyte count (IRF) and reticulocyte hemoglobin content (Ret-He) to establish deviations in hemoglobinization (5) and appropriate Hb discrimination levels.

Ret-He reflects a 'short term' indication concerning the status of reticulocytes hemoglobinization. In contrast zinc protoporphyrin (ZPP) reflects a 'long term' impression corresponding with the lifespan of red blood cells (RBCs) (6).

An appropriate biomarker for detection of iron deficient erytropoiesis (IDE) is zinc protoporphyrin. The parameter reflects the extent to which zinc rather than iron, has been chelated with protoporphyrin. This process functions as a homeostatic mechanism by inhibiting excretion of iron following haemolysis macrophages. Thus, it is a highly sensitive functional indicator of IDE (7).

Regarding clinical interpretation Ret-He is similar to ZPP. It is a cellular measure of IDE that on its own does not distinguish between functional and true iron deficiency. In contrast to ZPP in RBCs, Ret-He is measured in reticulocytes. Therefore it is considered to be a more sensitive marker for short term changes in Hb-production (6).

Pregnancy is associated with a physiological increase in inflammatory biomarkers, especially during the 1<sup>st</sup> and 3<sup>rd</sup> trimester. Serum ferritin will be elevated during inflammation because of its role as an acute-phase reactant, and therefore may overestimate body iron stores (7).

It should be stressed that functional and true IDE are not mutually exclusive processes and may co-exist, especially during late pregnancy when low-level inflammation with depleted iron stores is likely to occur (7).

Concerning ZPP/heme ratio results in our study we applicate an opinion based cut-off level of >75µmol/mol heme as an indication for iron deficient erytropoiesis.

# SUBJECTS, MATERIALS AND METHODS

Blood samples ( $K_2$ EDTA, Becton Dickinson, Plymouth UK) were selected from 114 pregnant women in the third trimester within a Hb range suspicious for anaemia in pregnancy (Hb  $\leq$ 7.0 mmol/l, MCV 80-100 fL). Apparently healthy women (n=35) were selected as a reference subjects' group. Hb, IRF and Ret-He were determined within 4 hours after sample collection on a Sysmex XE2100 hematology analyzer with additional dedicated software (Sysmex Corporation, Kobe, Japan).

The methodology of IRF and Ret-He measurements is based on application of automated fluorescent flowcytometry utilizing a polymethine dye for binding cytoplasmic RNA in reticulocytes. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure for volume and Hb content of red blood cells and reticulocytes. Several algorithms are applied in order to transform original data into IRF and RET-He dedicated results. Measurements of RBC zinc protoporphyrin heme ratio (ZPP) were performed on a haematofluorometer (AVIV Biochemical Inc., Lakewood, USA) using front surface illumination fluorometry.

## STATISTICAL EVALUATION

The statistical software package SPSS/PC, version 14.0 for Windows, was applied for statistical analysis of results (SPSS, Chicago, IL). Independent Samples T-test was performed in order to detect statistically significant deviations between the groups of subjects.

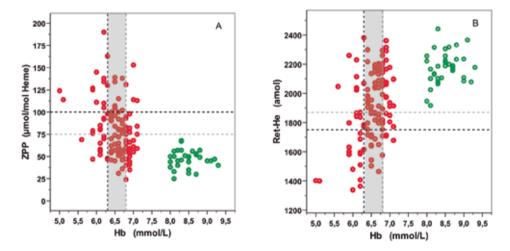


Figure 1. (A) Scatterplot representing Hb and ZPP concentrations. (B) Scatterplot representing Hb and RET-He content. Research group = red; Reference group = green; x-axis: ------ (left, black) KNOV diagnostic guideline, ----- (right, grey) WHO diagnostic guideline; y-axis: ------ (grey) upper (A) or lower (B) level of reference value; ------ (black) discrimination level for absolute iron deficiency.

## RESULTS

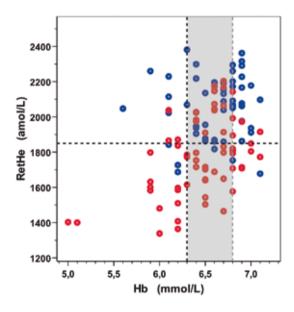
Discrimination concerning anaemia in pregnancy based on application of the KNOV guideline (Hb <6.3 mmol/l) resulted in 21% of subjects with decreased Hb concentrations and in case of application of the WHO guideline (Hb <6.8 mmol/l) even in 69%. Hb values in 48% of the subjects were in the debatable range of 6.3-6.8 mmol/l; in 33% of these

subjects poor RBC-hemoglobinization occurred (Ret-He <1850 amol). Establishment of ZPP/heme ratio revealed increased results (>75  $\mu$ mol/mol heme) in 45% of the subjects in the inconclusive Hb concentration range (Hb 6.3-6.8 mmol/l) (figure 1). IRF counts demonstrated increased results (0.011  $\pm$  0.007x 10<sup>12</sup>/L) if compared to the reference group (0.003  $\pm$  0.002 x 10<sup>12</sup>/L, p = <0.001).

Hemoglobinization parameters Ret-He, RBC-He and Ret-He/RBC-He ratio showed significantly decreased results if compared to the reference group (mean  $\pm$  standard deviation: 1921  $\pm$  240 amol (p = < 0.001), 1882  $\pm$  129 amol (p = <0.001) and 1.01  $\pm$  0.05 (p = < 0.001) respectively.

With respect to clinical interpretation of results in the indicated grey area (Hb 6.3-6.8 mmol/l) 4 groups are considered (figure 2):

- 1. ZPP <75 μmol/mol heme, Ret-He >1850 amol (n = 24, blue) Interpretation: normal hemoglobinization (low Hb due to haemodilution).
- 2. ZPP <75  $\mu$ mol/mol heme, Ret-He <1850 amol (n = 1, blue). Interpretation: ineffective hemoglobinzation? Follow-up after 2-4 weeks is advised.
- 3. ZPP >75  $\mu$ mol/mol heme, Ret-He <1850 amol (n = 17, red). Interpretation: ineffective hemoglobinization.
- 4. ZPP >75  $\mu$ mol/mol heme, Ret-He >1850 amol (n = 13, red). Interpretation: ineffective hemoglobinization due to iron suppletion or increased erytropoiesis. It is recommended to check Ret-He/RBC-He ratio in advance. Ret-He/RBC-He ratio > 1.05 is indicative for increased erytropoiesis.



**Figure 2.** Scatterplot representing Hb and Ret-He content. ZPP >75  $\mu$ mol/mol heme = red; ZPP  $\leq$  75  $\mu$ mol/mol heme = blue; x-axis: ----- (left, black) KNOV diagnostic guideline, (right, grey) WHO diagnostic guideline; y-axis: ----- (black) discrimination level for impaired hemoglobinization.

## DISCUSSION AND CONCLUSION

Anaemia is the most common haematological problem in pregnancy (8). What is referred to as the physiologic anaemia of pregnancy is a dilution process secondary to an increase in plasma volume. During pregnancy the demand for micronutrients, especially iron and folate, is increased and maternal body stores and dietary intake may be insufficient for adequate erytropoiesis (7). Appropriate assessment is complicated by disproportionate expansion of plasma volume compared with RBC mass (2, 3, 7).

Anaemia screening during pregnancy only based on Hb measurements is inappropriate and inconclusive in many subjects. A decreased Ret-He result is considered to be indicative for insufficient RBC hemoglobinization. Increased IRF counts are indicative for increased erytropoiesis during the third trimester of pregnancy (9). Increased IRF results combined with increased ZPP and decreased Ret-He results are indicative for functional iron deficiency. With establishment of ZPP iron deficient erytropoiesis can be detected with reasonable accuracy. However, ZPP cannot discriminate between true and functional iron deficiency. The latter phenomenon occurs when body iron stores are adequate but iron is not available to the bone marrow, such as may happen in case of infection and inflammation.

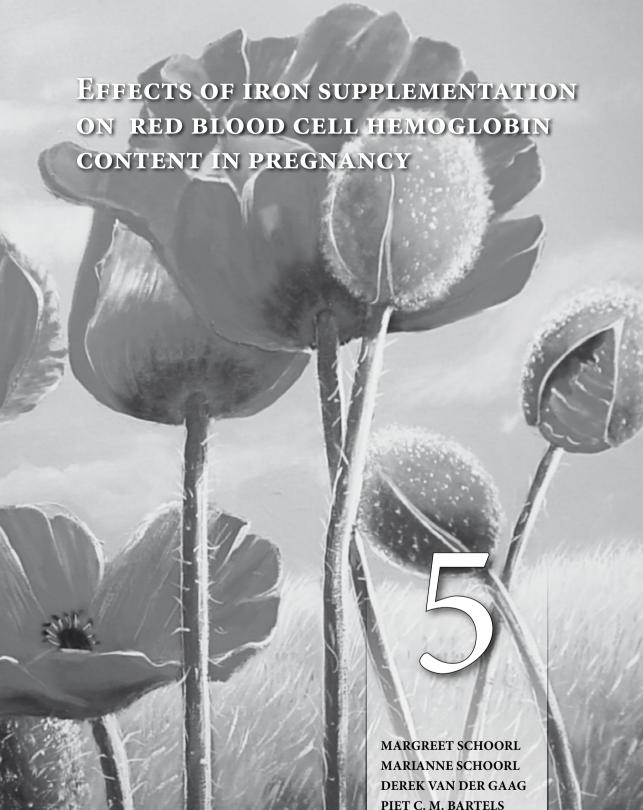
Many pregnant women demonstrate inconclusive Hb values between KNOV and WHO discriminant values (figures: grey area). In the grey area, Hb is an unreliable indicator for hemoglobinizaton. In pregnant women, MCV is a poor marker for detection of iron deficiency for at least two reasons. Firstly, the physiological increase in MCV during gestation will counterbalance the microcytosis resulting from iron deficiency in an early stage. Secondly, RBCs have a mean survival of approximately 120 days. Consequently, it takes a large number of RBCs with a small volume to reveal a decreased MCV value. Therefore, MCV reduction can be observed only in late pregnancy when the RBC population has been partially replaced by young RBCs (8). The opinion is favoured that iron deficiency anaemia during pregnancy is an unphysiologic event. That is the reason why discriminatory values for cut-off levels should be derived from an iron treated, iron depleted population (8).

Ret-He is considered to yield a useful tool for diagnostic screening and follow-up of iron availability during the second half of pregnancy. It is recommended to evaluate ZPP and Ret-He in addition.

# REFERENCES

- 1. Anemie in de eerstelijns verloskundige praktijk: praktijkkaart behorend bij de gelijknamige KNOV-standaard (Bilthoven 2000).
- 2. Milman N, Bergholt T, Byg KE et al. Reference intervals for haematological variables during normal pregnancy and postpartum in 434 healthy Danish women. Eur J Hematol 2007; 79: 39-46.
- 3. Milman N. Prepartum anaemia: prevention and treatment. Ann Hematol 2008: 87: 949-959.
- 4. Elion-Gerritzen WE et al. De standaard 'Anemie in de eerstelijns verloskundige praktijk' van de KNOV: risico voor het niet onderkennen van ijzergebrek en hemoglobinopathie. Ned Tijdschr Geneesk 2002; 146: 457-459.
- Bartels PCM, Schoorl M, Schoorl M. Haemoglobinization and functional availability of iron for erytropoiesis

- in case of thalassemia and iron deficiency anemia. Clin Lab 2006; 52: 107-114.
- Thomas C, Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002; 48: 1066-1076.
- 7. Wheeler S. Assessment and interpretation of micronutrient status during pregnancy. Proc Nutr Soc 2008; 67: 437-50.
- 8. Milmann N, Byg KE, Agger AO. Hemoglobin and erythrocyte indices during normal pregnancy and postpartum in 206 women with and without iron supplementation. Acta Obstet Gynecol Scand 2000; 79: 89-98.
- 9. Choi JW, Pai SH. Change in erytropoiesis with gestational age during pregnancy. Ann Hematol 2001; 80: 26-31.



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## **ABSTRACT**

Although a mild degree of anemia is common in the third trimester of pregnancy, it remains a challenge to establish whether a decrease in hemoglobin (Hb) concentration is physiological or pathological. The World Health Organization suggested a Hb concentration of 110 g/L to discriminate anemia. Several European investigators recommended Hb cut-off values of between 101-110 g/L. The aim of this study was to establish short-term effects of iron supplementation on the hemoglobin content of reticulocytes (Ret-He) and red blood cells (RBC-He) in case of suspected iron deficient erythropoiesis (IDE) in the third trimester of pregnancy. Twenty-five subjects with suspected IDE during pregnancy (Hb  $\leq$ 110 g/L, Ret-He <29.6 pg, zinc protoporphyrin >75 µmol/mol hem) participated in the study. After iron supplementation, reticulocyte counts increased from  $0.061 \pm 0.015 \times 10^{12}$ /L to  $0.079 \pm$  $0.026 \times 10^{12}$ /L and Ret-He increased from 23.6±2.8 pg to 28.3 ± 2.6 pg (p=<0.001). RBC-He increased from  $26.9 \pm 1.9$  pg to  $27.4 \pm 1.8$  pg (not significant, NS) and Ret-He/RBC-He ratio increased from  $0.97 \pm 0.06$  towards  $1.07 \pm 0.05$  (p=<0.001). Hb concentrations demonstrated an obvious increase from 105 ± 6 g/L towards 115 ± 5 g/L (p≤0.001) after supplementation. An obvious increase in RBC distribution width was observed from 45.0 ± 3.6 fL towards  $52.3 \pm 7.0$  fL (p $\leq 0.001$ ). We recommend that Ret-He and Ret-He/RBC-He ratio be integrated into the protocols for anemia screening and for monitoring effects of iron supplementation during pregnancy. In particular, the parameters should be considered in subjects with Hb results in the controversial range of 101-108 g/L.

# INTRODUCTION

A high prevalence of anemia during pregnancy has been reported worldwide, ranging from 2% to 30% in developed countries.<sup>1-3</sup> Anemia during pregnancy is partly due to physiological hemodilution and insufficient availability of essential nutrients for hemoglobin (Hb) synthesis and red blood cell (RBC) production in the erythron, such as iron, folic acid and vitamin B12.4 In the last trimester of pregnancy, decreased Hb concentrations as a result of functional iron deficiency may be associated with complications such as maternal infection, low birth weight and premature delivery.<sup>5,6</sup> Although a mild degree of anemia is common in the third trimester of pregnancy, it remains a challenge to establish whether a decreased Hb concentration is a physiological or pathological phenomenon due to iron deficient erythropoiesis (IDE).4,5,7 Disagreement in diagnostic guidelines in obstetric practice illustrates the complexity of establishing discriminating Hb levels for screening anemia during pregnancy. The World Health Organization (WHO) suggested a Hb concentration of 110 g/L to discriminate anemia. European investigators recommend Hb cut-off values of between 101-110 g/L. 4,9-13 The aim of this study was to evaluate the possible beneficial effects of iron supplementation on the hemoglobin content of reticulocytes and RBCs in subjects with inconclusive Hb concentrations in the third trimester of pregnancy.

Hemocytometric parameters such as Hb concentration and mean corpuscular volume (MCV) demonstrate poor sensitivity for the detection of short-term disturbances in erythropoiesis during pregnancy.<sup>5-7</sup> In addition, biomarkers reflecting iron status, i.e. serum concentrations of ferritin, transferrin receptor (TfR) and transferrin saturation (TfSat), reveal serious limitations concerning clinical interpretation. <sup>4,5</sup> It should be emphasized that functional IDE and definite IDE are not mutually exclusive phenomena. Both phenomena may co-exist, particularly during the last trimester of pregnancy when low-level inflammation together with depleted iron stores is likely to occur.<sup>10</sup> It is still a challenge to establish appropriate cut-off limits for evaluating the shift of hemoglobin content of RBCs in the course of pregnancy. A suitable biomarker for detection of *long-term* IDE is the zinc protoporphyrin hem ratio (ZPP/Hb ratio). In subjects without iron supplementation, ZPP will clearly increase in the last trimester of pregnancy. 10,14,15 Recently, new hemocytometric parameters such as erythrocyte hemoglobin content (RBC-He), reticulocyte hemoglobin content (Ret-He) and Ret-He/RBC-He ratio have been demonstrated to yield useful biomarkers for the detection of insufficient hemoglobinization in the third trimester of pregnancy. 5,16 In healthy subjects, Ret-He results exceed those of RBC-He, amounting to 5-15%. From corresponding shifts in decreased values for Ret-He and Ret-He/RBC-He ratios, respectively, a temporarily decreased degree of hemoglobinization is achieved. The RET-He/RBC-He ratio provides accurate and sensitive information concerning the deviation in hemoglobin content between the (normocytic) RBC population (RBC-He) and the (hypochromic) reticulocyte population (Ret-He).<sup>17</sup> In contrast to ZPP and RBC-He, Ret-He reflects a *short-term* indication corresponding to a lifespan of reticulocytes in the blood circulation of several days.18

## MATERIALS AND METHODS

## Study design

Subjects with inconclusive Hb concentrations in the range 101-110 g/L in the third trimester of pregnancy were selected for inclusion in the screening program. The subjects were subsequently supplemented with ferrous fumarate (200 mg 2 times a day, approx. 200 mg iron a day) according to local practice. From the first trimester of pregnancy, 400 µg folic acid was given as supplement in a multivitamin tablet (Centrum Materna). After four weeks of iron supplementation, blood samples were drawn to establish hemocytometric parameters to evaluate RBC and reticulocyte hemoglobin content.

## Hemocytometry

Hemocytometric analyses were performed within 4 h after collection of blood samples (K<sub>2</sub>EDTA, Becton Dickinson, Plymouth, UK) on a Sysmex XE2100 hematology analyzer (Sysmex Corporation, Kobe, Japan). Reticulocyte methodology of measurement is based on automated fluorescent flow cytometry utilizing a polymethine dye for binding cytoplasmic RNA. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure that reflects particle volume and Hb content of RBCs and reticulocytes, respectively. Hb content was initially reported as RBC-Y for RBCs and RET-Y for reticulocytes.

Subsequently, algorithms  $y=6.4 \text{ x e}^{0.0009 \text{ x Ret-Y}}$  and  $y=6.4 \text{ x e}^{0.0009 \text{ x RBC-Y}}$  were applied in order to transform arbitrarily reported channel numbers of the RET-Y and RBC-Y into hemoglobin content equivalents. How content in reticulocytes and RBCs is expressed in pg and denoted as RET-He and RBC-He, respectively.

## Zinc protoporphyrin hem ratio

Measurements of zinc protoporphyrin hem ratio (ZPP/Hb ratio) were performed on a hematofluorometer (AVIV Biochemical Inc., Lakewood, NJ, USA) using front surface illumination fluorometry.<sup>20</sup>

## Statistical analysis

SPSS/PC statistical software, version 14.0 for Windows, was applied for statistical analysis of results (SPSS, Chicago, IL, USA). Paired sample t-tests were performed to detect statistically significant deviations between results before and after iron supplementation. p<0.05 was considered statistically significantly different. Data are expressed as mean values±SD, unless specified otherwise.

#### RESULTS

The study included a group of 25 subjects during the third trimester of pregnancy. On suspicion of IDE, we selected parameters to discriminate subjects with Hb  $\leq$ 110 g/L. Additionally, MCV 80-100 fL, Ret-He <29.6 pg and ZPP >75  $\mu$ mol/mol hem were applied as initial screening parameters. <sup>16</sup> Results indicating deviations of erythropoiesis activity are listed in Table 1. Reticulocyte counts demonstrated a tendency towards increased levels

**Table 1.** Hemocytometric parameters before and after iron supplementation. Results are established in 25 subjects with Hemoglobin values in the range of 101-110 g/L.

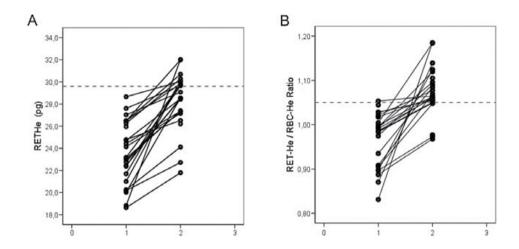
Iron supplementation					
Parameter	Before mean±SD (min-max)	After mean±SD (min-max)	р		
Hb (g/L)	105±6	114±5	<0.001		
6. /	(87-111)	(108-122)			
MCV (fL)	83.3±4.2	85.0±3.3	0.118 (NS)		
. ,	(72.5-90.9)	(78.7-89.7)	, ,		
MCHC (mmol/L)	20.3±0.5	20.4±0.6	0.599 (NS)		
	(19.3-21.2)	(19.4-21.8)			
RDW-SD (fL)	45.0±3.6	52.3±7.0	< 0.001		
	(37.8-52.0)	(40.1-69.2)			
Reti (x1012/L)	0.061±0.015	$0.079\pm0.026$	< 0.001		
	(0.024 - 0.089)	(0.025-0.150)			
Ret-He (pg)	23.6±2.8	28.3±2.6	< 0.001		
	(18.6-28.7)	(21.8-32.0)			
RBC-He (pg)	26.9±1.9	27.4±1.8	0.197 (NS)		
	(22.2-29.6)	(24.2-30.2)			
Ret-He / RBC-He ratio	0.97±0.06	1.07±0.05	< 0.001		
	(0.83-1.05)	(0.97-1.18)			
ZPP (μMol/Mol haem)	) 124±44	116±34	0.359 (NS)		
,	(77-246)	(63-207)			

SD, standard deviation; NS, non significant; Hb, Hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW-SD, red blood cell distribution width; Ret-He, hemoglobin content of reticulocytes: RBC-He, hemoglobin content of red blood cell; ZPP, zinc protoporphyrin.

after iron supplementation ( $0.079\pm0.026 \times 10^{12}/L$ ) compared with those before supplementation ( $0.061\pm0.015\times 10^{12}/L$ ) (p<0.001). Individual hemoglobin content of reticulocytes (Ret-He and Ret-He/RBC-He ratio) before and after iron supplementation are shown in Figure 1A and B. After iron supplementation, there was a clear increase in Ret-He content of 20% from 23.6  $\pm$  2.8 pg to 28.3  $\pm$  2.6 pg (p<0.001) and Ret-He/RBC-He ratio was increased by 10% from 0.97  $\pm$  0.06 to 1.07  $\pm$  0.05 (p<0.001).

There was only a slight (2%) increase in RBC-He from  $26.9 \pm 1.9$  pg to  $27.4 \pm 1.8$  pg (NS). Hb concentrations showed a tendency to increase from  $105 \pm 6$  g/L (mean $\pm$ SD) to  $115 \pm 5$  g/L (p<0.001) after iron supplementation. In order to evaluate the effect of iron supplementation on RET-He, deviations in Hb, RET-He and RET-He/RBC-He ratio, respectively, are shown in Figure 2A and B. Evaluation of RET-He and RET-He/RBC-He ratio provides a more sensitive measurement of shifts in values as a result of iron supplementation when compared with traditional Hb measurements. This is expected, because RET-He and RET-He/RBC-He ratio parameters reflect short-term deviations. No statistically significant deviations in MCV or mean corpuscular hemoglobin concentration (MCHC) were observed.

A statistically significant tendency towards increased red blood cell distribution width



**Figure 1.** Individual results for reticulocyte hemoglobin content (RET-He, pg, A) and RetHe/RBC-He ratio (B) established in 25 subjects during pregnancy before (1) and after (2) four weeks of iron supplementation. The horizontal line indicates the lower level of the reference range for apparently healthy subjects.

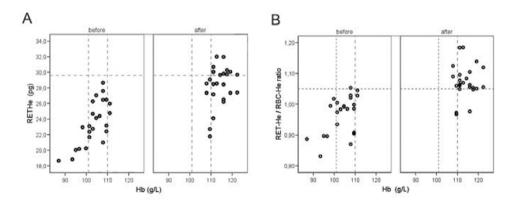
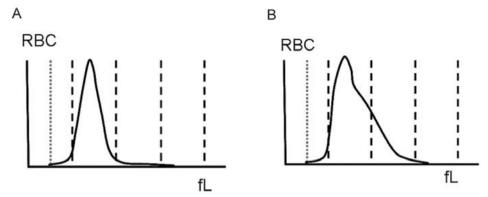


Figure 2. Changes in hemoglobin (Hb, g/L) and reticulocyte hemoglobin content (RET-He, pg, A), respectively, RET-He/RBC-He ratio (B) established in 25 subjects during pregnancy before (1) and after (2) four weeks of iron supplementation. The horizontal line indicates the lower level of the reference range for apparently healthy subjects. The vertical lines indicate the controversial Hb range of 101-110 g/L.



**Figure 3.** An example of a red blood cell (RBC) histogram before (A) and after (B) iron supplementation. The newly formed RBC population (B) is demonstrated on the right side of the curve. The x-axis demonstrates the RBC-volume (fL). The vertical dashed line (gray) reflects the lower discriminator of the RBC-volume (fL).

(RDW-SD) was observed showing a rise from  $45.0\pm3.6$  fL before supplementation to  $52.3\pm7.0$  fL after supplementation (p<0.001). An example of a representative shift in RBC histogram before and after iron supplementation is shown in Figure 3A and Figure 3B. After iron supplementation, the RBC histogram shows a dimorphic population, with a shoulder indicating the new RBC population on the right side.

There was a slight decrease in ZPP from 124  $\pm$  44  $\mu$ mol/mol hem to 116  $\pm$  34  $\mu$ mol/mol hem (NS) after iron supplementation.

### DISCUSSION

A mild degree of anemia is common in the third trimester of pregnancy. Additional supplementation of iron is a question for debate. The aim of this study was to establish short-term effects of iron supplementation on Ret-He and RBC-He of women with inconclusive Hb concentrations with suspected IDE in the third trimester of pregnancy.

Our study demonstrated that Ret-He levels clearly increased after four weeks of iron supplementation towards levels within the lower region of the reference interval  $30.4 \pm 36.8$  pg. <sup>17</sup> Ret-He/RBC-He ratio demonstrated a similar trend when compared with Ret-He. The observed shifts in Ret-He and Ret-He/RBC-He ratio reflect *short-term* alterations concerning the quality of erythropoiesis. <sup>17-25</sup> Our study revealed a clear increase in Hb concentrations and absolute reticulocyte counts after iron supplementation, in particular in the group of subjects with Hb in the controversial range of 101- 110 g/L. During pregnancy, it is difficult to assess whether an increase in Hb concentration and reticulocytes is the effect of increased activity of erythropoiesis after supplementation, or if this is due to a less rapid increase in plasma volume in late pregnancy. Several investigators reported a 6 g/L increase in Hb in late pregnancy without iron supplementation. <sup>4.5.7</sup> However, in

our study, after iron supplementation, an increase in Hb of approximately 10 g/L was observed.

According to an evaluation made in previous studies, nutrient supplementation did not reveal any significant changes in RBC-He content or ZPP/Hb ratio. <sup>10,14,15</sup> The lack of effect may be explained by the fact that RBC-He and ZPP reflect *long-term* impact on shifts in hemoglobinization, corresponding with the lifespan of circulating mature RBCs (100 days). <sup>18</sup> An increased degree of heterogeneity in the size of RBCs (anisocytosis), amongst others due to increased erythropoiesis, is reflected in RDW. After iron supplementation, RDW-SD increased in 88% of the subjects. In this study, increased RDW values are indicative of enhanced erythropoiesis as a response to iron supplementation. However, despite a positive response to erythropoiesis after iron supplementation, no definitive conclusions can be drawn concerning depletion of iron stores. During pregnancy, the need for micronutrients, in particular iron and folate, is increased. Maternal body stores and dietary intake may be insufficient for adequate erythropoiesis. Supplementary iron is needed for erythropoiesis to enhance increased production of RBC and to fulfill the additional iron demands of the fetus. The physiological mechanism for covering additional iron requirements is to release iron from the body stores.

However, many Western European women have an inadequate dietary iron intake which can not fulfill the increased demands in middle and late pregnancy.<sup>2,9,26</sup> Therefore, IDE is a frequent cause of anemia during pregnancy.

The Hb cut-off level for suspected IDE has been the subject of frequent discussion. <sup>4,9-11</sup> Approximately 10% of the pregnant women showed inconclusive Hb concentrations in the range between 101-110 g/L (Margreet Schoorl, 2012 personal communication). Anemia screening and monitoring based exclusively on Hb concentrations is considered to be inappropriate. It has been recommended ZPP and Ret-He should both also be assessed. <sup>5,10,16</sup> The appropriate dosage of prophylactic iron supplementation needs to be considered. Several investigators reported wide deviations in daily doses in the range of 40-200 mg. Regimens with less frequent iron supplementation, such as once or twice weekly, have been described as promising. <sup>11,13,21,24,27</sup>

Only marginal effects of iron supplementation on the newborn's birth weight or on prenatal morbidity or mortality in mother and child have been reported. However, positive effects have also been reported, such as increased physical fitness and well-being in pregnant women, prevention of postpartum iron deficiency due to blood loss at delivery, and enhanced iron reserves in the newborn to prevent iron deficiency in the first years of life. <sup>9,28</sup> In summary, clinical practice needs simple and reliable strategies for screening and monitoring IDE during pregnancy.

### Study limitations

In the present study, subjects with thalassemia were excluded. Although Ret-He is decreased in these subjects, results for Ret He/RBC-He ratio are within the reference range. Corresponding shifts in decreased values for Ret-He and Ret-He/RBC-He ratios, respectively, lead to the conclusion that there is a temporarily decreased degree of hemoglobinization. <sup>17</sup> In subjects with thalassemia, we also recommend measurement of Ret-He and Ret-He/RBC-He in IDE screening during pregnancy.

### **CONCLUSIONS**

We recommend that Ret-He and Ret-He/RBC-He ratio parameters should be integrated into the protocol for anemia screening and monitoring during pregnancy. Ret-He and Ret-He/RBC-He ratio are sensitive markers for screening when a decrease in red blood cell hemoglobin content is observed and for monitoring short-term effects of iron supplementation. The recommended parameters should be considered in particular in the group of subjects with Hb in the controversial range of 101- 110 g/L. Ret-He and Ret-He/RBC-He ratio may in future be a useful measurement to help optimize the dosage of prophylactic iron supplementation during pregnancy.

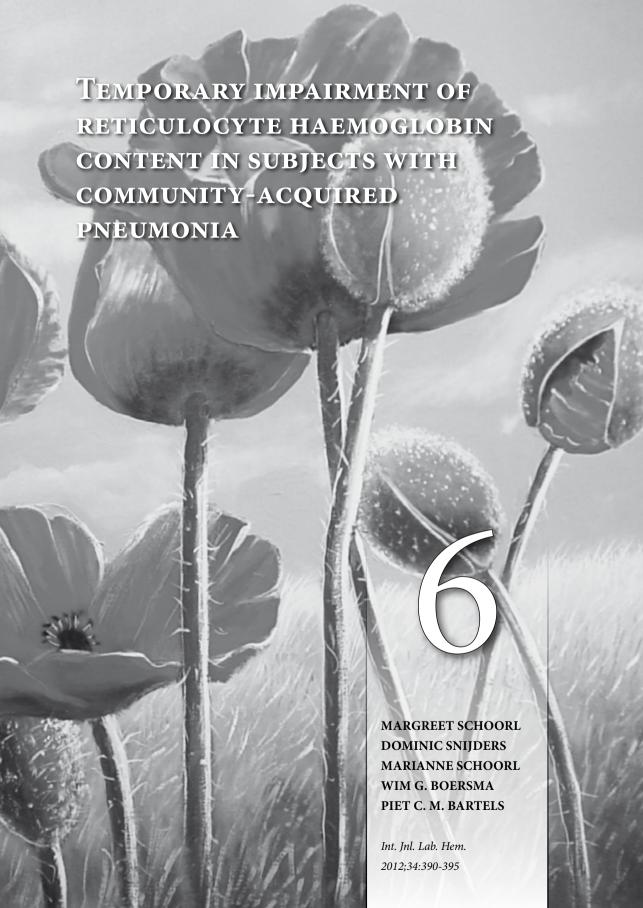
### REFERENCES

- Lindsay H. Pregnancy and iron deficiency: unresolved issues. Nutr Rev 1997;55:91-101.
- Hercberg S, Preziosi P, Galan P. Iron deficiency in Europe. Public Health Nutr 2001;4:537-45.
- 3. Nybo M, Friis-Hansen L, Felding P. Higher prevalence of anemia among pregnant immigrant women compared to pregnant ethnic Danish women. Ann Hematol 2007;86:647-51.
- 4. Milman N, Bergholt T, Byg K, et al. Reference intervals for haematological variables during normal pregnancy and postpartum in 434 healthy Danish women. Eur J Haematol 2007;79:39-46.
- Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Haematol 2007;79:539-45.
- Sant-Rayn P. Should we screen for iron deficiency anaemia? A review and recent recommendations. Pathology 2012;44:139-47.
- Klajnbard A, Szecsi PB, Colov NP, et al. Laboratory reference intervals during pregnancy, delivery and the early postpartum period. Clin Chem Lab Med 2010;48: 237-48.
- 8. WHO. Department of making pregnancy safer. Standards for maternal and neonatal care: iron and folate supplementation. Integrated management of pregnancy and childbirth (IMPAC). Geneva: World Health Organisation, 2006.
- 9. Milman N, Bergholt T, Byg K, et al. Iron status and iron imbalance during pregnancy. A critical reappraisal of iron supplementation. Acta Obstet Gyn Scan 1999;78:749-57.
- 10. Wheeler S. Assessment and interpreta-

- tion of micronutrient status during pregnancy. Proc Nutr Soc 2008;67:437-50.
- 11. Pavord S, Myers B, Robinson S, et al. UK guidelines on the management of iron deficiency in pregnancy. Br J Haematol 2012;156:588-600.
- Koninklijke Nederlandse Organisatie van Verloskundigen (Royal Dutch Organisation of Midwives). Anemie in de eerstelijns verloskundige praktijk. Praktijkkaart behorende bij de gelijknamige KNOV-standaard. Bilthoven. 2000.
- Koninklijke Nederlandse Organisatie van Verloskundigen (Royal Dutch Organisation of Midwives). Anemie in de verloskundige praktijk. Praktijkkaart behorende bij de gelijknamige KNOV-standaard 2010. Utrecht.
- 14. Romslo I, Haram K Sagan N, Augensen K. Iron requirement in normal pregnancy assessed by serum ferritin, serum transferrin saturation and erythrocyte protoporphyrin determinations. Br J Obstet Gynaec 1983;90:101-7.
- 15. Harthoorn EJ, Lindemans J, Langenhuijsen MMAC. Erythrocyte zinc protoporphyrin testing in pregnancy. Acta Obstet Gyn Scan 2000;79:660-6.
- Schoorl M, van der Gaag D, Schoorl M, Bartels PCM. Changes in red blood cell hemoglobinization during pregnancy. Ned Tijdschr Klin Chem Labgeneesk 2010; 35:206-8.
- 17. Bartels PCM, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-14.
- 18. Thomas C, Thomas L. Biochemical markers and haematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-76.
- 19. Franck S, Linssen J, Messinger M, Tho-

- mas L. Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. Clin Chem 2004;50:1240-2.
- Blumberg WE, Eisinger J. Principles and applications of haematofluorometry. J Clin Lab Autom 1984;4:29-42.
- 21. Milman N. Iron prophylaxis in pregnancy general or individual and in which dose? Ann Hematol 2006;85:821-8.
- 22. Milman N. Targeted (not global) iron supplementation/fortification is the issue! Ann Hematol 2012;91:959-60.
- 23. Ghio AJ. Anemia and global iron fortification and supplementation. Ann Hematol 2012;91:957-8.
- 24. Ribot B, Aranda N, Viteri F, et al. Depleted iron stores without anaemia early in pregnancy carries increased risk of

- lower birthweight even when supplemented daily with moderate iron. Hum Reprod 2012; 27:1260-6.
- 25. Brugnara C. Reticulocyte haemoglobin equivalent (Ret-He) and assessment of iron-deficient states. Clin Lab Haematol 2006;28:303-8.
- 26. Svandberg B. Absorption of iron in pregnancy. Acta Obstet Gyn Scan 1975;48 (Suppl):1-108.
- Beentjes M, Jans S. Revised practice Guideline Anaemia in midwifery practice. Ned Tijdschr Geneeskd. 2012; 156:A3711.
- 28. Wiegerinck MM, Mol BWJ. Insufficient evidence supporting iron supplementation in anaemia during pregnancy. Ned Tijdschr Geneeskd. 2012;156:A4293.



### **ABSTRACT**

*Introduction*: In the case of inflammation, imbalance of iron homoeostasis is caused by increased retention of iron within cells of the reticuloendothelial system. Iron-restricted erythropoiesis occurs because of decreased availability of iron for haemoglobin (Hb) synthesis in erythroid progenitor cells.

Deviations in reticulocyte haemoglobin (Ret-He) content are investigated together with inflammation markers in subjects with community-acquired pneumonia (CAP). Short-term alterations with regard to Ret-He during and after completing antibiotic treatment are investigated.

*Methods*: A total of 75 patients, classified into three subgroups with CURB-65 scores of  $\leq 1$ , 2 and  $\geq 3$ , participated in the study.

**Results:** Within the three subgroups, Hb results demonstrate a decline from the day of admission until day 4. From day 4, an increase towards higher values is observed at day 14. Within 24 h after admission, Ret-He results are situated within the lower quartile region of the reference range interval. Until day 4 of hospital admission, a steady trend towards a decline of 3-8% is established. During antibiotic treatment, an increase in reticulocyte count occurs from  $0.039 \pm 0.014 \times 10^{12}/L$  at day 4 to  $0.057 \pm 0.020 \times 10^{12}/L$  at day 14 (mean  $\pm$  SD). Recovery of Hb and Ret-He occurs towards values within the reference range.

**Conclusion:** In subjects with CAP, acute inflammation results in impairment of Ret-He at an early stage. After onset of pneumonia, decreased results of Ret-He and Ret-He/RBC-He ratio are demonstrated, reflecting acute erythropoietic dysfunction, which are amongst others caused by functional iron depletion.

### INTRODUCTION

The condition indicated as the *anaemia of inflammation* refers to subjects with acute or chronic immune activation. A protective mechanism including retention of iron from the blood circulation as an essential growth factor, preventing pathogens from invading organs, whilst increasing the efficacy of cell mediated immunity (Brittenham *et al.*, 2000). Cytokines and cells of the reticuloendothelial system are able to induce changes in pathophysiology concerning iron homoeostasis. Phenomena reflecting the pathophysiology of anaemia are decreased proliferation of erythropoietic progenitor cells, reduction in erythropoietin stimulation and a shorter lifespan of red blood cells (RBCs) (Weiss & Goodnough, 2005).

In healthy subjects, reticulocyte maturation occurs with progressive reduction in RBC size and haemoglobin content (RBC-He). Haemoglobin content of reticulocytes (Ret-He) yields actual information concerning the functional availability of iron for haemoglobinization in erythroid precursors (Hinzman, 2003).

In the case of inflammation, imbalance of iron homoeostasis is because of increased retention of iron within cells of the reticuloendothelial system. As a result, iron-restricted erythropoiesis occurs because of decreased availability of iron for Hb synthesis in erythroid progenitor cells (Weiss & Goodnough, 2005). The amount of studies concerning the impact of infection on erythropoiesis is limited (Banfi *et al.*, 2006).

Innovation of haematology analysers yields procedures for classification of RBCs and reticulocytes. In the case of subjects with an anaemia of inflammation, results of Ret-He and Ret-He/RBC-He ratio combined with conventional parameters like mean cell volume (MCV), haemoglobin (Hb) and mean cell haemoglobin concentration (MCHC) should be considered to achieve an appropriate clinical interpretation with regard to deviations in Hb synthesis.

With the application of a longitudinal study design, deviations of Ret-He are investigated and combined with simultaneous monitoring of inflammatory markers in subjects with community-acquired pneumonia (CAP). The aim of the study is to look for short-term alterations with regard to Ret-He during and after completing antibiotic treatment.

# **SUBJECTS AND METHODS**

## Study design

The study protocol was approved by the local medical ethics committee of the Medical Center Alkmaar.

Seventy-five patients (men: n = 48; women: n = 27) with a medical history and clinical and radiological findings consistent with CAP requiring hospitalization were enrolled in the study. Inclusion criteria were written informed consent, age  $\ge 18$  years, new consolidation(s) on the chest radiograph and clinical presentation of acute illness with one or more of the following symptoms indicating CAP: temperature ( $\ge 38^{\circ}$ C), dyspnoea, cough (with or without expectoration of sputum) and chest pain. Exclusion criteria were pregnancy or lactation, severe immunosuppression, neoplastic disease, any condition requiring corticosteroid treatment prior to admission for pneumonia (as in the case of



**Table 1.** Evaluation of haemocytometric parameters, including mean value, standard deviation (SD), minimum–maximum values, is established in 75 subjects with CURB-65 scores ≤1, 2 and ≥3. The longitudinal study concerns results at hospital admission (day 1), during antibiotic treatment (day 4) and after antibiotic treatment (day 14).

	CLIDD	Day 1	Day 4	Day 14
Parameter	CURB score	Mean ± SD (min-max)	Mean ± SD (min-max)	Mean ± SD (min-max)
RBC (10 <sup>12</sup> /L)	≤1	$4.48 \pm 0.50 \ (2.91 - 5.40)$	4.21 ± 0.40 (3.52-5.18)	$4.48 \pm 0.47 (3.55 - 5.42)$
	2	$4.29 \pm 0.48 (2.91 - 4.87)$	$3.89 \pm 0.46  (2.47 - 4.53)$	$4.17 \pm 0.25 (3.80 - 4.70)$
	≥3	$4.09 \pm 0.52 (3.26 - 5.18)$	$3.76 \pm 0.39 (3.13 - 4.38)$	$3.86 \pm 0.41 (3.12 - 4.54)$
Hb (g/L)	≤1	137 ± 13 (105–163)	127 ± 11 (106–156)	$137 \pm 13 (108 - 164)$
	2	$129 \pm 14 (89-153)$	$118 \pm 14 (76 - 135)$	$126 \pm 10 (113 - 143)$
	≥3	$127 \pm 18 (100 - 171)$	$116 \pm 13 (95-137)$	$121 \pm 13 (95-134)$
MCV (fL)	≤1	$89.6 \pm 5.0 (78.1 - 101.4)$	$89.6 \pm 4.6 \ (78.2 - 98.3)$	$90.6 \pm 5.2 (79.2 - 104.2)$
	2	$90.0 \pm 3.7 (83.8 - 98.3)$	$90.6 \pm 3.8 \ (84.0 - 99.2)$	$91.5 \pm 3.8 (85.1 - 99.5)$
	≥3	$92.0 \pm 5.1 \ (83.5 - 103.9)$	$91.2 \pm 6.1 \ (82.0 - 102.9)$	93.1 ± 5.9 (84.8–103.5)
MCHC (g/L)	≤1	$341 \pm 13 (317 - 372)$	$340 \pm 11 (319 - 362)$	$336 \pm 10 (319 - 357)$
	2	$336 \pm 11 (317 - 356)$	$332 \pm 10 (314 - 348)$	$328 \pm 10 (314 - 345)$
	≥3	341 ± 11 (319–357)	$336 \pm 10 \ (324 - 356)$	$330 \pm 8 \ (322 - 348)$

MCHC, mean cell haemoglobin concentration; MCV, mean corpuscular volume.

COPD and asthma), pneumonia which developed within 8 days after hospital discharge, and obstruction pneumonia (e.g. caused by lung cancer). At hospital admission, severity of illness was scored with application of the CURB-65 score. CURB-65 score is a severity index for CAP classification, evaluating degree of confusion, blood urea nitrogen, respiratory rate, blood pressure and age 65 or older (Fine *et al.*, 1997; Lim *et al.*, 2003). The group of patients was subdivided into three groups with CURB-65 scores  $\leq 1$  (n = 38), 2 (n = 21) and  $\geq 3$  (n = 16), respectively.

Patients received antibiotic treatment for 7 days according to locally used guidelines (Schouten et al., 2005). Subjects were monitored daily with regard to clinical stability. Time to clinical stability was scored according to the criteria from Halm *et al.* (1998). Laboratory investigations were performed at hospital admission and subsequently once a day until day 7. The final measurement was taken on day 14 (Snijders *et al.*, 2010).

#### Preparation of blood samples

Blood samples were drawn into Vacutainer® tubes, anticoagulated with  $K_2$ EDTA (Becton Dickinson, Plymouth, UK), and plain tubes (Vacutainer SST, Becton Dickinson, Plymouth, UK). Blood samples were analysed within 4 h after collection. For additional investigations of biochemical parameters reflecting activity state of infection, serum samples were stored in aliquots at -20°C until analysis.

### Haemocytometry

Haemocytometric parameters were established by measurement on a Sysmex XE-2100 Haematology Analyzer (Sysmex Corporation, Kobe, Japan). Reticulocyte methodology

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of measurement is based on automated fluorescent flow cytometry utilizing a polymethine dye for binding cytoplasmic RNA. The mean forward, light scatter intensity in the reticulocyte channel is estimated as a measure reflecting the particle volume and Hb content of RBCs and reticulocytes, respectively. Results concerning Hb content are initially reported as RBC-Y for RBCs and RET-Y for reticulocytes. Subsequently, algorithms  $y = 6.4xe^{0.0009\,x\,\text{Ret-Y}}$  and  $y = 6.4xe^{0.0009\,x\,\text{Ret-Y}}$  are applied to transform arbitrarily reported channel numbers of the RET-Y and RBC-Y into haemoglobin content equivalents (Franck *et al.*, 2004). Hb content in reticulocytes and RBCs is expressed in picograms and denoted as Ret-He and RBC-He, respectively.

### Parameters reflecting inflammatory state of infectious disease

Procalcitonin (PCT) concentration was established by applying Kryptor B.R.A.H.M.S. PCT Sensitive assay (Brahms AG, Hennigsdorf, Germany) on the Kryptor Compact analyser (Brahms AG).

#### Statistical evaluation

Statistical evaluation of analytical results is performed with SPSS software 14.0 for Windows (SPSS, Chicago, IL, USA). Paired-samples t-tests are performed to detect statistically significant deviations between the days of hospital admission. Independent-sample t-tests are performed between the subgroups; P-values <0.05 are considered to be statistically significant. Data are expressed as mean values  $\pm$  SD, unless specified otherwise.

### RESULTS

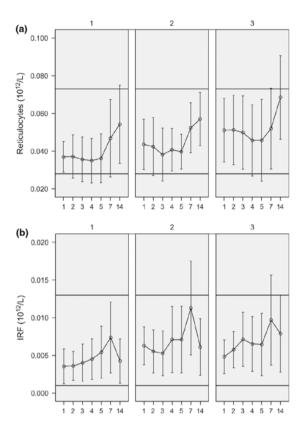
Results for conventional haemocytometric parameters are listed in Table 1. Within 24 h after hospital admission, results for Hb are situated within the lower region of the reference range interval (men, 137–177 g/L; women, 121–161 g/L). Until day 4 of hospital admission, a steady trend towards a decline of 3–8% is established.

Within the three subgroups (CURB-65  $\leq$ 1, 2 and  $\geq$ 3), results for Hb concentration demonstrate a decline from admission (137  $\pm$  13, 129  $\pm$  14 and 127  $\pm$  18 g/L, respectively) until day 4 (127  $\pm$  11 (P = 0.000), 118  $\pm$  14 (P = 0.000) and 116  $\pm$  13 (P = 0.026) g/L, respectively). Subjects with higher CURB-65 scores demonstrate a tendency towards lower Hb concentrations.

From day 4, a steady trend towards increasing Hb concentrations amounting to  $137 \pm 13$  (P = 0.000),  $126 \pm 10$  (P = 0.006) and  $121 \pm 13$  (NS) g/L, respectively, at day 14 is observed. Decreasing Hb concentrations may be explained amongst others by haemodilution. No significant deviations are observed for results of MCHC.

Fluctuations of erythopoïesis activity over time are depicted in Figure 1 (mean values  $\pm$  SD). In the subgroups (CURB-65  $\leq$ 1, 2 and  $\geq$ 3), results for absolute reticulocyte counts demonstrate a tendency towards increase from day 4 towards 0.055  $\pm$  0.021 (P = 0.001), 0.057  $\pm$  0.014 (*P* = 0.000) and 0.065  $\pm$  0.023 (NS) x 10<sup>12</sup>/L, respectively, at day 14.

Results for immature reticulocyte fraction (IRF) increase steadily from admission towards 0.0076  $\pm$  0.0044 (P = 0.005), 0.0113  $\pm$  0.0062 (P = 0.002) and 0.0104  $\pm$  0.0066 (NS), respectively, at day 7. At day 7, results for IRF decrease slightly towards 0.0043  $\pm$  0.0029



Results (mean  $\pm$  SD) for reticulocytes count (1012/L, a) and immature reticulocyte fraction (IRF, 1012/L, b) have been established in 75 subjects with CURB-65 scores  $\leq 1$  (=1), 2 (=2) and  $\geq 3$  (=3) at hospital admission (day 1), at several days during antibiotic treatment (day 2, 3, 4, 5 and 7) and after antibiotic treatment (day 14). The horizontal lines indicate the lower and upper levels of the reference range for apparently healthy subjects.

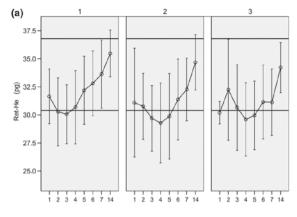
Figure 1.

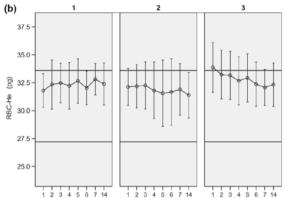
(P=0.000), 0.0061  $\pm$  0.0038 (NS) and 0.0083  $\pm$  0.0052 (NS) x  $10^{12}$ /L, respectively, at day 14. Results concerning Ret-He fluctuations are represented in Figure 2 (mean values  $\pm$  SD). With regard to initial measurements within 24 h after hospital admission, Ret-He values are situated within the lower region of the reference range interval (30.4–36.8 pg). Results for Ret-He in the subgroups demonstrate a decline of 3–8% (NS) within the time period elapsing from hospital admission (31.6  $\pm$  2.4, 30.8  $\pm$  2.9 and 32.3  $\pm$  4.5 pg, respectively) until day 4 [30.7  $\pm$  3.2 (NS), 29.3  $\pm$  3.6 (NS) and 29.6  $\pm$  3.4 pg (NS), respectively]. After reaching a minimum Ret-He value at day 4, an increasing trend towards values within the reference range of 35.5  $\pm$  2.0 (P=0.000), 34.7  $\pm$  2.5 (P=0.000) and 34.0  $\pm$  2.3 (P=0.001) pg, respectively, is observed at day 14.

In contrast, no shift is detected when concerning results for RBC-He content (Figure 2). For results concerning Ret-He/RBC-He ratio, a similar longitudinal pattern is demonstrated. Within 24 h after admission results of Ret-He/RBC ratio are decreased (0.99  $\pm$  0.07, 0.97  $\pm$  0.16 and 0.97  $\pm$  0.11, respectively). Until day 4, a further decrease to 0.95  $\pm$  0.07 (NS), 0.92  $\pm$  0.08 (NS) and 0.91  $\pm$  0.08 (NS), respectively, is observed. From day 4, a steady trend to increase towards 1.10  $\pm$  0.02 (P = 0.000), 1.10  $\pm$  0.02 (P = 0.000) and 1.06  $\pm$  0.05 (P = 0.000), respectively, at day 14 is observed.

At hospital admission, PCT concentrations are obviously increased (2.6  $\pm$  6.2, 6.2  $\pm$  5.5 and 16.1  $\pm$  11.6  $\mu g/L$  respectively). Owing to treatment with antibiotics, a steady trend to







1 2 3 30.0 - 25.0 - 20.0 - 15.0 - 10.0 - 5.0 - 10.0 - 12.3 4 5 7 14 1 2 3 4 5 7 14

Figure 3. Results (mean  $\pm$  SD) for concentrations of procalcitonin (µg/L) have been established in 75 subjects with CURB-65 scores  $\leq 1$  (=1), 2 (=2) and  $\geq 3$  (=3) at hospital admission (day 1), at several days during antibiotic treatment (day 2, 3, 4, 5 and 7) and after antibiotic treatment (day 14). The horizontal line indicates the upper level of the reference range for apparently healthy subjects.

Figure 2.

Results (mean ± SD) for reticulocyte haemoglobin content (Ret-He,

picograms, a) and red blood cell haemoglobin

content (RBC-He, picograms, b) have been established in 75 subjects

with CURB-65 scores  $\leq 1$  (=1), 2 (=2) and  $\geq 3$ 

(=3) at hospital admission (day 1), at several days during antibiotic treatment (day 2, 3, 4, 5

and 7) and after antibi-

otic treatment (day 14). The horizontal lines in-

dicate the lower and up-

per levels of the reference range for apparently

healthy subjects.

decrease towards the reference range (<0.05  $\mu$ g/L, Christ-Crain et al., 2006) is demonstrated for PCT results at day 14 (0.05  $\pm$  0.03 (P = 0.012), 0.06  $\pm$  0.03 (P = 0.036) and 0.06  $\pm$  0.03  $\mu$ g/L (P = 0.036), respectively (Figure 3). Declining results are indicative of reduction in inflammation activity because of beneficial response to treatment with antibiotics.

## **DISCUSSION**

The anaemia of inflammation is in part because of a disturbance of systemic iron utilization. In pathological conditions, temporarily decreased haemoglobin synthesis may be due to reduced iron availability to the developing erythron (Weiss, 1999; Brittenham et al., 2000; Thomas & Thomas, 2005; Ganz, 2011).

Pro-inflammatory cytokines and cells of the reticuloendothelial system induce changes in iron homoeostasis. Availability of iron is thought to be of vital importance for tissue pro-liferation of micro organisms because it is an essential nutrient for enzyme activity in the citric acid cycle, mitochondrial respiration, DNA synthesis and oxygen transport systems. Sequestration of iron from micro organisms into the reticuloendothelial system implies a protective strategy for inhibiting the growth of pathogens.

Haemocytometric parameters assessing the effects of poor iron supply for Ret-He have been recently introduced. Ret-He is demonstrated to be a sensitive indicator of monitoring short-term deteriorations in functional iron supply for erythropoiesis (Bartels, Schoorl & Schoorl, 2006; Brugnara, Schiller & Moran, 2006). In the case of apparently healthy subjects, Ret-He results exceed the results of RBC-He amounting to 5–15%.

A temporarily decreased degree of haemoglobinization is inferred from corresponding shifts in MCV values with decreased values for Ret-He and Ret-He/RBC-He ratios, respectively (Schoorl et al., 2006). If compared with interpretation of RBC counts, Ret-He yields an immediate indication, concerning the actual shift of iron availability for haemoglobinization in erythroid precursors.

In the case of immune activation, iron suppletion induces production of highly toxic hydroxyl radicals, which may cause tissue damage and endothelial dysfunction, and an increased risk of acute cardiovascular events (Weiss & Goodnough, 2005). Iron suppletion is inappropriate in the case of subjects with acute inflammation (Brittenham *et al.*, 2000; Fleming, 2008).

### **CONCLUSION**

Results of our study demonstrate a temporary impairment of Ret-He in subjects with community-acquired pneumonia. After onset of pneumonia, decreased results of Ret-He and Ret-He/RBC-He ratio occur, reflecting acute erythropoietic changes that may be due to functional iron deficiency. Recently, it has been established that iron redistribution during inflammation is primarily regulated by the hepatocellular peptide hormone hepcidin (Fleming, 2008). The effect of hepcidin will be investigated in a next study.

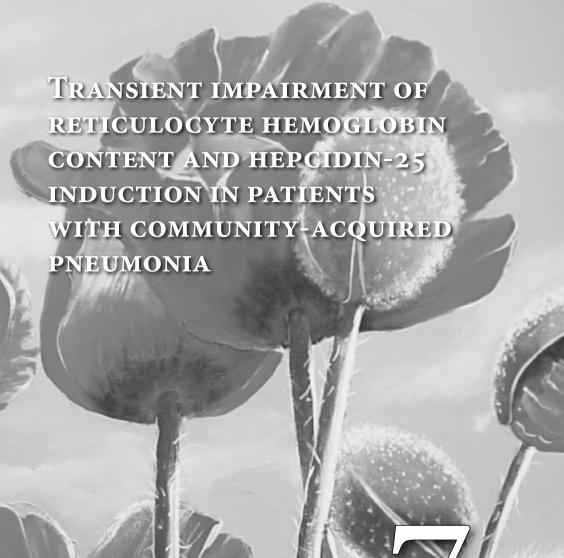
### REFERENCES

- Banfi G., Mauri C., Morelli B., Di Gaetano N., Malgeri U. & Melegati G. (2006) Reticulocyte count, mean reticulocyte volume, immature reticulocyte fraction and mean sphered cell volume in elite athletes: reference values and comparison with the general population. Clinical Chemistry and Laboratory Medicine 44, 616–622.
- Bartels P.C.M., Schoorl M. & Schoorl M. (2006) Haemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clinical Laboratory 52, 107–114.
- Brittenham G.M., Weiss G., Brissot P., Lainé
  F., Guillygomarc'h A., Guyader D., Moirand
  R. & Deugnier Y. (2000) Clinical consequences of new insights in the pathophysiology of disorders of iron and heme metabolism. Hematology American Society of Hematology Education Program, 39–50.
- Brugnara C., Schiller B. & Moran J. (2006)
  Reticulocyte hemoglobin equivalent (Ret-He) and assessment of iron-deficient states.
  Clinical and Laboratory Haematology 28,
  303–308.
- Christ-Crain M., Stolz D., Bingisser R., Müller C., Miedinger D., Huber P.R., Zimmerli W., Harbarth S., Tamm M. & Müller B. (2006) Procalcitonin guidance of antibiotic therapy in Community-Acquired Pneumonia. American Journal of Respiratory and Critical Care Medicine 174, 84–93.
- Fine M.J., Auble T.E., Yealy D.M., Hanusa B.H., Weissfeld L.A., Singer D.E., Coley C.M., Marrie T.J. & Kapoor W.N. (1997) A prediction rule to identify low-risk patients with community-acquired pneumonia. New England Journal of Medicine 336, 243–250.
- Fleming R.E. (2008) Iron and inflammation: a cross-talk between pathways regulating hepcidin. Journal of Molecular Medicine 86, 491–494.

- Franck S., Linssen J., Messinger M. & Thomas L. (2004) Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. Clinical Chemistry 50, 1240–1242.
- Ganz T. (2011) Hepcidin and iron regulation, ten years later. Blood 117, 4425–4433.
- Halm E.A., Fine M.J., Marrie T.J., Coley C.M., Kapoor W.N., Obrosky D.S. & Singer D.E. (1998) Time to clinical stability in patients hospitalized with community-acquired pneumonia: implications for practice guidelines. Journal of the American Medical Association 279, 1452–1457.
- Hinzman R. (2003) Iron metabolism, iron deficiency and anaemia. From diagnosis to treatment and monitoring. Sysmex Journal International 13, 65–73.
- Lim W.S., van der Eerden M.M., Laing R., Boersma W.G., Karalus N., Town G.I., Lewis S.A. & Macfarlane J.T. (2003) Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. Thorax 58, 377–382.
- Schoorl M., Schoorl M., Nubé M.J. & Bartels P.C.M. (2006) Erythropoiesis activity, iron availability and reticulocyte haemoglobinization during treatment with hemodialysis and in subjects with uremia. Clinical Laboratory 52, 621–629.
- Schouten J.A., Prins J.M., Bonten M., Degener J.E., Janknegt R., Hollander J.M.R., Jonkers R., Wijnands W., Verheij T., Sachsen A. & Kullberg B.J. (2005) Optimizing the antibiotics policy in the Netherlands. VIII. Revised SWAB guidelines for antimicrobial therapy in adults with community acquired pneumonia. Nederlands Tijdschrift Voor Geneeskunde 149, 2495–2500.
- Snijders D., Daniels J.M.A., de Graaff C.S., van der Werf J.S. & Boersma W.G. (2010) Efficacy of corticosteroids in communityacquired pneumonia. American Journal



- of Respiratory and Critical Care Medicine 181, 975–982.
- Thomas C. & Thomas L. (2005) Anemia of chronic disease: pathophysiology and laboratory diagnosis. Laboratory Haematology 11, 14–23.
- Weiss G. (1999) Iron and anemia of chronic disease. Kidney International 55, s12–s17.
- Weiss G. & Goodnough L.T. (2005) Anemia of chronic disease. New England Journal of Medicine 352, 1011–1023.



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Scand J Clin Lab Invest, 2013; 73: 54-60

### **ABSTRACT**

*Introduction.* Patients with community-acquired pneumonia (CAP) often exhibit a declining hemoglobin (Hb) concentration. During inflammation pro-inflammatory cytokines and cells of the reticuloendothelial system induce disturbances in iron homeostasis. In this study inflammation markers and hepcidin-25 concentrations were monitored together with short-term alterations in reticulocyte hemoglobinization (RET-He).

*Methods.* A total of 25 patients with CAP participated in the study. The assay for serum hepcidin-25 is based on a combination of weak cation exchange chromatography and time-of-flight mass spectrometry.

**Results.** At hospital admission serum hepcidin-25 concentrations (14.6  $\pm$  6.9 nMol/L, mean  $\pm$  SD) were established in the upper level of the reference range (0.5-13.9 nMol/L). Results for C-reactive protein (CRP) and Interleukin-6 (IL-6) were obviously increased compared to the reference ranges. From admission until day 14 hepcidin-25, CRP and IL-6 steadily decreased towards the reference ranges. Hb concentrations declined from admission until day 4 from 8.1  $\pm$  1.0 mMol/L to 7.4  $\pm$  0.9 mMol/L. At admission Ret-He results were within the lower region of the reference range (1900-2300aMol) and results demonstrated a decline during admission from 1931  $\pm$  241 aMol until 1845  $\pm$  199 aMol (NS) at day 4. From a minimum Ret-He value at day 4 results increased towards 2129  $\pm$  136 aMol at day14.

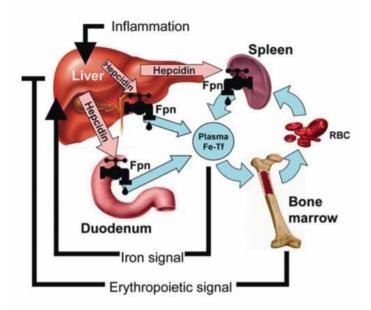
**Conclusion.** A transient increase of cytokine-stimulated serum hepcidin-25 in combination with a temporary decrease of Hb and Ret-He is demonstrated in patients with CAP. Our results support the hypothesis that hepcidin-25 induces transient impairment of reticulocyte hemoglobin content (Ret-He).

### INTRODUCTION

The condition indicated as the *anemia of inflammation* refers to subjects with acute or chronic immune activation. A protective mechanism, including withdrawal of iron from the blood circulation as an essential growth factor of pathogens, is hypothesized to prevent pathogens from invading organs while increasing the efficacy of cell-mediated immunity [1-3].

In the pathophysiology of inflammation, iron homeostasis is deteriorated by increased uptake and increased storage of iron within cells of the reticuloendothelial system. As a result, iron restricted erythropoiesis occurs because of decreased availability of iron for hemoglobin (Hb)-synthesis in the erythron [2]

Iron redistribution during inflammation is amongst others affected by the hepatocellular peptide hormone hepcidin-25. The name implicates an indication for the antimicrobial activity of the peptide [4]. In case of inflammation hepcidin-25 synthesis is superseded by the upregulatory effects of several cytokines. The most important cytokine inducing hepcidin-25 synthesis is IL-6. Increased storage of reticuloendothelial macrophage iron coincides with an increased serum hepcidin-25 concentration, decreased dietary iron absorption and decreased circulating iron. Hepcidin-25 inhibits the release of iron in reticulo endothelial macrophages by binding to the cellular iron exporter ferroportin (Figure 1) [3,5,6].



**Figure 1.** The role of hepcidin in iron metabolism. Hepcidin-ferroportin interaction determines the flow of iron into the plasma. Hepcidin concentration is in turn regulated by iron, erythropoietic activity and inflammation. From: Nemeth E, Ganz T: Acta Haematol 2009; 122:78-86 (DOI: 10.1159/000243791). (Copyright permission obtained from S. Karger AG, Basel).



The anemia of inflammation is in part due to a disturbance of systemic iron utilization. In pathological conditions, temporarily decreased hemoglobin synthesis is due to reduced iron availability to the developing erythron [1,3,7].

Recently new hemocytometric parameters like reticulocyte hernoglobin content (Ret-He), erythrocyte haemoglobin content (RBC-He) and Ret-He/RBC-He ratio have been introduced. Ret-He is demonstrated to be a sensitive indicator for monitoring short-term deteriorations of functional iron supply in erythropoiesis [8-10]. In the case of healthy subjects, Ret-He results exceed the results of RBC-He by 5-15%. From corresponding shifts in decreased values for Ret-He and Ret-He/RBC-He ratios respectively, a temporarily decreased degree of hemoglobinization is concluded. The RET-He/RBC-He ratio provides accurate and sensitive information of the deviation in hemoglobinization between the (normocytic) red blood cell (RBC) population (RBC-He) and the (hypochromic) reticulocyte population (Ret-He) [9].

We hypothesized that transient impairment of hemoglobin synthesis in patients with acute inflammation due to community-acquired pneumonia (CAP) corresponds with increased hepcidin-25 concentrations.

The aim of this study was to elucidate the effects of iron deprivation yielding short term alterations of hemoglobinization by simultaneously monitoring hepcidin-25 and biomarkers regarding activity of infection in patients with CAP.

## SUBJECTS AND METHODS

## Study design

Patients with a medical history combined with clinical and radiological findings consistent with CAP requiring hospitalization were enrolled in the study. Inclusion criteria were written informed consent, age ≥ 18 years, new consolidation(s) on the chest radiograph and clinical presentation of acute illness with one or more of the following symptoms indicating CAP: temperature ≥38°C, dyspnoea, cough (with or without expectoration of sputum), chest pain. Exclusion criteria were pregnancy or lactation, severe immunosuppression, neoplastic disease, any condition requiring corticosteroid treatment prior to admission for pneumonia, pneumonia which developed within 8 days after hospital discharge and obstruction pneumonia (e.g. due to lung cancer). At hospital admission severity of illness was evaluated by application of the CURB-65 score. Patients were classified into three subgroups, with CURB-65 scores  $\leq 1$  (n = 12), 2 (n = 8), and  $\geq 3$  (n = 5) respectively. The CURB-65 score concerns an index for CAP severity classification considering degree of confusion, blood urea nitrogen concentration, respiratory rate, blood pressure and age 65 or older [11]. During hospitalization patients received antibiotic treatment for 7 days according to locally applicable guidelines [12]. Patients were monitored daily with regard to clinical stability. The time period until clinical stability was assessed according to the criteria of Halm et al. [13]. Laboratory investigations were performed at hospital admission and subsequently once a day until day 7. The final measurement was performed at day 14. The present study is part of a larger study [14]. The study protocol was approved by the local medical ethics committee.

## Preparation of blood samples

The total amount of blood drawn for laboratory tests during the integral study is estimated to be approximately 100 mL. Blood samples were drawn into Vacutainer® tubes, anticoagulated with K<sub>2</sub>EDTA (Becton Dickinson, Plymouth, UK) and plain tubes (Vacutainer SST, Becton Dickinson, Plymouth, UK). Blood samples were analyzed within 4 h after collection. Serum samples were stored in aliquots at - 20°C until analysis for investigation of biochemical parameters reflecting activity state of infection.

### Serum hepcidin-25

Serum hepcidin-25 concentrations were established by application of a combined methodology of weak cation exchange chromatography and time-of-flight mass spectrometry (TOF MS). An internal standard (synthetic hepcidin-24; Peptide International Inc.) was applied for quantification [15]. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). The median level of serum hepcidin-25 in a reference subjects group, obtained with the same equipment and methodology, amounted to 4.2 nMol/L, range 0.5-13.9 nMol/L [16]. The analyses were performed in the Department of Laboratory Medicine of the Radboud University (Nijmegen, The Netherlands). This laboratory is particularly renowned for its outstanding expertise with regard to hepcidin-25.

## Biomarhers reflecting the inflammatory activity state of infectious disease

Concentrations of C-reactive protein (CRP) were established on a ProSpec nephelometer according to the manufacturer's instructions (Siemens Healthcare Diagnostics, Deerfield IL, USA). Serum IL-6 concentrations were established on a Beckman Access analyzer using the Access IL-6 reagent (Beckman Coulter Inc., Fullerton, USA).

### Hemocytometry

Hemocytometric parameters were established by measurement on a Sysmex XE-2100 Hematology Analyzer (Sysmex Corporation, Kobe, Japan). Methodology of reticulocyte counting is based on automated fluorescent flowcytometry utilizing a polymethine dye for binding cytoplasmic RNA. The mean forward light scatter intensity in the reticulocyte channel is estimated as a measure for the particle volume and Hb content of RBCs and reticulocytes respectively. Results concerning Hb content are initially reported as RBC- Y for RBCs and RET- Y for reticulocytes. Subsequently, algorithms  $y = 400 \times e^{0.0009 \times Ret-Y}$  and  $y = 400 \times e^{0.0009 \times RBC-Y}$  are applied to transform arbitrarily reported channel numbers of the RET-Y and RBC-Y into hemoglobin content equivalents [17]. Hemoglobin contents in reticulocytes and RBCs are expressed in attomol and denoted as RET-He and RBC-He respectively.

### Statistical evaluation

Statistical evaluation of results was performed with application of SPSS software 14.0 for Windows (SPSS, Chicago, USA). Data were expressed as mean values  $\pm$  SD, unless specified otherwise. Paired-samples t-tests were performed to detect statistically significant deviations in results of analyses between the days of hospital admission. Independent-sample t-tests were performed between the subgroups with increasing CURB-65 scores, p-value < 0.05 was considered to be statistically significant.

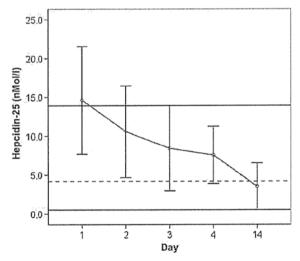


### **RESULTS**

Twenty-five patients (male n = 12, female n = 13) were enrolled in the study. Classification for severity of illness resulted in three subgroups with CURB-65 scores  $\leq 1$  (n = 12), 2 (n = 8), and  $\geq 3$  (n = 5) respectively. Parameters including hepcidin-25 concentrations, actual state of inflammation and the amount of RBC and reticulocyte hemoglobin content were not significantly different between the subgroups with increasing CURB-65 scores (data not shown).

With regard to initial measurements at hospital admission mean values for hepcidin-25 concentrations were situated near the upper level of the reference range (0.5-13.9 nMol/L). Results for hepcidin-25 decreased steadily from 14.6  $\pm$  6.9 nMol/L on the day of hospital admission towards 7.6  $\pm$  3.7 nMol/L (p = 0.024) at day 4 and 3.6  $\pm$  3.1 nMol/L (p = 0.037) at day 14 (Figure 2). For the calculation of p values for day 4 and 14 dates were compared to the day of admission.

At hospital admission CRP and IL-6 concentrations were obviously increased (Table I). CRP- and IL-6 concentrations demonstrated a steadily ongoing decrease from the day of admission until day 14 towards the reference ranges (CRP < 5 mg/L, IL-6 < 10 ng/L). Results for hemocytometric parameters are listed in Table II. With regard to the evaluation of Hb concentration, results below the reference range were observed in 30% of the patients. For Hb concentrations no statistically significant deviation between the subgroups could be detected within 24 h after admission. Results for Hb concentration demonstrated a decline from  $8.1 \pm 1.0$  mMol/L at admission to  $7.4 \pm 0.9$  mMol/L on day 4 (p < 0.001).



**Figure 2.** Results (Mean ± SD) for serum hepcidin-25 concentration (nMol/L) which was established in 25 patients at hospital admission (day 1), at several days during antibiotic treatment (days 2, 3, 4) and after completion of antibiotic treatment (day 14). The horizontal lines indicate the lower and upper level of the reference range for apparently healthy subjects (0.5-13.9 nMol/L). The horizontal dashed line indicates the median value for apparently healthy subjects (4.2 nMol/L).

**Table I.** Longitudinal evaluation of infection biomarkers CRP and IL-6, including median value, standard deviation (SD), minimum-maximum values. Results are established in 25 patients with CAP at hospital admission (day 1) and after antibiotic treatment (day 14). Statistically significant changes from day I until day 14 are shown; p-values < 0.05 are statistically significant; NS = not statistically significant. Reference range for CRP < 5 mg/L and for IL-6 < 10 ng/L.

Parameter	Day 1 ( = hospital admission) Median (min-max)	Day 4 Median (min-max)	Day 14 Median (min-max)	Statistical significance <i>p</i> -value day 1-14
CRP (mg/L)	244	108	7	0.008
IL-6 (ng/L)	(4-528) 54 (5-681)	(11-327) 36 (2-339)	(1-44) 5 (1-113)	0.006

From day 4 onwards a steady trend towards higher concentrations was observed, reaching a level of  $8.0 \pm 0.8$  (p < 0.001) mMol/L at day 14.

Results concerning fluctuations of Ret-He within a time period of 2 weeks are represented in Figure 3A (mean values  $\pm$  SD). With regard to initial measurements within 24 h after hospital admission, mean Ret-He values were situated within the lower region of the reference range interval (1900-2300 aMol) [9]. Results for Ret-He demonstrated a decline amounting to 3-8% (NS) within the time interval from admission 1931  $\pm$  241 aMol until 1845  $\pm$  199 aMol (NS) at day 4. After achievement of a minimum RET-He value at day 4 results steadily increased towards the reference range reaching 2129  $\pm$  136 aMol (p<0.001) at day 14.

In contrast, for RBC-He content no shift was detected within the time interval between admission (2020  $\pm$  135 aMol) and day 14 (1972  $\pm$  120) aMol (NS) (Figure 3B).

Within 24 h after admission results for RET-He/RBC-He ratio were obviously decreased (0.96  $\pm$  0.12). Until day 4 a further decrease to 0.93  $\pm$  0.07 (NS) was observed. From day 4 onwards a steady trend to increase was observed reaching a mean level amounting to 1.08±0.04 (p<0.00l) at day 14 (Figure 3C) .

### **DISCUSSION**

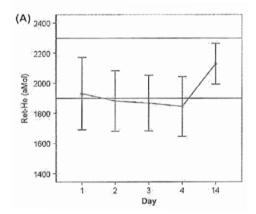
Anemia during acute systemic inflammation is a frequently encountered clinical problem which to a certain extent is caused by disturbance of iron utilization. Iron is sequestered in activated macrophages, enterocytes and hepatocytes. Hb synthesis is impaired due to limited availability of iron to maturing reticulocytes.

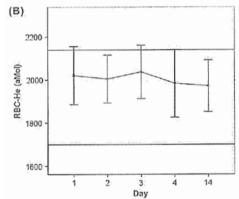
sults are established in 25 patients with CAP at hospital admission (day 1) and after antibiotic treatment. Statistically significant changes from Longitudinal evaluation of hemocytometric parameters, including mean value, standard deviation (SD), and minimum-maximum values. Reday 1 until day 4 and from day 4 until day 14 are shown; p-values < 0.05 are statistically significant; NS = not statistically significant. Table II.

Parameter	$\begin{array}{c} \text{Day 1} \\ \text{Mean $\pm$ SD$} \\ \text{(min-max)} \end{array}$	Day 4 Mean ± SD (min-max)	Day 14 Mean ± SD (min-max)	Statistical significance <i>p</i> -value Day 1-4	Statistical significance <i>p</i> -value Day 4-14	Reference range
RBC (10 <sup>12</sup> /L)	$4.33 \pm 0.45$ (2.91-5.14)	$3.98 \pm 0.50$ (2.47-4.84)	4.27±0.37 (3.80-5.25)	<0.001	<0.001	Male: 4.50-5.50 Female: 4.00-5.00
Hb (mMol/L)	$8.1\pm1.0$ (5.5-10.0)	7.4±0.9 (5.0-8.9)	$8.0\pm0.8$ (6.9-9.8)	<0.001	<0.001	Male: 8.5-11.0 Female: 7.5-10.0
MCV (fL)	90.0±5.3 (82.3-103.9)	$90.5\pm4.9$ (82.2-102.9)	$91.5\pm4.6$ (85.1-103.5)	NS	NS	80-100
MCHC (mMol/L)	$20.9 \pm 0.8$ (19.7-22.6)	$20.7\pm0.7$ (19.5-22.4)	$20.5\pm0.5$ (19.7-21.4)	0.019	NS	19.0-22.5
RDW-SD (fL)	$46.7\pm4.3$ (39.9-53.4)	47.2±3.9 (42.0-54.0)	$47.5\pm3.9$ (41.5-56.5)	NS	NS	38-48
RDW-CV (%)	$14.2\pm0.8$ $(12.8-16.2)$	$14.2\pm0.8$ (11.7-16.3)	$14.2\pm0.9$ $(12.8-17.0)$	NS	SN	12-15
Reticulocytes (10 <sup>12</sup> /L)	$0.044\pm0.015$ $(0.015-0.080)$	$0.041 \pm 0.017$ (0.016-0.082)	$0.055 \pm 0.021$ (0.016-0.120)	NS	0.020	Male: 0.035- 0.090 Female: 0.030-0.085

The aim of the study was to elucidate a shortterm alteration in hemoglobinization by the simultaneous monitoring of biomarkers regarding severity of infection and hepcidin-25 concentrations in patients with CAP. Our study revealed that results for CRP, IL-6 and hepcidin-25 concentrations were obviously increased at hospital admission. Due to treatment with antibiotics CRP, IL-6 and hepcidin-25 results steadily declined until day 14, indicating reduction of inflammation activity and physiological stress. After hospital admission results for Ret-He and Ret-He/RBC-He ratio decreased within several days after onset of pneumonia. After reaching a minimum RET-He value at day 4 an increasing trend towards results within the reference range was observed. These findings reflect a transient impairment of hemoglobin synthesis in erythropoiesis which might partly be due to functional iron depletion during inflammation.

In the present study results for mean hepcidin-25 concentrations were situated near the upper level of the reference range. Results for hepcidin-25 declined steadily until day 14. In case of inflammation hepcidin-25 release is induced by cytokines, in particular IL-6.





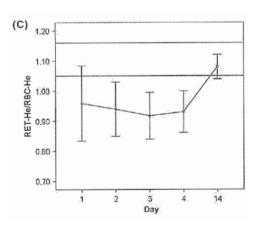


Figure 3. Results (Mean ± SD) for (A) reticulocyte hemoglobin content (RET-He, aMol), (B) red blood cell hemoglobin content (RBC-He, aMol), and (C) Ret-He/RBC ratio) which were established in 25 patients at hospital admission (day 1), at several days during antibiotic treatment (days 2, 3,4) and after completion of antibiotic treatment (day 14). The horizontal lines indicate the lower and upper level of the reference range for apparently healthy subjects.



An increase in hepcidin-25 concentration will induce an increase of reticuloendothelial macrophage iron. Redistribution of iron is initiated by cytokine-stimulated hepcidin-25 synthesis which induces loss of ferroportin from macrophage and enterocyte cell membranes and trapping of iron. Sequestration of iron restricts its availability for hemoglobin synthesis [3,5,18]. Recently Van Eijk et al. suggested that hepcidin-25 release is a modulator of anemia in septic patients with systemic inflammation. Hepcidin-25 induces a direct inhibitory effect on erythropoiesis [4].

At admission, results for CRP and IL-6 serum concentrations were obviously increased compared to the reference ranges. From the day of admission until day 14 CRP and IL-6 concentrations demonstrated a steady trend to decrease towards the reference range. Declining values are indicative for reduced inflammatory activity indicating a positive response to treatment with antibiotics.

Obviously decreased RBC and Hb concentrations were observed within the subgroup of patients with CURB-score  $\geq 3$ , which might be associated with the severity of inflammation [19]. Several investigators reported decreased Hb concentrations in up to 30% of patients with CAP [4,20-22]. The phenomenon of decreased Hb concentrations is associated with poor prognosis and increased mortality in patients admitted with CAP [23]. In another study a decrease in Hb concentration amounting to 0.3 mMol/L/day has been demonstrated during the first days of stay in the intensive care unit [24]. Steadily decreasing RBC and Hb concentrations during hospitalization are probably due to the combined effects of hemodilution, inhibition of erythropoietin production, blunted erythropoietic response, reduced lifespan of RBCs during inflammation and frequent phlebotomy for diagnostic testing [23-25].

With regard to the evaluation of the initial measurements within 24 h after hospital admission, mean Ret-He results were situated within the lower region of the reference range interval. Results for Ret-He demonstrated a short-term decline starting at admission. After day 4 a steady trend towards values within the reference range was observed until day 14 [19]. RET-He has been demonstrated to be a sensitive indicator for monitoring short-term deteriorations in functional iron supply for erythropoiesis [9,26]. Results for absolute reticulocyte counts decreased from admission towards day 4 and increased significantly until day 14. The observed changes of absolute reticulocyte count and Ret-He reflect short-term alterations of quantity and quality of erythropoiesis [19].

Mean results for red blood cell distribution width (RDW) were situated near the upper level of the reference range and demonstrated a slight but not statistically significant increase from admission until day 14.

Results for RDW were more pronounced in the patients with the highest category of CURB-scores. Increased levels of RDW may indicate an inflammatory state, which is associated with complications during hospitalization and increased mortality rates regardless of hemoglobin concentrations [27-29]. Increased RDW values occur as a result of ineffective RBC production due to cytokines blocking the activity of erythropoietin [27]. However, in our study we were not able to confirm the hypothesis probably because of the limited amount of patients.

In the case of subjects with an anemia of inflammation, results of Ret-He and Ret-He/RBC-He ratio combined with conventional parameters like mean cell volume (MCV), Hb, mean cell hemoglobin concentration (MCHC) and RDW should be considered in

order to achieve an appropriate clinical interpretation with regard to deviations in Hb synthesis [9,19,26].

Our findings contribute to understanding the limiting factors of Hb-synthesis in patients with CAP and may have important clinical implications. Awareness that Hb concentrations may decline even without active bleeding or hemodilution may eliminate the need for repeated diagnostic testing in patients with CAP.

## **CONCLUSION**

A temporary increase of cytokine-stimulated serum hepcidin-25 concentration is demonstrated in combination with a temporary decrease of Hb and Ret-He immediately after onset of pneumonia. Our results support the hypothesis that hepcidin-25 induces transient impairment of reticulocyte hemoglobin content (Ret-He).



### REFERENCES

- Brittenham GM, Weiss G, Brissot P, Lainé F, Guillygomarc'h A, Guyader D, Moirand R, Deugnier Y. Clinical consequences of new insights in the pathophysiology of disorders of iron and heme metabolism. Am Soc Hematol Educ Program. 2000;39-50.
- 2. Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med 2005;352:1011-23.
- 3. Ganz T. Hepcidin and iron regulation, ten years later. Blood 2011;117:4425-33.
- Van Eijk LT, Kroot JJC, Tromp M, Van der Hoeven JG, Swinkels DW, Pickkers P. Inflammation-induced hepcidin-25 is associated with the development of anemia in septic patients: an observational study. Crit Care 2011;15:R9.
- Fleming RE. Iron and inflammation: a cross-talk between pathways regulating hepcidin. J Mol Med 2008;86:491-4.
- Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol 2009;122:78-86.
- 7. Weiss G. Iron and anemia of chronic disease. Kidney Int 1999;55:s12-s17.
- 8. Thomas C, Thomas L. Anemia of chronic disease: pathophysiology and laboratory diagnosis. Lab Hematol 2005; 11:14-23.
- Bartels PCM, Schoorl M, Schoorl M. Haemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-14.
- Brugnara C, Schiller B, Moran J. Reticulocyte hemoglobin equivalent (Ret-He) and assessment of iron-deficient states. Clin Lab Haematol 2006;28:303-8.
- Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, Lewis SA, Macfarlane JT. Defining community acquired pneumonia severity on presentation to hospital: an international

- derivation and validation study. Thorax 2003;58:377-82.
- Schouten JA, Prins JM, Bonten M, Degener E, Janknegt R, Hollander JM, Jonkers R, Wijnands W, Verheij T, Sachs A, Kullberg BJ. Optimizing the antibiotics policy in the Netherlands. VIII. Revised SWAB guidelines for antimicrobial therapy in adults with community acquired pneumonia. Ned Tijdschr Geneesk 2005;49:2495-500.
- 13. Halm EA, Fine MJ, Marrie TJ, Coley CM, Kapoor WN, Obrosky DS, Singer DE. Time to clinical stability in patients hospitalized with community-acquired pneumonia: implications for practice guidelines. JAMA 1998;279:1452-7.
- Snijders D, Daniels JMA, De Graaff C, van der Werf TS, Boersma WG. Efficacy of corticosteroids in communityacquired pneumonia. Am J Respir Crit Care Med 2010; 181:975-82.
- Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H. Advances in quantitative hepcidin measurements by time-offlight mass spectrometry. PLoS ONE 2008;3:e2706.
- Kroot JJ, Hendriks JC, Laarakkers CM, Klaver SM, Kemna EH, Tjalsma H, Swinkels DW. (Pre)analytical imprecision, between-subjects variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem 2009;389:124-9.
- Franck S, Linssen J, Messinger M, Thomas L. Potential utility of Ret-Y in the diagnosis of iron restricted erythropoiesis. Clin Chem 2004;50: 1240-2.
- 18. Goodnough LT, Nemeth E, Ganz T. Detection, evaluation and management of iron-restricted erythropoiesis. Blood 2010;116:4754-61.

- Schoorl M, Snijders D, Schoorl M, Boersma W, Bartels PCM. Temporary impairment of reticulocyte haemoglobin content in subjects with communityacquired pneumonia. Int Jnl Lab Hem 2012;34:390-5.
- Musher DM, Alexandrandraki I, Graviss EA, Yanbeiy N, Eid A, Inderias LA, Phan HM, Solomon E. Bacteremic and nonbacteremic pneumococcal pneumonia. A prospective study. Medicine (Baltimore) 2000;79:210-21.
- Moore MA, Merson MH, Charache P, Shepard RH. The characteristics and mortality of outpatient-acquired pneumonia. Johns Hopkins Med J 1977;140:9-14.
- 22. Doshi S, Rueda AM, Corrales-Medina VF, Musher DM. Anemia and community-acquired pneumococcal pneumonia. Infection 2011;39:379-83.
- 23. Reade MC, Weissfeld L, Angus DC, Kellum JA, Milbrandt EB. The prevalence of anaemia and its association with 90-day mortality in hospitalized community-acquired pneumonia. BMC Pulm Med 2010;10-5.
- 24. Nguyen BY, Bota DP, Mélot C, Vincent

- J-L. Time course of haemoglobin concentrations in nonbleeding intensive care unit patients. Crit Care med 2003;31: 406-10.
- Jurado RL. Iron, infection, and anemia of inflammation. Clin Infect Dis 1997;25:888-95.
- 26. Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003;49:1573-8.
- 27. Braun E, Domany E, Kenig Y, Mazor Y, Makhoul BF, Azzam ZS. Elevated red blood cell distribution width predicts poor outcome in young patients with community acquired pneumonia. Crit Care 2011; 15:R194.
- 28. Hunziker S, Celi LA, Lee J, Howell MD. Red cell distribution width improves the simplified acute physiology for risk prediction in unselected critically ill patients. Crit Care 2012;16:R89.
- Lippi G, Filippozzi L, Montagnana M, Salvagno GL, Franchini M, Guidi GC, Targher G. Clinical usefulness of measuring red blood cell distribution width on admission in patients with acute coronary syndromes. Clin Chem Lab Med 2009;47:353-7.





A multicenter evaluation

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### **ABSTRACT**

For many years, application of RBC indices has been recommended for discriminating between subjects with iron deficiency from those with thalassemia. However, application of the algorithms resulted in only 30% to 40% of subjects being appropriately classified. The aim of the study was to establish the efficacy of algorithms for anemia screening including new hematologic parameters such as percentage of hypochromic and microcytic RBCs and hemoglobin content of reticulocytes. Subjects with iron deficiency anemia (IDA) (n = 142) and subjects with  $\beta$ -thalassemia (n = 34) were enrolled in a European multicenter study. Apparently healthy subjects were used as a reference group (n = 309).

Hemocytometric investigations were performed on a Sysmex XE5000 hematology analyzer. The algorithms for IDA discrimination yielded results for area under the curve, sensitivity, specificity, and positive and negative predictive values of 0.88, 79%, 97%, 74%, and 98%, respectively. The algorithms for  $\beta$ -thalassemia discrimination revealed similar results (0.86, 74%, 98%, 75%, and 99%, respectively). We conclude that the advanced algorithms, derived from extended RBC parameters provided by the Sysmex XE5000 analyzer, are useful as laboratory anemia screening devices.

### INTRODUCTION

The application of RBC indices has been recommended for discriminating between subjects with iron deficiency and subjects with thalassemia. However, application of the England and Fraser formula (mean corpuscular volume  $[MCV] - 5 \times hemoglobin$  [Hb] - RBC-3.4) and Mentzer formula (MCV/RBC) resulted in only 30% to 40% of subjects being appropriately classified.

Additional application of protoporphyrin content in RBCs was recommended for classifying microcytic RBC disorders. Multivariant discriminant analysis of algorithms including MCV, mean corpuscular hemoglobin (MCH), RBC, and red cell distribution width (RDW) was useful for the differential diagnosis of  $\alpha$ - or  $\beta$ -thalassemia and irondeficiency, but resulted in an inconclusive diagnosis in several cases. All 2

Measurement of the percentage of hypochromic and microcytic RBCs (%HypoHe and %MicroR) has demonstrated usefulness for detecting small changes in the amount of RBCs with inadequate hemoglobinization. <sup>13-15</sup>

Iron deficient erythropoiesis was characterized by the production of RBCs with decreased hemoglobin content resulting in an increased result for %HypoHe. As severity of anemia progresses, results for %MicroR will increase. However, subjects with  $\beta$ -thalassemia showed erythrocytosis and a high score for microcytosis. RBCs in case of subjects with  $\beta$ -thalassemia have a decreased volume because of impaired hemoglobin synthesis.

We decided to study the efficacy of new hematologic parameters for RBCs, like %HypoHe and %MicroR, and parameters for hemoglobinization of reticulocytes (Ret-He and  $\delta$ -He) to validate the application of discriminating algorithms for screening subjects for iron deficiency anemia (IDA) and  $\beta$ -thalassemia.

The study objectives included (1) establishing the sensitivity and specificity of new algorithms in a cohort of subjects with IDA, a group of subjects confirmed to have  $\beta$ -thalassemia, and a control group of healthy subjects, and (2) comparing the algorithms with current existing formulas for discrimination.

### MATERIALS AND METHODS

### Study Design

Our European multicenter study included 3 subject groups, namely, subjects with IDA, subjects with  $\beta$ -thalassemia, and apparently healthy hospital employees.

### Hemocytometry

Blood samples were collected in Vacutainer® tubes with  $K_2$ EDTA as anticoagulant (Becton Dickinson, Plymouth, England) and analyzed within 4 hours after collection. For hemocytometric investigations, we used the Sysmex XE5000 automated hematology analyzer (Sysmex, Kobe, Japan). Samples were selected from the daily workload and analyzed with full hemocytometric parameter profile (CBC + differential count + nucleated RBCs + reticulocytes) to ensure that results of all parameters were available. Parameters of particular interest were hemoglobin, RBC, MCV, RDW–standard deviation (SD), RDW–coefficient of variation (CV), reticulocytes Ret–hemoglobin equivalent (He), and  $\delta$ -He. In addition,



Preconditions and algorithms for screening for microcytic anemia in patients with IDA and β-thalassemia. Table 1.

No.	No. Precondition 1	Precondition 2	Algorithm	Condition
1	%MicroR≥3	MCV <85 and $\geq$ 75 and %MicroR $\geq$ 5	%MicroR/%HypoHe <4 and [(MCV² × RDW-CV)/ (Hb*100)] ≥75 and Ret <0.08	IDA
7	%MicroR≥3	MCV <75 and ≥65	%MicroR/%HypoHe <3.4 and [(MCV² × RDW-CV)/(Hb*100)] ≥77 and Ret <0.08	
8	%MicroR≥3	MCV <65	%MicroR – %HypoHe – RDW-CV < -5.2	IDA
4	%MicroR≥3	MCV <85 and ≥75 and RDW-SD <44.7 and RBC ≥3.50	[(MCV <sup>2</sup> × RDW-CV)/(Hb*100)] <75 and [(MCV-RBC −3.4 − (5*Hb)] <4 and Ret < 0.08 and δ-He ≥0	Thal
5	%MicroR≥3	MCV <75 and $\geq$ 65 and RBC $\geq$ 3.50	[(MCV <sup>2</sup> × RDW-CV)/(Hb*100)] $<$ 77 and %MicroR/%HypoHe $\geq$ 2.0	Thal
9	%MicroR≥3	MCV <65	(%MicroR – %HypoHe – RDW-CV) ≥ –5.2	Thal

CV, coefficient of variance; Hb, hemoglobin; IDA, iron-deficiency anemia; MCV, mean corpuscular volume; %MicroR, percentage of microcytic RBCs; %HypoHe, percentage of hypochromic RBCs, RDW, red cell distribution width; Ret, reticulocytes; SD, standard deviation.

<sup>\*</sup> Parameters used in the algorithms are expressed in the following units: RBC (x10<sup>12</sup>/L), Hb (g/dL), MCV (fL), RDW-SD (fL), RDW-CV (%), Ret (x10<sup>12</sup>/L).

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%HypoHe and %MicroR were quantified, indicating the percentage of hypochromic RBCs with a hemoglobin content of less than 17 pg and the percentage of microcytic RBCs with a volume of less than 60 fL.

#### ZPP/Hb Ratio

Zinc protoporphyrin (ZPP/Hb ratio) was measured with front surface illumination fluorometry using a dedicated hematofluorometer (Aviv Biomedical, Lakewood, NJ).<sup>18</sup>

### Hemoglobin Electrophoresis

Hemoglobin electrophoresis was used to diagnose subjects with  $\beta$ -thalassemia. Increased HbA<sub>2</sub> content (>3.2%) was considered to confirm  $\beta$ -thalassemia. In case of an abnormal hemoglobin variant and an increased fetal hemoglobin fraction in the electropherogram (>1.5%), the sample was excluded from the study.

#### Statistical Evaluation

The statistical software package MedCalc, version V11.5.1 for Windows, was applied for statistical analysis of results (MedCalc, Mariakerke, Belgium).

Receiver operating characteristic (ROC) curve analysis, including calculation of the area under the curve (AUC), was used to evaluate the diagnostic performance of the algorithms. Cut-off values were established based on the optimal combination of sensitivity and specificity. Sensitivity (sens%), specificity (spec%), positive predictive value (PPV), and negative predictive value (NPV) were calculated as follows:

Sensitivity =  $[true positive/(true positive + false negative)] \times 100$ Specificity =  $[true negative/(true negative + false positive)] \times 100$ Positive predictive value =  $[true positive/(true positive + false positive)] \times 100$ Mathematical formulas and cut-offs were previously published elsewhere. 1-3

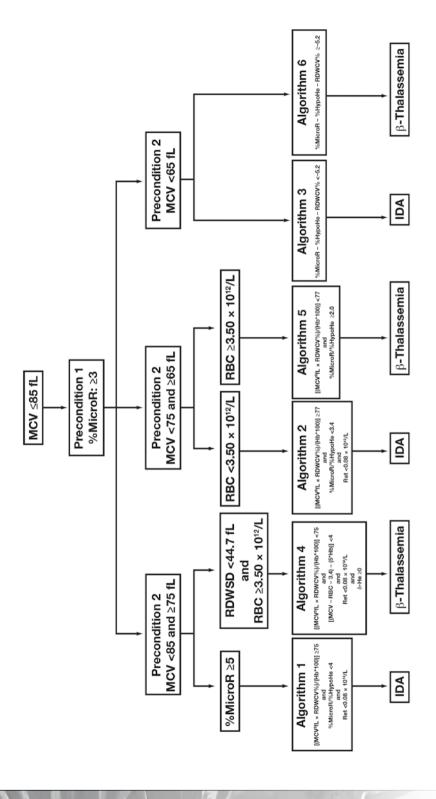
### **RESULTS**

### Subjects with IDA

One hundred forty-two subjects with IDA (6 male and 135 female) were enrolled in the study based on suspect results for MCV (n = 132 MCV  $\leq$ 80 fL; n = 9 MCV 80-86 fL) and ZPP/Hb ratio greater than or equal to 100 µmol per mol of heme. All selected subjects demonstrated results for %MicroR of 3 or higher. This limit was used as the *first precondition step* for discrimination using microcytic erythropoiesis.

The subjects were subsequently divided into 3 groups with normal to slightly decreased MCV (<85 and  $\ge75$  fL), moderately decreased MCV (<75 and  $\ge65$  fL), and severely decreased MCV (<65 fL). These subgroups were used in the *second precondition step*.

In addition, for the subgroup with MCV (<85 and ≥75 fL), ROC curve analysis (AUC) for %MicroR demonstrated that the optimal cut-off for %MicroR was 5 (Table 1), numbers 1-3). As shown in Table 1, new algorithms, including conventional and advanced hematologic parameters, were created for these subgroups. Results of ROC curve analysis (AUC) were applied to establish optimal cut-offs for concerning parameters.



Abbreviations: CV, coefficient of variation; Hb, hemoglobin; IDA, iron-deficiency anemia; MCV, mean corpuscular volume; MicroR, percentage of microcytic RBCs; %HypoHe, percentage of hypochromic RBCs; RDW, red cell distribution width; Ret, reticulocytes; SD, standard deviation. Flow diagram for screening on IDA and  $\beta$ -thalassemia. Figure 1.

Reference	Discrimination Function	Cut-off Level for IDA	Cut-off Level for β-Thalassemia
England and Fraser <sup>1</sup>	MCV – RBC-5 x Hb – 3.4	>0	<0
Green and King <sup>2</sup>	$[(MCV^2 \times RDW\text{-}CV)/(Hb^*100)]$	>65	<65
Mentzer <sup>3</sup>	MCV/RBC	>13	<13
Urrechaga <sup>20</sup>	%MicroR/%HypoHe	<2	>2
Urrechaga et al <sup>21</sup>	%MicroR – %HypoHe – RDW-CV	<-7.6	>-7.6

CV, coefficient of variation; Hb, hemoglobin; IDA, iron-deficiency anemia; MCV, mean corpuscular volume; %MicroR, percentage of microcytic RBCs; %HypoHe, percentage of hypochromic RBCs; RDW, red cell distribution width; SD, standard deviation.

### Subjects with $\beta$ -Thalassemia

Thirty-four subjects with  $\beta$ -thalassemia (18 male and 16 female) were enrolled in the study. We used an increased HbA2 content (>3.2%) to confirm  $\beta$ -thalassemia. Subjects with results for iron metabolism markers beyond the reference range were excluded. All subjects demonstrated %MicroR results of 3 or higher. This limit was used as the *first precondition step* for discrimination of microcytic erythropoiesis.

The subjects were subsequently divided into 3 subgroups as follows: (1) normal to slightly decreased MCV (<85 and  $\geq$ 75 fL), RDW-SD values less than 44.7 fL, and RBC values greater than or equal to  $3.50 \times 10^{12}$ /L; (2) moderately decreased MCV (<75 and  $\geq$ 65 fL) and RBC values greater than or equal to  $3.50 \times 10^{12}$ /L; and (3) severely decreased MCV (<65 fL). These subgroups were used in the *second precondition step* (Table 1, numbers 4-6).

As shown in Table 1 (numbers 4-6) new algorithms, including conventional and advanced hematologic parameters, were created for these subgroups. Results of ROC curve analysis (AUC) were applied to establish optimal cut-offs for concerning parameters.

To clarify the decision-making process for screening for IDA and  $\beta$ -thalassemia, we used the preselection criteria and algorithms depicted in (Figure 1). Our algorithms for IDA and  $\beta$ -thalassemia discrimination, including the cut-off levels, were compared with previously published discriminating algorithms listed in (Table 2).

#### **Apparently Healthy Subjects**

Three hundred nine apparently healthy hospital employees (133 male and 176 female, 16-63 years of age) without any clinical symptoms of disease were used as a reference group. Of these subjects, 3 scored a positive result for one of the IDA algorithms. These subjects demonstrated ferritin results of less than  $10 \mu g/L$ . All other subjects showed results within the reference ranges for hemocytometric parameters, including %MicroR less than 3, and

<sup>\*</sup> Parameters used in the algorithms are expressed in the following units: RBC (×10<sup>12</sup>/L), Hb (g/dL), MCV (fL), RDW-CV (%), Ret (×10<sup>12</sup>/L).

**Table 3.** Efficacy of the newly developed algorithms compared with the previously published conventional formulas.

		Iror	n Deficie	ncy				Thalasse	mia	
			]		ease nce 10%					ease ence 4%
Reference	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
England and Fraser <sup>1</sup>	0.696	97	45	10	90	0.918	51	96	36	98
Green and King <sup>2</sup>	0.691	97	56	15	90	0.945	64	97	44	99
Mentzer <sup>3</sup>	0.613	94	47	4	90	0.854	48	95	30	98
Urrechaga <sup>20</sup>	0.791	76	83	53	93	0.806	84	68	10	99
Urrechaga et al <sup>21</sup>	0.828	90	68	54	93	0.893	69	89	21	99
Algorithm 1-3 for discrimination of ID	0.878 OA	79	97	74	98					
Algorithm 4-6 for disc of β-thalassemia	criminat	tion				0.860	74	98	75	99

AUC, area under the curve; IDA, iron-deficiency anemia; NPV, negative predictive value; PPV, positive predictive value.

iron metabolism markers (data not shown).  $^{19}$  None of the subjects scored a positive result with the algorithms for  $\beta$ -thalassemia.

The new algorithms for IDA discrimination demonstrated results for AUC, sensitivity, specificity, and positive and negative predictive values of 0.88, 79%, 97%, 74%, and 98%, respectively. The algorithms for  $\beta$ -thalassemia discrimination showed comparable results of 0.86, 74%, 98%, 75%, and 99%, respectively (Table 3).

#### **DISCUSSION**

Application of RBC indices has been recommended for discriminating between subjects with IDA and subjects with thalassemia. In this study, application of these formulas resulted in only 30% to 40% of subjects with  $\beta$ -thalassemia in a proper classification.

More recently, Urrechaga et al<sup>17,20,21</sup> reported discriminating formulas with novel hematologic parameters such as %HypoHe and %MicroR combined with RDW-CV. Measurement of %HypoHe and %MicroR was demonstrated to be useful for detecting small changes in the amount of RBCs with inadequate hemoglobinization.

Because of the lifespan of circulating mature RBCs, %HypoHe yields information on iron status in the preceding 2 to 3 months; this parameter is shown to be a sensitive indicator for detecting functional iron deficiency.

In subjects with  $\beta$ -thalassemia, %HypoHe results are below the reference range because of ineffective erythropoiesis resulting from reduced production of intact hemoglobin.

%HypoHe results in subjects with  $\beta$ -thalassemia are not different from those observed in subjects with IDA.

Results for %MicroR are increased in  $\beta$ -thalassemia subjects compared with those with IDA.

A European multicenter evaluation was performed to establish advanced algorithms for anemia discrimination. The study revealed algorithms with advanced hematologic parameters, such as %HypoHe, %MicroR, Ret-He, and  $\delta$ -He, for discrimination of IDA and  $\beta$ -thalassemia. The algorithms demonstrated excellent diagnostic efficacy, compared with conventional discriminating formulas. In our study, application of the Urrechaga formulas resulted in about 50% of subjects with IDA and only 10% to 20% of subjects with  $\beta$ -thalassemia being appropriately classified. 17,20,21

The purpose of using formulas in anemia discrimination is to detect subjects who have a high probability of requiring appropriate follow-up to reduce unnecessary investigations and costs. In future, screening and reporting results of various parameters will be insufficient. Reduction of healthcare budgets and increasing numbers of parameters available in laboratory hematologic analyses make it necessary to provide support and interpretation for a correct clinical diagnosis. Algorithms for discrimination purposes must have *high sensitivity* scores to detect the maximum number of subjects of interest. On the other hand, adequate screening algorithms should be able to eliminate as many "other" subjects (*high specificity*) as possible to avoid further analysis (*false positives*). Using our recently developed algorithms for anemia screening, about 75% of subjects with IDA and subjects with  $\beta$ -thalassemia were classified properly. High sensitivity and specificity scores were demonstrated compared with conventional formulas.<sup>1-3</sup> The high sensitivity and specificity scores of the algorithms demonstrate that the discriminating algorithms are appropriate devices for microcytic anemia screening.

None of the algorithms had 100% sensitivity and 100% specificity in discriminating between subjects with iron deficiency from subjects with  $\beta$ -thalassemia. Therefore, after screening with the 6 algorithms, confirmatory testing should be performed for proper diagnosis. The efficacy of the algorithms should be confirmed in a subsequent study with prospectively selected subjects with microcytic anemia because of other causes such as  $\alpha$ -thalassemia or combinations of IDA with thalassemia.

We conclude that the advanced algorithms, derived from extended RBC parameters provided by the Sysmex XE5000 analyzer, are useful as laboratory devices for anemia screening.



### REFERENCES

- England JM, Fraser PM. Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet 1973;i:449-452.
- Green R, King R. Red cell discriminant function, β-thalassaemia minor versus iron deficiency. Blood Cells 1989;15:481-495.
- Mentzer WC. Differentiation of iron deficiency from thalassaemia trait. Lancet 1973;i:882.
- Bartels PCM, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocyte count and red cell zinc protoporphyrin for appropriate classification of microcytic anemia. Sysmex J Int 2003;13:78-82.
- Stockman JA, Weiner LS, Simon GE, et al. The measurement of free erythrocyte protoporphyrin (FEP) as a simple means of distinguishing iron deficiency from beta-thalassaemia trait in subjects with microcytosis. J Lab Clin Med 1975;85:113-119.
- Koenig HM, Lightsey AL, Schanberger JE.
   The micro-measurement of free erythrocyte protoporphyrin as a means of differentiating alpha thalassaemia trait from iron deficiency anemia. J Pediatr 1975;86:539-541.
- Han P, Fung KP, Teo CP, et al. Alpha-thalassaemia and beta-thalassaemia traits: biological difference based on red cell indices and zinc protoporphyrin. Clin Lab Haematol 1990;12:169-176.
- Haska J, Lasserre JJ, Schwarzbeck A, et al. Laboratory test of iron status: correlation or common sense? Clin Chem 1996;42:718-724.
- 9. Labbé RF, Vreman HJ, Stevenson DK. Zinc protoporphyrin: a metabolite with a mission. Clin Chem 1999;45:2060-2072.
- Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM. Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassaemia from iron deficiency anemia. Eur J Hematol 1998;60:245-251.
- 11. Akai Y, Kubota F, Nakamoto H, et al. A study

- of  $\beta$ -thalassaemia screening using an automated haematology analyzer. Sysmex J Int 1998:8:110-114.
- Eldibany MM, Totonchi KF, Joseph NJ, et al. Usefulness of certain red blood cell indices in diagnosing and differentiating thalassaemia trait from iron deficiency. Am J Clin Pathol 1999:111:676-682.
- Navarro JF, Macia ML. Hypochromic red cells as an indicator of iron deficiency. J Rheumatol 1997;24:804-805.
- Macdougall IC. Merits of hypochromic red cells as a marker of functional iron deficiency. Nephrol Dial Transplant 1998;13:847-849.
- Thomas C, Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.
- Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003;49:1573-1578.
- 17. Urrechaga E, Borque L, Escanero JF. Potential utility of the new Sysmex XE5000 red blood cell extended parameters in the study of disorders of iron metabolism. Clin Chem Lab Med 2009;47:1411-1416.
- 18. Blumberg WE, Eisinger J, Lamola AA, et al. Principles and applications of haematofluorometry. J Clin Lab Autom 1984;4:29-42.
- 19. Pekelharing JM, Haus O, de Jonge R, et al. Haematology reference intervals for established and novel parameters in healthy individuals. Sysmex Diagn Perspect 2011;1:1-11.
- Urrechaga E. Discriminant value of % microcytic/% hypochromic ratio in the differential diagnosis of microcytic anemia. Clin Chem Lab 2008;46:1752-1758.
- Urrechaga E, Borque L, Escanero F. The role of automated measurement of RBC subpopulations in differential diagnosis of microcytic anemia and β-thalassemia. Am J Clin Pathol 2011;135:374-379.



Mini REVIEW

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### **ABSTRACT**

Haemocytometric parameters like red blood cell [RBC] count, mean red blood cell volume [MCV], reticulocyte count, red blood cell distribution width [RDW-SD] and zinc protoporphyrin [ZPP] are frequently established for discrimination between iron-deficiency anaemia and thalassaemia in subjects with microcytic erythropoiesis. However, no single marker or combination of tests is optimal for discrimination between iron-deficiency anaemia and thalassaemia. This is the reason why algorithms have been introduced. However, application of algorithms, like Mentzer, England&Fraser or Green&King indexes only resulted in appropriate classification of 30-40% of subjects.

The efficacy of innovative haematological parameters for detection of alterations in RBCs have been studied. It refers to parameters for haemoglobinisation of RBCs and reticulocytes and the percentages microcytic and hypochromic RBCs, for discrimination between subjects with iron-deficiency anaemia [IDA] or thalassaemia as well as a combination of both.

A new discriminating tool including the above mentioned parameters was developed, based on two precondition steps and discriminating algorithms. The percentage microcytic RBCs is considered in the first precondition step. MCV, RDW-SD and RBC count are applied in the second precondition step. Subsequently, new algorithms, including conventional as well as innovative haematological parameters, were assessed for subgroups with microcytic erythropoiesis. The new algorithms for IDA discrimination yielded results for sensitivity of 79%, specificity of 97%, positive and negative predictive values of 74% and 98% respectively. The algorithms for  $\beta$ -thalassaemia discrimination revealed similar results (74%, 98%, 75% and 99% respectively).

We advocate that innovative algorithms, including parameters reflecting haemoglobinisation of RBCs and reticulocytes, are integrated in an easily accessible software program linked to the haematology equipment to improve the discrimination between IDA and thalassaemia.

### INTRODUCTION

Anaemia is a global public health problem affecting populations in both developing and developed countries. According to the World Health Organisation [WHO] anaemia affects 1.62 billion people, which corresponds to approximately 25% of the world population. It is assumed that 50% of the cases of anaemia are due to insufficient iron content in the diet, especially in women in child-bearing age with increased menstrual blood loss or during pregnancy, young children and vegetarians.<sup>1</sup>

Iron-deficient erythropoiesis and thalassaemia are both associated with mild to moderate microcytic anaemia, which frequently results in an incorrect diagnosis.

It is important to discriminate between IDA and thalassaemia, in order to avoid unnecessary iron therapy and to prevent the development of haemosiderosis, which may result in serious complications like cardiomyopathy, liver fibrosis or endocrine dysfunctions.<sup>23</sup> In this mini review the expedience of innovative haematological parameters concerning RBC haemoglobinisation as well as RBC production are considered regarding discrimination between iron deficiency and thalassaemia.

#### 1. IRON DEFICIENCY

Nutritional deficiency as a result of inadequate iron, folic acid and vitamin B12 intake, will contribute to the development of anaemia. Iron deficiency is considered to be the main cause for anaemia: it is responsible for more than 50% of all cases of anaemia. Iron deficiency results, amongst others, in impaired activity of several enzymes. As a consequence, iron depletion may result in serious health problems such as retarded growth, mental irritability, reduced resistance to infection and impaired intellectual development, particularly in case of infants in the growth phase. The causes for iron deficiency are decreased resorption of haeme iron, increased consumption of phytate and phenolic compounds which inhibit iron absorption, increased requirement, and blood loss. 4,5,6

According to WHO publications, the highest number of pre-school children, pregnant women and non-pregnant women suffering from IDA live in the Eastern Mediterranean countries, North-Africa and the Middle East.<sup>7</sup> Despite the start of iron fortification, the prevalence of iron deficiency is still rather high in the Middle East countries. The prevalence of IDA in the Middle Eastern countries is equal to the prevalence in developing countries (25-35%), which is much higher than in industrial countries (5-8%).<sup>8,9</sup>

#### 2. DISORDERS OF HAEMOGLOBIN SYNTHESIS

Haemoglobinopathy encompasses a group of genetic disorders which involve an abnormal structure of one of the globin chains of the haemoglobin molecule. In contrast, thalassaemias are disorders of haemoglobin synthesis that usually result in decreased production of normal globin proteins, often due to mutations in regulatory genes.<sup>2,3</sup>

It is estimated that approximately 7% of the world's population carries mutations of globin genes. In several regions of the world the prevalence of thalassaemia is even higher. With increasing migration of people, a genetic disease which was initially rare in northern Europe, Australia and North America is more common at the present time in these regions. The actual number of subjects with haemoglobinopathy and thalassaemia worldwide is unknown and is probably underestimated.<sup>3,7,10</sup>

#### α-thalassaemia

α-thalassaemias are the most prevalent disorders of haemoglobin synthesis.<sup>3</sup>

Subjects with  $\alpha$ -thalassaemia 2 heterozygosity (with three functional  $\alpha$ -globin genes:  $\alpha\alpha/-\alpha$ ) are characterised by normal to moderately decreased mean cell volume (MCV) values with normal or slightly decreased haemoglobin concentrations. Subjects with homozygous  $\alpha$ -thalassaemia 2 (- $\alpha$ /- $\alpha$ ) and heterozygous  $\alpha$ -thalassaemia 1 ( $\alpha\alpha$ /--) are diagnostically very similar and cannot be distinguished by blood count markers. Erythropoiesis with reduced MCV, frequently associated with mild anaemia, is frequently observed.

### β-thalassaemia

Mutations causing  $\beta$ -thalassaemia result in a lack of  $\beta$ -globin production, which ranges from minimal (mild  $\beta$ +-thalassaemia alleles) to a complete absence ( $\beta$ 0- alleles).

Almost all heterozygous conditions (except the so-called normal HbA2-thalassaemias, e.g.  $\delta\beta$ -thalassaemia), are characterised by an increased HbA2 content ( $\geq 3.2\%$ ) and a marked degree of microcytosis, which is frequently associated with mild anaemia. Subjects with homozygous  $\beta$ -thalassaemia suffer from severe microcytic anaemia.

## Haemoglobinopathies

Haemoglobinopathies encompasses a heterogeneous group of inherited disorders which affect the structure of the haemoglobin molecule, resulting in microcytic erythropoiesis. Four abnormal haemoglobins, in particular HbS, HbC, HbE and HbD<sup>Punjab</sup> are rather common in various parts of the world, such as Africa, the Mediterranean area and Southeast Asia. Carriers of haemoglobinopathies, if not associated with  $\alpha$ -thalassaemia, usually do not suffer from anaemia while results of MCV and mean red blood cell haemoglobin [MCH] are situated in the lower part of the reference range.

### Discrimination between iron deficiency and thalassaemia syndromes

The discrimination between IDA and thalassaemia has important clinical implications. A reliable diagnosis is important in order to reduce unnecessary laboratory testing and to avoid inappropriate treatment. A wide range of laboratory parameters is available to facilitate the discrimination between IDA and thalassaemia. However, no single marker or combination of tests is optimal for discrimination between iron deficiency and thalassaemia. In addition, iron deficiency often occurs in combination with other diseases which complicates the differential diagnosis. Diagnosing subjects with combined thalassaemia minor and iron deficiency is even more challenging.

In order to facilitate the discrimination procedure a number of discriminating algorithms have been described, which combine routine RBC parameters and indices obtained from routine complete blood count (Table I). <sup>13,14,15</sup> Application of these algorithms only resulted in appropriate classification of 30-40% of subjects with  $\beta$ -thalassaemia.

In the late 80s, the determination of ZPP in RBC was recommended to appropriately discriminate subjects with iron deficiency from subjects with thalassaemia. ZPP is a biomarker for the detection of long term iron-deficient erythropoiesis. <sup>16</sup> ZPP results are obviously

increased in patients with iron deficiency and, to a lesser degree, in subjects with  $\alpha$ - or  $\beta$ -thassaemia trait.  $^{17,18,19}$ 

In addition, introduction of discriminating algorithms including MCV, MCH, RBC and RDW has advanced the differential diagnosis of iron deficiency and  $\alpha$ - or  $\beta$ -thalassaemia, but in several cases these algorithms still resulted in an inappropriate diagnosis.<sup>20</sup>

**Table I.** Overview of discrimination formulas including cut-off levels for iron deficiency anaemia and for  $\beta$ -thalassaemia.

		Iron deficiency anaemia	Thalassaemia
England & Fraser <sup>14</sup> Green & King <sup>15</sup>	$MCV - RBC - 5xHb - 3.4$ $(MCV2 \times RDW-CV) / (Hbx100)$	>0 >65	<0 <65
Mentzer <sup>13</sup>	MCV/RBC	>13	<13

Over the past decade, discriminating procedures and algorithms have been improved *step-by-step*.

In 2003 the efficacy of a discriminating algorithm including the haematological parameters RBC, RDW-SD, ZPP and reticulocyte count has been established. MCV is recommended as an initial test. Subsequently, appropriate classification was achieved in 90% of the subjects with iron deficiency,  $\alpha$ -thalassaemia or  $\beta$ -thalassaemia by application of the algorithm 2x RDW (fL) - 5x RBC(x10<sup>12</sup>/L) - 250x reticulocytes (x10<sup>12</sup>/L) + 30x ZPP ( $\mu$ mol/mol Hb). 21

The introduction of new generations of haematology equipment enabled the use of innovative haemocytometric parameters for haemoglobinisaton in reticulocytes and RBCs. Haemoglobin content in reticulocytes reflects a *short term* indication of the availability of iron for erythropoiesis and the response to iron supplementation. Reticulocyte maturation occurs with progressive decrease in RBC volume and haemoglobin [Hb] content. If compared with interpretation of Hb content of mature RBCs, interpretation of Hb content of reticulocytes yields additional information concerning decreased functional availability of iron for haemoglobinisation of erythroid precursors. <sup>22,23</sup>

Thomas et al demonstrated a diagnostic model including the haemoglobin content of reticulocytes in combination with the soluble transferrin receptor / log ferritin ratio [sTfR-F index] for the purpose of monitoring the progression of iron deficiency, regardless of acute phase response.<sup>22</sup>

Hb content of RBCs and reticulocytes and parameters indicating reticulocyte maturity are established with advanced haematology analysers such as the Sysmex XE2100 haematology analyser (Sysmex Corporation, Kobe, Japan). The parameters are respectively denoted as RBC-He, Ret-He, Ret-He/RBC-He ratio, Delta-He (Ret-He minus RBC-He) and immature reticulocyte fraction [IRF].

 Table II.
 Preconditions and algorithms for discrimination microcytic anaemia on IDA and beta-thallassaemia.

	Precondition 1	Precondition 2	Algorithm	Condition
-	%MicroR ≥ 3	MCV <85 and ≥75 AND %MicroR≥5	$\label{eq:main_model} $$ \%MicroR / \%HypoHe < 4 \ AND \ [(MCV^2 \times RDW-CV) / (Hb^*100)] \ge 75 \ AND \ Reti# < 0.08$	IDA
7	%MicroR ≥ 3	<b>MCV &lt;75 and ≥65</b>	%MicroR / %HypoHe < 3.4 AND [(MCV² x RDW-CV) / (Hb*100)] ≥ 77 AND Reti# < 0.08	IDA
3	%MicroR ≥ 3	MCV <65	%MicroR - %HypoHe - RDW-CV < -5.2	IDA
4	%MicroR ≥ 3	MCV <85 and $\geq$ 75 AND RDW-SD < 44.7 AND RBC $\geq$ 3.50	$[(MCV^2 \times RDW-CV) / (Hb*100)] < 75 \text{ AND}$ [(MCV-RBC - 3.4 - (5*Hb)] < 4 AND Reti# < 0.08 AND Delta-He $\ge 0$	THAL
ro	%MicroR ≥ 3	MCV <75 and ≥65 AND RBC≥ 3.50	$[(MCV^2 \times RDW-CV) / (Hb^*100)] < 77 \text{ AND}$ %MicroR / %HypoHe $\geq 2.0$	THAL
9	%MicroR ≥ 3	MCV <65	$(\%MicroR - \%HypoHe - RDW-CV) \ge -5.2$	THAL

Parameters used in the algorithms are expressed in the following units: RBC (10 $^{12}$ /L), Hb (g/dL), MCV (fL), RDW-SD (fL), RDW-CV (%), Reti# (10 $^{12}$ /L).

In 2006 the additional value of these innovative haematological parameters in case of subjects with IDA and thalassaemia has been explored. Ret-He and RBC-He results were decreased in the subgroups with IDA and  $\alpha$ - or  $\beta$ -thalassaemia, if compared with the group of apparently healthy subjects. For the Ret-He/RBC-He ratio obviously decreased results were demonstrated in subjects with IDA (1.02  $\pm$  0.08, mean  $\pm$  SD) and to a lesser extent in subjects with  $\beta$ -thalassaemia (1.06  $\pm$  0.04) if compared with the group of healthy reference subjects (1.11  $\pm$  0.02) and subjects with  $\alpha$ -thalassaemia (1.11  $\pm$  0.07). A combination of results for Ret-He, Ret-He/RBC-He ratio in relation to MCV results yields an appropriate method for obtaining insight into the degree of haemoglobinisation and RBCs characteristics and proper clinical interpretation in the case of subjects with IDA or thalassaemia. Cut-off levels for Ret-He of <29.5 pg and for Ret-He/RBC-He ratio of <1.02 are advocated as indicators of iron-deficient erythropoesis.²4

More recently, innovative methods for establishment of microcytic and hypochromic RBCs have been introduced. Measurement of the percentage hypochromic and microcytic RBCs, has demonstrated to be useful for detection of rather small changes in the amount of RBCs with inadequate haemoglobinisation.<sup>22,25,26</sup>

In subjects with  $\beta$ -thalassaemia results for the percentage hypochromic RBCs are below the reference range, indicating decreased production of haemoglobin. Results for the percentage hypochromic RBCs are not different from those observed in subjects with IDA. Results for the percentage microcytic RBCs are increased in  $\beta$ -thalassaemia subjects if compared with results obtained in subjects with IDA.

Already in 1992 d'Onofrio et al reported on the ratio between the percentage of microcytes and the percentage of hypochromic cells, the so-called M-H ratio.<sup>27</sup> More recently, Urrechaga et al reported on improved discriminating formulas including the percentage hypochromic and microcytic RBCs (indicated as %Hypo-He and %MicroR respectively) combined with RDW-CV.<sup>28</sup>

The parameter %HypoHe reflects the percentage of hypochromic RBCs with a Hb content of less than 17 pg. %MicroR indicates the percentage of microcytic RBCs with a volume of less than 60 fL.<sup>28,29</sup>

In cases of  $\beta$ -thalassaemia, obviously increased results for %MicroR are observed (mean±SD, 37.8±11.4) if compared with the groups of healthy subjects (1.1±0.44) and subjects with IDA (19.1±9.7). In cases of IDA, results of %Hypo-He are increased (mean±SD, 15.7±11.5) compared with the groups of healthy subjects (0.3±0.16) and subjects with  $\beta$ -thalassaemia (11.9±7.2). In the same study, the efficacy of application of algorithms are explored such as %MicroR - %Hypo-He (M-H). The cut off value which provides the best results for application of Youden index for M-H was 11.5 ( $\beta$ -thalassaemia >11.5, IDA <11.5).<sup>28,29</sup>

An European multicentre study established the additional value of new discriminating algorithms that include the parameters Ret-He and Delta-He (RET-He minus RBC-He), %HypoHe and %MicroR for discrimination between IDA and  $\beta$ -thalassaemia. <sup>30</sup> The study established the sensitivity and specificity of the novel discriminating algorithms between these groups and a control group of healthy subjects and compared the algorithms with currently available algorithms for discrimination. To illustrate the decision making pro-

cess for discriminating between IDA and  $\beta$ -thalassaemia, preselection criteria and algorithms are depicted in Table II. Application of the new algorithms for discriminating IDA yielded in an area under the curve 0.88, a sensitivity of 79%, a specificity of 97% and positive and negative predictive values of 74% and 98%, respectively. For the detection of  $\beta$ -thalassaemia similar results were achieved (0.86, 74%, 98%, 75%, and 99%, respectively). Evaluation of the diagnostic efficacy of the algorithms demonstrated excellent performance, if compared with conventional discriminating algorithms described by England&Fraser, Green&King and Mentzer. Nevertheless, none of the algorithms provided 100% sensitivity and 100% specificity in discrimination between subjects with iron deficiency and subjects with  $\beta$ -thalassaemia. Therefore, after screening with the six algorithms, confirmatory testing should be performed for proper diagnosis.  $\beta$ 0

### Anaemia discrimination in subjects with combinations of IDA and thalassaemia

When facing the challenge of a discriminating algorithm for subjects with combinations of  $\beta$ -thalassaemia and IDA, application of Ret-He and Ret-He/RBC-He ratio as additional parameters is recommended. In this particular group of subjects, Ret-He and Ret-He/RBC-He ratio are obviously decreased (respectively 29.5 pg and <1.02).<sup>24</sup>

In subjects with combinations of  $\beta$ -thalassaemia and IDA, also a combination of %Hypo-He and M-H index seems to be promising. In this group of subjects markedly increased %Hypo-He (>20) occur in combination with a decreased M-H index (<11.5) (own observation, not published). Further investigation is required.

#### Critical remark

Several manufacturers of automated haematology equipment facilitate analysis of innovative parameters like haemoglobin content of red blood cells and reticulocytes. However, the comparability of these parameters is a matter of concern. Particularly in multicentersettings, calibration and adjustment of the measuring channels is an important issue. In case of multicenter evaluation studies involving haematology equipment from different suppliers, validation and mutual alignment is required.

#### CONCLUSIONS

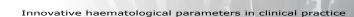
It is strongly advised to use discriminating algorithms as a tool for anaemia discrimination in order to reduce diagnostic testing for confirmation and to properly diagnose the underlying cause(s) in the patients.

It is advocated that innovative algorithms, including parameters reflecting haemoglobinisation of RBCs and reticulocytes, are integrated in an easily accessible software program linked to the haematology equipment, preferably provided by the manufacturer of the equipment to facilitate this interaction, to improve the discrimination between IDA and thalassaemia.

### REFERENCES

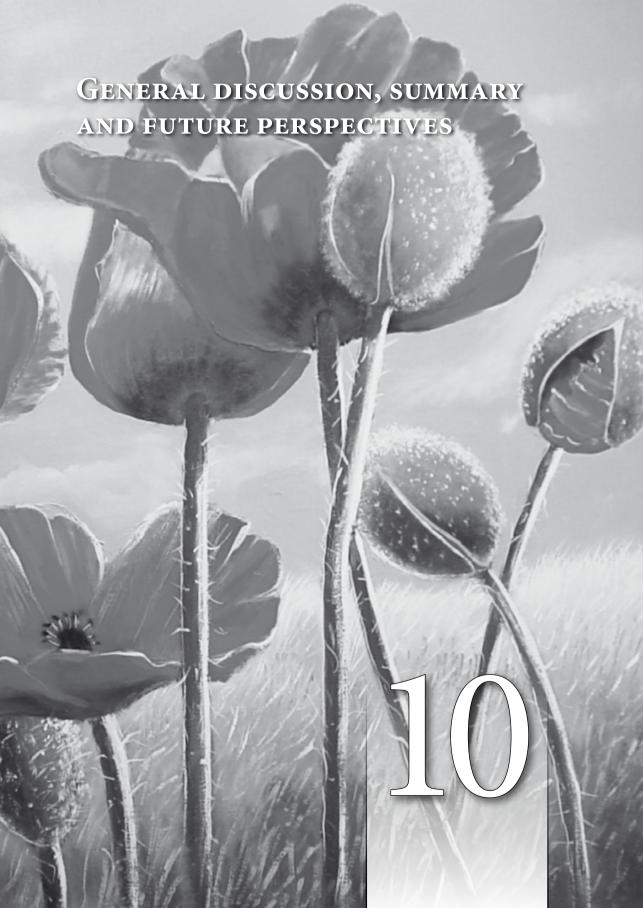
- De Benoise B, McLean E, Egli I, Cogswell M. Worldprevalence of anaemia 1993 – 2005; WHO Global Database on anaemia. ISBN 978 92 4 159665 7.
- Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th edition Blackwell Science: Oxford. 2001.
- 3. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. The Lancet 2012;379:373-383.
- Fairbanks VF, Beutler E. Iron deficiency.
   In: Beutler E., Lichtman MA, Coller B,
   Kipps TJ, Seligsohn U (eds) Williams
   Hematology, 6th edition. New York: Mc
   Graw-Hill, 2001:447-470.
- Hurrell R, Ranum P, de Pee S, et al. Revised recommendations for iron fortification of wheat flour and an evaluation of the expected impact of current national wheat flour fortification programs. Food Nutr Bull 2010;31:S7-21.
- Research for universal health coverage: World health report 2013. Poverty. Geneva, World Health organization, 2013. ISBN: 978 92 4 156459 5.
- Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ 2008;86:480-487.
- 8. Stolzfus RJ. Iron deficiency: global prevalence and consequences. Food Nutr Bull 2003;24:S99-103.
- 9. Mirmiran P, Golzarand M, Serra-Majem L, Azizi F. Iron, Iodine and Vitamin A in the Middle East; A systematic review of deficiency and food fortification. Iranian J Publ Health 2012;41:8-19.
- Weatherall DJ. The inherited diseases of haemoglobin are an emerging global health burden. Blood 2010;115:4331-4336.
- 11. Maude GH, Higgs DR, Beckford M, et al. Alpha thalassaemia and the haematology

- of normal Jamaican children. Clin Lab Haematol 1985;7:289-295.
- Pornpatkul M, Wasi P, Na-Nakorn S. Haematologic parameters in obligatory alpha thalassaemia traits. J Med Assoc Thai 1969;52:801-809.
- Mentzer WC. Differentiation of iron deficiency from thalassaemia trait. Lancet 1973:i:882.
- 14. England JM, Fraser PM. Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet 1973; i:449-452.
- Green R, King R. Red cell discriminant function, β-thalassaemia minor versus iron deficiency. Blood cells 1989;15:481-495.
- Blumberg WE, Eisinger J, Lamola AA, et al. Principles and applications of haematofluorometry. J Clin Lab Autom 1984; 4:29-42.
- 17. Han P, Fung KP, Teo CP, et al. Alpha-thalassaemia and beta-thalassaemia traits: biological difference based on red cell indices and zinc protoporphyrin. Clin Lab Haematol 1990;12:169-176.
- Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM. Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassaemia from iron deficiency anemia. Eur J Hematol 1998;60:245-251.
- Labbé RF, Vreman HJ, Stevenson DK. Zinc protoporphyrin: a metabolite with a mission. Clin Chem 1999;45:2060-2072.
- Eldibany MM, Totonchi KF, Joseph NJ, et al. Usefulness of certain red blood cell indices in diagnosing and differentiating thalassaemia trait from iron deficiency. Am J Clin Pathol 1999;111:676-682.
- Bartels P C M, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocytes count and red



- cell zinc protoporphyrin for appropriate classification of microcytic anaemia. Sysmex J Int 2003;13:78-82.
- 22. Thomas C, Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.
- Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003:49:1573-1578.
- Bartels PCM, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-114.
- Navarro JF, Macia ML. Hypochromic red cells as an indicator of iron deficiency. J Rheumatol 1997;24:804-805.
- Macdougall IC. Merits of hypochromic red cells as a marker of functional iron deficiency. Nephrol Dial Transplant 1998;13:847-849.

- d'Onofrio G, Zini G, Ricerca BM, et al.
   Automated meausument of red blood cell microcytosis and hypochromia in iron deficiency and β-thalassemia trait. Arch Pathol Lab Med 1992;116:84-89.
- 28. Urrechaga E. Discriminant value of % microcytic / % hypochromic ratio in the differential diagnosis of microcytic anemia. Clin Chem Lab 2008;46:1752-8.
- 29. Urrechaga E, Borque L, Escanero F. The role of automated measurement of RBC subpopulations in differential diagnosis of microcytic anemia and β-thalassaemia. Am J Clin Pathol 2011;135:374-379.
- Schoorl M, Schoorl M, Linssen J, et al. Efficacy of advanced discriminating algorithms for screening on iron-deficiency anemia and β-thalassemia trait. Am J Clin Pathol 2012;138:300-304.
- 31. Schoorl M. Combined Iron deficiency and Thalassemia Minor. Author reply on comment Burdick C. Am J Clin Pathol 2013;139;259-262.



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In this thesis, several aspects of red blood cell haemoglobinisation for the diagnosis of iron-restricted erythropoiesis in subjects with microcytic anaemia, haemodialysis treatment, community-acquired pneumonia and pregnancy were investigated. Subsequently, the discrimination for anaemia was improved by developing advanced discriminating algorithms for the diagnosis of iron-deficient erythropoiesis and thalassaemia. Clinical studies revealed the usefulness of innovative haemocytometric parameters for haemoglobinisation of red blood cells (RBC) and reticulocytes, RBC-He and Ret-He.

#### 1 IRON-DEFICIENCY ANAEMIA AND THALASSAEMIA

As discrimination between thalassaemia trait and iron-deficiency anaemia has important clinical implications a reliable diagnosis is important for reducing redundant testing and preventing inappropriate treatment. To facilitate discrimination between iron-deficiency anaemia and thalassaemia trait, a wide range of laboratory parameters is available. In addition, a number of algorithms has been described which combine routine red blood cell parameters (Table 1). However, application of these algorithms only resulted in appropriate classification of 30 – 40% of subjects with  $\beta$ -thalassaemia. The introduction of discriminating algorithms including MCV, MCH, RBC and RDW has advanced the differential diagnosis of iron deficiency and  $\alpha$ - or  $\beta$ -thalassemia, but in several cases these algorithms still resulted in an inappropriate diagnosis.

**Table 1.** Overview of discriminating algorithms

Cut-off levels for discrimination between iron-deficiency anaemia and  $\beta$ -thalassaemia.

		Iron-deficiency anaemia	Thalassaemia
England & Fraser <sup>1</sup>	MCV - RBC - 5xHb - 3.4	>0	<0
Green & King <sup>2</sup>	$(MCV^2 \times RDW-CV) / (Hb \times 100)$	>65	<65
Mentzer <sup>3</sup>	MCV/RBC	>13	<13

Additional assessment of zinc protoporphyrin (ZPP) content in RBC was recommended for the discrimination of microcytic RBC disorders. Section Bartels et al demonstrated an increase of the efficacy of a discriminating algorithm including the haematological parameters RDW-SD, RBC, ZPP/Hb ratio and reticulocyte count, in which MCV is recommended as an initial test. Subsequently, appropriate classification was achieved in 90% of subjects with iron deficiency,  $\alpha$ -thalassaemia or  $\beta$ -thalassaemia by application of the algorithm 2x RDW (fL) - 5x RBC(x10^{12}/L) - 250x reticulocytes (x10^{12}/L) + 30x ZPP (µmol/mol Hb), the so called MCA-index. Section Section 1.

In this algorithm ZPP was included as an additional parameter. ZPP is a biomarker for the detection of *long term* iron-deficient erythropoiesis.<sup>6,10</sup> Application of ZPP was already recommended many years ago in order to appropriately discriminate subjects with iron

Overview of results for biomarkers concerning haemoglobinisation of red blood cells and reticulocytes. Table 2.

Parameter	Reference group healthy subjects	Reference group of healthy subjects	Iron- deficiency anaemia	β-thalassaemia	α-thalassaemia	CKD	Uraemia
	Median	Median (p2.5 - p97.5)					
MCV (fL)	88.6	(80.2 - 97.1)	<b>→</b>	<b>;</b>	→ <u>'</u> Z	Z	Z
RDW-SD (fL)	42.3	(37.5 - 48.1)	<b>←</b> - Z	⇉	→ - Z	←	<b>←</b>
ZPP (μMol/Mol Heme)	45	(32 - 73)	<b>‡</b>	<b>V</b> - <b>V</b>	<b>←</b> - <b>Z</b>	←	<b>←</b>
Reticulocytes (10 <sup>12</sup> /L)	0.043	(0.028 – 0.073)	Z	<b>←</b>	z	←	<b>←</b>
IRF (10 <sup>12</sup> /L)	0.003	(0.001 – 0.013)	<b>←</b> - Z	<b>←</b>	z	←	<b>←</b>
Ret-He (aMol)	2060	(1897 - 2309)	<b>→</b>	<b>→</b>	<b>→</b>	<b>→</b>	<b>→</b>
RBC-He (aMol)	1876	(1698 - 2083)	<b>→</b> - →	<b>→</b>	<b>→</b>	→ 'Z	→ · Z
Ret-He / RBC-He ratio	1.10	(1.05 - 1.16)	<b>⇒</b>	→ - N	Z	<b>→</b>	<b>→</b>

Abbreviations: MCV=mean cell volume, RDW-SD=red cell distribution width, ZPP=zinc protoporphyrin, IRF=immature reticulocyte fraction, Ret-He=reticulocyte haemoglobin equivalent, RBC-He=red blood cell haemoglobin equivalent, CKD= chronic kidney disease, N=normal / within the reference range, 1 = slightly increased,  $\uparrow\uparrow=$  obviously increased,  $\downarrow=$  slightly decreased,  $\downarrow\downarrow=$  obviously decreased,

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deficiency from subjects with thalassaemia. ZPP results are obviously increased in patients with iron deficiency and, to a lesser degree, in subjects with  $\alpha$ - or  $\beta$ -thassaemia trait. 7.8,11,12,13

The introduction of new generations of haematology equipment enabled the use of innovative haemocytometric parameters for haemoglobinisaton in reticulocytes and RBC. Haemoglobin content in reticulocytes (Ret-He) is considered to be a sensitive indicator for monitoring *short term* deteriorations in iron and vitamin availability for erythropoiesis. The measurement is not affected by physiologic interferences, except in cases of thalassaemia<sup>14,15</sup> and macrocytosis. Reticulocyte maturation coincides with progressive decrease in RBC volume and Hb content. If compared with the interpretation of haemoglobin content of mature RBC, the interpretation of haemoglobin content of reticulocytes yields additional information concerning various states of decreased availability of iron for the haemoglobinisation of erythroid precursors. <sup>16,17</sup> If iron supplements are administered in subjects with depleted iron stores, Ret-He will already increase within a few days. <sup>18</sup>

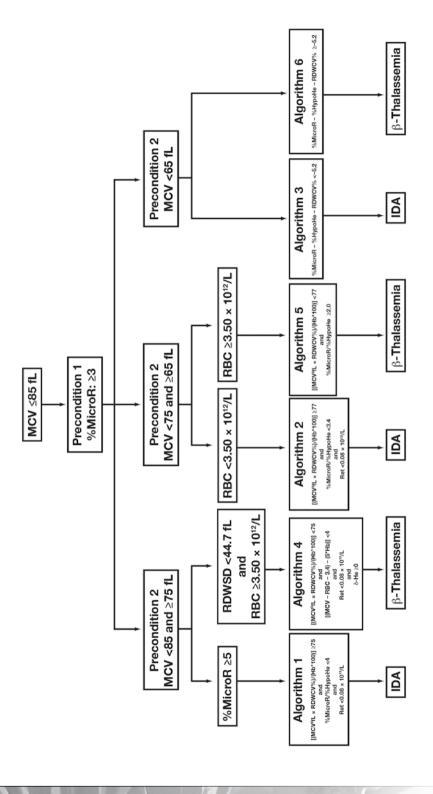
Haemoglobin content of RBC and reticulocytes and parameters indicating reticulocyte maturity are established with application of advanced haematology analysers such as the Sysmex XE2100 haematology analyser (Sysmex Corporation, Kobe, Japan). The parameters involved are respectively denoted as RBC-He, Ret-He, Ret-He/RBC-He ratio, Delta-He (Ret-He minus RBC-He) and IRF (immature reticulocyte fraction).

Chapter 2 described deviations with regard to the degree of haemoglobinisation in reticulocytes and mature red blood cells. Iron availability was studied in subjects with anaemia resulting from iron deficiency and  $\alpha$ - or  $\beta$ -thalassaemia by application of conventional and innovative haematological parameters. In addition, results of a group of healthy subjects were used to establish reference ranges for the haemoglobin content of RBC and reticulocytes (RBC-He and Ret-He respectively) and parameters indicating reticulocyte maturity (IRF) (Table 2).

Ret-He and RBC-He results were decreased in the subgroups with iron-deficiency anaemia and  $\alpha$ - or  $\beta$ -thalassaemia, if compared with the group of apparently healthy subjects. For the Ret-He/RBC-He ratio, obviously decreased results were demonstrated in subjects with iron-deficiency anaemia  $(1.02 \pm 0.08, mean \pm SD)$  and to a lesser extent in subjects with  $\beta$ -thalassaemia  $(1.06 \pm 0.04)$  if compared with the group of healthy reference subjects  $(1.11 \pm 0.02)$  and subjects with  $\alpha$ -thalassaemia  $(1.11 \pm 0.07)$ . Subjects with iron-deficiency anaemia are appropriately discriminated from the reference group based on routine parameters such as erythrocyte count, RDW-SD, ZPP, MCV, Ret-He and the ratio of Ret-He/RBC-He (Table 2).

We recommend cut-off values for Ret-He (<1850 aMol) and Ret-He/RBC-He ratio (<1.02), as indicators for iron-deficient erythropoiesis.

A combined interpretation of results for Ret-He, Ret-He/RBC-He ratio in relation to MCV results yields an appropriate method for obtaining insight into the degree of haemoglobinisation and RBC characteristics and proper clinical interpretation in case of subjects with iron-deficiency anaemia and thalassaemia. The Department of Clinical Chemistry, Haematology and Immunology of the Medical Center Alkmaar already uses this approach for many years in diagnostic tests for initial screening in subjects with microcytic erythropoiesis, pregnant women with normocytic anaemia and monitoring of iron therapy.



Abbreviations: CV = coefficient of variation; Hb = haemoglobin; IDA = ivon-deficiency anaemia; MCV = mean cell volume; MicroR = percentage of microcytic RBC; %HypoHe = percentage of hypochromic RBC; RDW = red blood cell distribution width; RBC = red blood cells; RET Flow diagram for discrimination on IDA and \(\beta\)-thalassemia. = reticulocytes; SD = standard deviation. Figure 1.

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More recently, haemocytometric methods became available for establishing the percentage of hypochromic and microcytic RBC, denoted as %HypoHe and %MicroR respectively. The %HypoHe indicates the percentage of hypochromic red blood cells with a haemoglobin content of less than 17 pg (= 1062 aMol). The %MicroR indicates the percentage of microcytic red blood cells with a volume of less than 60 fL. Measurement of %HypoHe and %MicroR has demonstrated to be useful for the detection of small changes in the amount of red blood cells with inadequate haemoglobinisation.  $^{16,19,20}$  In subjects with  $\beta$ -thalassaemia results for %HypoHe are below the reference range as a result of decreased production of haemoglobin. Results for %HypoHe are not different from those observed in subjects with iron-deficiency anaemia. Results for %MicroR are increased in  $\beta$ -thalassaemia subjects if compared with results obtained in subjects with iron-deficiency anaemia.

In an European multicentre study the additional value is established of new discriminating algorithms which include the parameters Ret-He and Delta-He (Ret-He minus RBC-He) and %HypoHe, %MicroR, for discrimination between iron-deficiency anaemia and  $\beta$ -thalassaemia (**Chapter 8**). The study established the sensitivity and specificity of the novel discriminating algorithms between these groups and a control group of healthy subjects and compared the algorithms with currently available algorithms for discrimination. To illustrate the process for discrimination between iron-deficiency anaemia and  $\beta$ -thalassaemia, preselection criteria and algorithms are depicted in Figure 1.

Application of the flow diagram for discriminating iron-deficiency anaemia yielded an area under the curve of 0.88, a sensitivity of 79%, a specificity of 97%, and positive and negative predictive values of 74% and 98%, respectively. For the detection of  $\beta$ -thalassemia similar results were achieved (0.86, 74%, 98%, 75%, and 99%, respectively). Evaluation of the diagnostic efficacy of the algorithms in the flow diagram demonstrated excellent performance compared with the conventional discriminating algorithms described by England&Fraser, Green&King and Mentzer. 1.2.3

We recommend the integration of discriminating algorithms, derived from a comprehensive collection of red blood cell parameters, in laboratory devices for differential diagnosis of iron-deficiency anaemia and thalassaemia. In this context it should be mentioned that the Sysmex company will soon include the parameter Delta-He as an official parameter in its testmenu.

### Combined iron-deficiency anaemia and thalassaemia

When facing the challenge of a discriminating algorithm for subjects with a combination of  $\beta$ -thalassaemia and iron-deficiency anaemia, we recommend the application of RetHe and Ret-He/RBC-He ratio as additional parameters. In this particular group of subjects, Ret-He and Ret-He/RBC-He ratio are obviously decreased (<1850 aMol and <1.02, respectively). The M-H ratio is the ratio between the percentage of microcytes and the percentage of hypochromic red blood cells. In patients with a combined  $\beta$ -thalassaemia and iron-deficiency anaemia a combination of the % Hypo-He and the M-H ratio is promising. In this group of subjects a markedly increased % Hypo-He (> 20) occurs, in combination with a decreased M-H ratio (<11.5) (own observation in a pilot study, data not published). However, further investigation is required.

## 2 ANAEMIA OF CHRONIC DISEASE (ANAEMIA OF INFLAMMATION)

In **Chapter 3, 6 and 7** deviations in erythropoiesis activity, functional iron deficiency and haemoglobinisation of red blood cells and reticulocytes in subjects with chronic kidney disease and in subjects with community-acquired pneumonia are presented.

## 2.1 Chronic kidney disease

Anaemia is rather common in subjects affected by chronic kidney disease, especially in end stage renal disease subjects with haemodialysis treatment. Blood loss related to haemodialysis treatment and poor iron absorption in the gastrointestinal tract frequently lead to the depletion of iron stores. In addition, the risk of iron deficiency is increased by blood loss due to blood sampling for laboratory analyses (approximately 500 mL in a year) and reduced lifespan of red blood cells, which were found to be adverse factors in subjects with renal disease and uraemia. The pathogenesis of anaemia is multifactorial, but the main factor is a low kidney production of erythropoietin.

In haemodialysis subjects correction of anaemia is facilitated by combined supplementation of recombinant human erythropoietin and intravenous administration of iron. After administration of erythropoietin, an increase in reticulocyte count, immature reticulocytes and reticulocyte mean cell volume are detected, together with a decrease in Ret-He independent of the iron status.<sup>25</sup> As a result of accelerated erythropoiesis, the need for iron increases. Supplementation of iron, however, has potential risks, such as a decrease of polymorphonuclear leucocyte function, infections, and organ damage due to iron overload. Appropriate anaemia management improves quality of life as well as cognitive- and cardiovascular functions and reduces mortality.<sup>26,27</sup>

Biochemical markers such as ferritin and transferrin are not ideal for assessing iron status as they are affected by biological variability and inflammation. Revised European Best Practice Guidelines (EBPG) for the management of anaemia in patients with chronic renal failure recommend haematological parameters, such as the haemoglobin content of reticulocytes (Ret-He) and the percentage of hypochromic red blood cells (%Hypo), to assess functional iron availability. According to these guidelines, Ret-He of <1812 aMol or %Hypo >10% are indications for iron supplementation.<sup>28</sup>

**Chapter 3** described a study of erythropoiesis activity, functional iron deficiency and haemoglobinisation of RBC and reticulocytes in subjects with chronic kidney disease. The results demonstrated that reticulocyte counts and results concerning immature reticulocyte fraction (IRF) were obviously increased in subjects with haemodialysis treatment and in subjects with uraemia when compared to a reference group of healthy subjects. If compared with the reference group of subjects, the results for RBC counts and MCHC were statistically decreased in subjects with haemodialysis and in subjects with uraemia, whereas increased results were determined with regard to RDW-SD values. At a definite MCV value, both groups of subjects demonstrated decreased levels of haemoglobin content of reticulocytes and red blood cells (Ret-He and RBC-He respectively). For the ratio of Ret-He and RBC-He, obviously decreased results were demonstrated in subjects treated with haemodialysis (1.05  $\pm$  0.05, mean  $\pm$  SD) and uraemia (1.02  $\pm$  0.10) if compared with the reference subjects (1.11  $\pm$  0.02) (Table 2).

The combined interpretation of MCV values within the reference range and decreased values for Ret-He and Ret-He/RBC-He ratios respectively, revealed a decreased degree of haemoglobinisation in subjects with haemodialysis or uraemia. Decreased haemoglobinisation is supported by the presence of increased results for serum transferrin receptor (sTfR) and ZPP concentrations. In combination, these results imply a reduced availability of iron for haemoglobin synthesis.

We conclude that monitoring of functional iron availability for erythropoiesis requires biomarkers reflecting the iron status as well as longitudinal follow-up of Ret-He. Moreover, such a combined evaluation procedure will facilitate short-term corrections of intravenous iron supplementation at an individual level.

# 2.2 Community-acquired pneumonia

Anaemia of inflammation is the most prevalent anaemia in hospitalized subjects. It occurs in patients with acute or chronic inflammatory conditions, including infections, cancer, rheumatoid arthritis, and chronic kidney disease. Anaemia is not only frequent among critically ill subjects, anaemia is also associated with increased transfusion rates and worse clinical outcomes (increased length of stay, increased mortality). <sup>29,30,31,32</sup> Anaemia of inflammation is associated with immune cell activation and inflammatory cytokine response which blunts erythropoietin production, impairs erythropoiesis, decreases red cell life span and deregulates iron homeostasis. <sup>33</sup>

The discovery of hepcidin has shed a new light on iron homeostasis. Hepcidin is a key regulatory protein that controls intestinal iron absorption and distribution of iron from body stores, including reticuloendothelial macrophages.<sup>34</sup> Hepcidin has increased understanding of complex clinical situations, such as those observed in critically ill patients, where several regulatory circuits interfere with iron metabolism.<sup>35,36</sup> Synthesis of hepcidin is stimulated by Interleukin-6. By degrading ferroportin, hepcidin is able to decrease the availability of iron from macrophages. When functional iron deficiency and inflammatory disease coexist, increased hepcidin synthesis will restrict the absorption of oral iron. Supplementation of intravenous iron preparations will bypass the blockade.<sup>37,38</sup>

In **Chapter 6 and 7** results of investigating functional iron deficiency in subjects with community-acquired pneumonia are presented. Deviations in reticulocyte haemoglobin content (Ret-He) occurred simultaneously with increases of inflammation markers.

Within 24 hours after admission, Ret-He results were situated within the lower quartile region of the reference range interval. Until day 4 of hospital admission, a steady trend towards a decline of 3-8% was established. During antibiotic treatment, reticulocyte count increased from day 4 to day 14. In a similar pattern, the decreased haemoglobin concentration and Ret-He recovered to values within the reference range.

Acute inflammation in subjects with community-acquired pneumonia results in impairment of Ret-He at an early stage. After onset of pneumonia decreased results of Ret-He and Ret-He/RBC-He ratios were demonstrated, reflecting an acute haemoglobinisation arrest, which could be explained by functional iron depletion (**Chapter 6**).

At hospital admission serum hepcidin concentrations were in the upper level of the reference range. The levels of C-reactive protein (CRP) and Interleukin-6 were also increased

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compared to the reference ranges. From admission to day 14, hepcidin, CRP and Interleukin-6 decreased towards the reference ranges.

The temporary increase of hepcidin, along with the increase of Interleukin-6 and CRP, supports the hypothesis that hepcidin induces inflammation-dependent transient impairment of reticulocyte haemoglobin content in subjects with community-acquired pneumonia (**Chapter 7**).

#### 3 PREGNANCY

In pregnant women, the prevalence of iron-deficiency anaemia ranges from 6 to 30%. The highest prevalences are observed in countries where routine iron supplementation is not usually given during pregnancy.<sup>39</sup> Anaemia during pregnancy is partially due to physiological haemodilution and insufficient availability of essential nutrients for haemoglobin synthesis and red blood cell production in the erythron, such as iron, folic acid and vitamin B12. As the degree of haemodilution demonstrates a wide inter-individual variation, haemoglobin concentrations show comparable variations. Hence, it is complicated to establish whether a decreased haemoglobin concentration is a physiological condition or a pathological phenomenon due to iron-deficient erythropoiesis.

During pregnancy, about 1000 mg additional iron is needed to ensure a sufficient supply to the expanding red blood cell mass of mother and fetus.<sup>40</sup> This implies a two to threefold increase of the iron requirements.<sup>39</sup> Maternal body stores and dietary intake may be insufficient for adequate erythropoiesis. Supplementary iron is needed for erythropoiesis to enhance the increased production of RBC and to fulfil additional iron demands of the foetus. The extra demand on maternal iron stores makes iron-deficiency anaemia a common complication during pregnancy.

In the last trimester of pregnancy, decreased Hb concentrations as a result of functional iron deficiency may be associated with various complications, such as maternal infections, low birth weight and premature delivery. Reported positive effects of iron supplementation include increased physical fitness and well-being in pregnant women, prevention of postpartum iron deficiency due to blood loss at delivery, and enhanced iron reserves in the new-born, which prevent iron deficiency in the first years of life. 41.42

Pregnancy is associated with a physiological increase in inflammatory biomarkers, particularly during the 1st and 3rd trimester. During inflammation, the serum ferritin concentration increases as a result of an acute phase response, which can lead to an overestimation of the iron stores if the results of the assay are wrongly interpreted. A3,44 It should be emphasized that functional and absolute iron-deficient erythropoiesis may co-exist, especially during late pregnancy when low-level inflammation with depleted iron stores is likely to occur. Hence, studies reveal serious limitations concerning clinical interpretation of biomarkers reflecting iron status, i.e. serum concentrations of ferritin, transferrin receptor and transferrin saturation.

Recommendations vary among the diagnostic guidelines applied in obstetric practice. The World Health Organization proposes haemoglobin values of 6.8 mMol/L for anaemia discrimination and as an indication for subsequent iron supplementation, whereas 6.3 mMol/L is recommended by the Dutch organization of midwifes ('Koninklijke Neder-

landse Organisatie voor Verloskundigen, KNOV). Evidently, anaemia diagnostics during pregnancy was still in need of improvement.

Chapter 4 described deviations in red blood cell haemoglobinisation during pregnancy. The aim of the study was to gain insight into the additional value of advanced RBC parameters, in particular immature reticulocyte count (IRF) and reticulocyte haemoglobin content (Ret-He), for establishing deviations in the haemoglobinisation to benefit appropriate discrimination levels and thus as an indication for iron supplementation. Results demonstrated that haemoglobin measurements alone are inappropriate for anaemia screening during pregnancy. In accordance with Ervasti and Wheeler, Ret-He is considered to be a useful tool for diagnostic screening and for follow-up monitoring of iron availability during pregnancy.<sup>43,46</sup>

We recommend application of Ret-He with a cut off level off <1850 aMol as a useful tool for screening on decreased iron availability during the third trimester of pregnancy. A decreased Ret-He result is considered to be indicative for insufficient red blood cell haemoglobinisation, especially within a haemoglobin range suspicious for anaemia in pregnancy (Hb  $\leq$ 6.8 mMol/L, MCV 80-100 fL).

Chapter 5 presented the effects of iron supplementation on RBC and reticulocytes haemoglobin content during pregnancy. The aim of this study was to evaluate the beneficial effects of iron supplementation on the haemoglobin content of reticulocytes (Ret-He) and red blood cells (RBC-He) in subjects with borderline haemoglobin concentrations in the third trimester of pregnancy. Discrimination criteria for iron-deficient erythropoiesis included Hb  $\leq$  6.8 mMol/L, Ret-He <1850 aMol and, zinc protoporphyrin (ZPP) >75  $\mu$ Mol/Mol heme.

Our study demonstrated that Ret-He results, after 4 weeks of iron supplementation, obviously increased towards the lower region of the reference interval, which is 1900-2300 aMol.<sup>21</sup> Results for Ret-He/RBC-He ratio demonstrated a similar trend when compared with Ret-He results. The observed shifts in Ret-He and Ret-He/RBC-He ratio reflect *short term* alterations concerning the *'quality'* of erythropoiesis.<sup>21,47</sup>

Our study yielded obviously increased results for haemoglobin concentration and absolute reticulocyte counts after iron supplementation, in particular in subjects with haemoglobin results in the controversial range of 6.3-6.8 mMol/L.

Previous studies demonstrated that nutrient supplementation did not reveal significant changes in RBC-He content and ZPP.<sup>43,48</sup> The lack of effect may be explained by the fact that RBC-He and ZPP reflect rather *long term* effects for shifts in haemoglobinisation, corresponding with the lifespan of circulating mature RBC (100 days).<sup>16</sup>

We advocate state-of-the-art modifications in the recently revised guideline 'Anaemia in the midwife practice', which recommends the MCV as a conclusive marker in subjects with haemoglobin concentration  $< 6.5 \, \text{mMol/L}.^{49,50}$  Our results have demonstrated that MCV alone is inappropriate for diagnosis of iron-deficient erythropoiesis in the third trimester of pregnancy. We therefore recommend the integration of Ret-He and Ret-He/RBC-He ratio parameters in the protocol for anaemia screening and monitoring. These parameters should be considered in particular in subjects with haemoglobin results in the controversial range of  $6.3-6.8 \, \text{mMol/L}.$ 



### 4 CLOSING REMARKS AND FUTURE PERSPECTIVES

Anno 2015 iron metabolism is still very relevant in the discrimination of anaemia. The theme of this thesis refers to the establishment of the additional value of innovative parameters reflecting haemoglobin content of RBC and reticulocytes in various groups of subjects. We strongly advise the discriminating algorithms as a tool for anaemia discrimination in order to reduce diagnostic testing for confirmation and to properly diagnose the underlying cause(s) in the patients.

Diagnostic guidelines are applied for the discrimination and follow-up monitoring in case of anaemia. The Dutch organization for general practitioners and the Dutch Society for Clinical Chemistry (NHG-Standaard Anemie and NVKC Reflex diagnostiek Anemie) recommend haemoglobin concentrations below the reference range for anaemia discrimination. MCV and ferritin are recommended as reflex-test for evaluation of the iron status. <sup>51,52</sup> However, the relationship between serum ferritin and iron stores is disturbed by acute and chronic infections and in case of inflammatory disorders, liver disease, and malignancy. In our opinion, a state-of-the-art update is required for the above mentioned guidelines. We recommend that manufacturers of haematology equipment develop software applications including new algorithms for anaemia discrimination and follow-up monitoring in case of therapy.

Several manufacturers of automated haematology equipment facilitate analysis of innovative parameters like haemoglobin content of red blood cells and reticulocytes. However, the comparability of these parameters is a matter of concern. Particularly in multicentersettings, calibration and adjustment of the measuring channels is an important issue. In case of multicenter evaluation studies involving haematology equipment from different suppliers, validation and mutual alignment is required.

Incidence of anaemia is known to increase with advancing age.<sup>53,54</sup> According to WHO criteria (male: Hb <8.1 mMol/L, female: Hb <7.5 mMol/L), approximately 16% of men and 10% of women aged 75-85 years would have anaemia.<sup>55,56</sup> Among people aged >85 years, the prevalence of anaemia increases even more. In elderly people a clear relationship is established between haemoglobin concentration and mortality. Data collected from the 'Leiden 85-plus study' have demonstrated that the mortality rate increases, in both men and women, with decreasing haemoglobin concentrations.<sup>54,57</sup> In this category of subjects, anaemia is frequently due to renal failure or anaemia of chronic disease (ACD). Where renal failure is frequently associated with anaemia due to decreased EPO production, ACD is characterized by hypoferremia which results in iron-restricted erythropoiesis.

In addition, deficiency of several nutrients necessary for optimal haemoglobinisation of RBC frequently occurs in the elderly. The deficiency could be the result of an insufficient intake in the diet, or the result of malabsorption. It should be noted that in the case of a vitamin B12 or folic acid deficiency the Ret-He will increase, as a result of an increased red blood cell volume (MCV). For the purpose of routinely monitoring of the nutrient status in elderly people, we recommend to incorporate the innovative haematological parameters RBC-He, Ret-He, Ret-He/RBC ratio or delta-He in a flow diagram. However, further investigation is required for establishment of appropriate cut-off levels for discrimination.

Increasing interest is focused on diagnosis and management of anaemia prior to surgery in order to minimize RBC transfusions.<sup>59,60</sup> The reported prevalence of preoperative anaemia in elective surgical patients is variable but may be as high as 40%.<sup>61,62</sup> Iron deficiency and chronic inflammation amongst others due to mild to moderate renal failure, with or without iron deficiency, are common causes for pre-operative anaemia, while deficiencies of iron, folic acid and/or vitamin B12 without anaemia also frequently occur, especially in an elderly population.<sup>59</sup>

Early detection and treatment of pre-operative anaemia is required because of its association with adverse post-operative outcomes. <sup>61,63</sup> Implementation of blood management strategies, including pre-operative administration of erythropoietein (EPO), not only reduces transfusion requirements but also improves post-operative outcomes. <sup>64,65</sup> Haemoglobin levels at admission have been shown to have impact on postoperative functional recovery in an elderly population with hip fractures <sup>66,67</sup> and on the quality of life after total hip arthroplasty. <sup>68</sup>

EPO suppletion stimulates RBC production with additional iron requirements. In order to meet the increased iron demands, EPO application is usually combined with oral iron supplementation. However, in many conditions iron uptake is diminished, e.g. in case of bowel diseases, Helicobacter pylori infection, inflammation or with medication such as antibiotics, proton pump inhibitors and Vitamin K antagonists, which may result in ineffective erythropoiesis. <sup>69,70</sup> Because of the limitations of oral iron supplementation in the group of patients with elective surgery supplementation with intravenous iron is preferred. <sup>71,72,73</sup>

We recommend application of RBC-He, Ret-He, Ret-He/RBC-He ratio or Delta-He in the pre-operative anaemia screening and monitoring, in order to improve the pre-operative EPO and iron therapy at an individual level.

In conclusion, this thesis provides a basis for improved diagnostics of anaemia using specific parameters or advanced algorithms, depending on the patient group involved. Incorporating such data in an easily accessible software program linked to the haematology equipment, preferably provided by the manufacturer of the equipment to facilitate this interaction, could improve the patient care.



### REFERENCES

- England JM, Fraser PM: Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet 1973; i:449-452.
- Green R, King R: Red cell discriminant function, β-thalassaemia minor versus iron deficiency. Blood cells 1989;15:481-495.
- Mentzer WC: Differentiation of iron deficiency from thalassaemia trait. Lancet 1973;i:882.
- Eldibany MM, Totonchi KF, Joseph NJ, Rhone D. Usefulness of certain red blood cell indices in diagnosing and differentiating thalassaemia trait from iron deficiency. Am J Clin Pathol 1999;111: 676-682.
- Bartels PCM, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocyte count and red cell zinc protoporphyrin for appropriate classification of microcytic anemia. Sysmex J Int 2003;13:78-82.
- 6. Haska J, Lasserre JJ, Schwarzbeck A, Reiter A, Hehlmann R. Laboratory test of iron status: correlation or common sense? Clin Chem 1996;42:718-724.
- 7. Labbé RF, Vreman HJ, Stevenson DK: Zinc protoporphyrin: a metabolite with a mission. Clin Chem 1999;45:2060-2072.
- Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM: Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassaemia from iron deficiency anemia. Eur J Hematol 1998;60:245-251.
- Akai Y, Kubota F, Nakamoto H, et al.: A study of β-thalassaemia screening using an automated haematology analyzer. Sysmex J Int 1998;8:110-114.
- 10. Blumberg WE, Eisinger J, Lamola AA, Zuckerman DM, Murray. Principles and applications of haematofluorometry. J Clin Lab Autom 1984;4:29-42.

- Stockman JA, Weiner LS, Simon GE, et al.
   The measurement of free erythrocyte protoporphyrin (FEP) as a simple means of distinguishing iron deficiency from beta thalassaemia trait in subjects with microcytosis. J Lab Clin Med 1975;85:113-119.
- Koenig HM, Lightsey AL, Schanberger JE.
   The micro-measurement of free erythrocy-te protoporphyrin as a means of differentiating alpha thalassaemia trait from iron deficiency anemia. J Pediatr 1975;86:539-541.
- Han P, Fung KP, Teo CP, Tam LP. Alphathalassaemia and beta-thalasaemia traits: biological difference based on red cell indices and zinc protoporphyrin. Clin Lab Haematol 1990;12:169-176.
- 14. Skarmoutsu C, Papassotiriou I, Traeger-Synodinos JT, Stamou H, Ladis V, Metaxotou-Mavrommati A, Stamoulakatou A, Kanavakis E. Erythroid bone marrow activity and red cell hemoglobinization in iron-sufficient β-thalassemia heterozygotes as reflected by soluble transferrin receptor and reticulocyte haemoglobin content. Correlation with genotypes and HbA2 levels. Haematologica 2003;88:631-636.
- 15. Mast AE, Blinder MA, Lu Q, Flax S, Dietzen DJ. Clinical utility of the reticulocyte hemoglobin content in the diagnosis of iron deficiency. Blood 2002;99:1489-1491.
- Thomas C, Thomas L. Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.
- 17. Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003;49:1573-1578.
- Buttarello M, Temporin V, Ceravolo R, Farina G, Bulian P. The new reticulocyte parameter (RET-Y) of the Sysmex XE 2100: its use in the diagnosis and monitoring of posttreatment sideropenic anemia. Am J Clin Pathol. 2004;121:489-95.

- Navarro JF, Macia ML. Hypochromic red cells as an indicator of iron deficiency. J Rheumatol 1997;24:804-805.
- Macdougall IC. Merits of hypochromic red cells as a marker of functional iron deficiency. Nephrol Dial Transplant 1998;13:847-849.
- Bartels PC, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-114.
- d'Onofrio G, Zini G, Ricerca BM, Mancini S, Mango G. Automated measurement of red blood cell microcytosis and hypochromia in iron deficiency and beta-thalassemia trait. Arch Pathol Lab Med 1992 Jan;116(1):84-9.
- 23. Marticorena LJ, Donnely S. Red blood cell survival in chronic renal failure Am J Kidney Dis 2004;44:715-719.
- 24. Jonckheere S, Dierick J, Vanhouteghem H, Devleeschouwer M, Stove V. Erythrocyte indices in the assessment of iron status in dialysis-dependent patients with end-stage renal disease on continuous erythropoietin receptor activator versus epoetin β therapy. Acta Haematol 2010;124:27-33.
- 25. Sowade O, Sowade B, Brilla K, Franke W, Stephan P, Gross J, Scigalla P, Warnke H. Kinetics of reticulocyte maturity fractions and indices and iron status during therapy with epoetin beta (recombinant human erythropoietin) in cardiac surgery patients. Am J Haematol 1997;55:89-96.
- Rao M, Pereira BJ. Optimal anemia management reduces cardiovascular morbidity, mortality, and costs in chronic kidney disease. Kidney Int 2005;68:1432-1438.
- 27. Buttarello M, Pajola R, Novello E, Rebeschini M, Cantaro S, Oliosi F, Naso A, Plebani M. Diagnosis of iron deficiency in patients undergoing hemodialysis. Am J Clin Pathol 2010;133:949-954.
- 28. Locatelli F, Aljama P, Bárány P, Canaud B,

- Carrera F, Eckhardt KU, Hörl WH, Macdougal IC, Macleod A, Wiecek A, Cameron S. Revised European best practice guidelines for management of anaemia in patients with chronic renal failure. Nephrol Dial Transplant 2004;19(suppl 2):ii1-ii47.
- Doshi SM, Rueda AM, Corrales-Medina VF, Musher DM. Anemia and communityacquired pneumococcal pneumonia. Infection 2011;39:379-383.
- Reade M, Weissfeld L, Angus D, Kellum J, Milbrandt E. The prevalence of anemia and its association with 90-day mortality in hospitalized community-acquired pneumonia. BMC Pulm Med 2010;10:15.
- Vincent JL, Baron JF, Reinhart K, Gattinoni L, Yhijs L, Webb A, Meier-Hellmann A, Nollet G, Peres-Bota D, ABC (Anemia and Blood transfusion in critical care) Investigators. Anemia and blood transfusion in critically ill patients. JAMA 2002;288:1499-1507.
- 32. Corwin HL, Gettinger A, Pearl RG, Fink MP, Levy MM, Abraham E, MacIntyre NR, Shabot MM, Duh MS, Shapiro MJ. The CRIT Study; Anemia and blood transfusion in the critically ill-current clinical practice in the critically ill: current clinical practice in the United States. Crit Care Med 2004;32:39-52.
- Weiss G. Goodnough LT. Anemia of chronic disease. N Engl J Med. 2005;352:1011-1023.
- 34. Ganz T. Hepcidin and iron regulation, 10 years later. Blood 2011;117:4425-4433.
- 35. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037-1044.
- Heming N, Montravers P, Lasocki S. Iron deficiency in critically ill patients highlighting the role of hepcidin. Crit Care 2011;15:210.



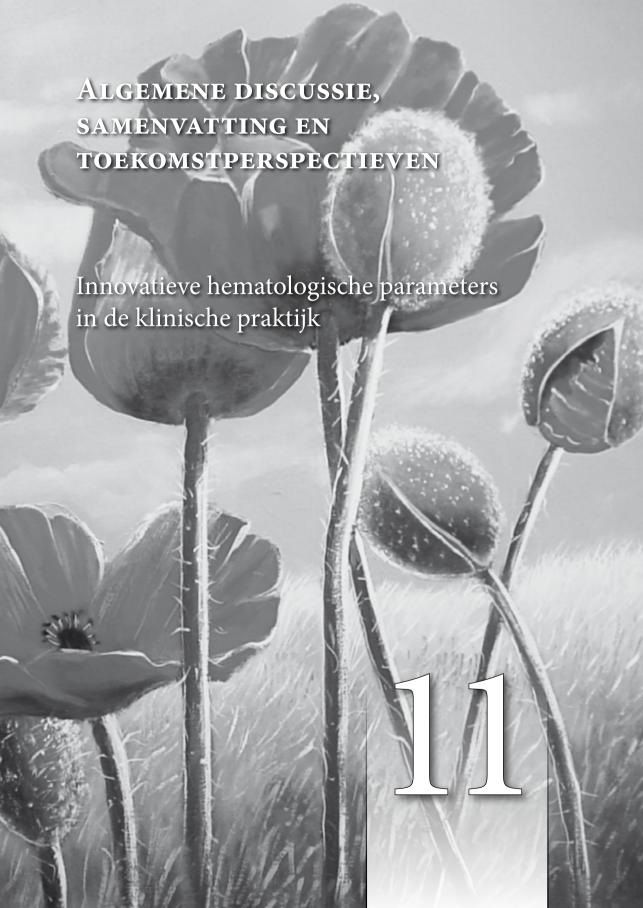
- 37. Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol 2009;122:78-86(DOI:10.1159/000243791).
- 38. Kroot JJC, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-1669.
- 39. Hercberg S, Preziosi P, Galan P. Iron deficiency in Europe. Public Health Nutrition 2001;4:537-545.
- Bentley DP. Iron metabolism and anaemia in pregnancy. In: Letsky EA, guest ed. Haematological disorders in pregnancy Clinics in Haematology vol.14. London. W.B. Saunders Comp 1985;613-628.
- Milman N, Bergholt T, Byg K, Eriksen L, Graudal N. Iron status and iron imbalance during pregnancy. A critical reappraisal of iron supplementation. Acta Obstet Gynecol Scand 1999;78:749-57.
- Wiegerinck MM, Mol BWJ. Insufficient evidence supporting iron supplementation in anaemia during pregnancy. Ned Tijdschr Geneeskd. 2012;156:A4293.
- 43. Wheeler S. Assessment and interpretation of micronutrient status during pregnancy. Proc Nutr Soc 2008;67:437-450.
- 44. Rogers JT, Bridges KR, Durnowicz GP, Glass J, Auron PE, Munro HN. Translational control during the acute phase response. Ferritin synthesis in response to interleukin-1. J Biol Chem 1990;265:14572-14578.
- 45. Milman N, Bergholt T, Byg K, Eriksen L, Hvas A. Reference intervals for haematological variables during normal pregnancy and postpartum in 434 healthy Danish women. Eur J Hematol 2007;79: 39-46.
- 46. Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Hematol 2007;79:539-45
- 47. Brugnara C, Schiller B, Moran J. Reticulocyte haemoglobin equivalent (Ret-He) and

- assessment of iron-deficient states. Clin Lab Haematol 2006;28:303-308.
- 48. Harthoorn EJ, Lindemans J, Langenhuijsen MMAC. Erythrocyte zinc protoporphyrin testing in pregnancy. Acta Obstet Gynecol Scand 2000;79:660-66.
- KNOV 2010 Anemie in de verloskundige praktijk. Praktijkkaart behorende bij de gelijknamige KNOV-standaard 2010. Utrecht: Koninklijke Nederlandse Organisatie voor Verloskundigen.
- Beentjes M, Jans S. Revised practice Guideline 'Anaemia in midwifery practice'. Ned Tijdschr Geneeskd. 2012;156: A3711.
- Verboeket-van de Venne WPHG, Oosterhuis WP, Keuren JFW, Ulenkate HJLM, Leers MPG. Richtlijn NVKC Reflexdiagnostiek bij anemie. NVKC 2012.
- NHG werkgroep Anemie, oktober 2014.
   NHG Richtlijn Anemie. www.nhg.org/ standaarden/volledig/nhg-standaard-anemie.
- Beghe C, Wilson A, Ershler W. Prevalence and outcomes of anaemia in geriatrics: a systemetic review of the literature. Am J Med 2004;116:3S-10S.
- 54. Izaks GJ, Westendorp RGJ. Grenswaarden voor anemie bij oude mensen. Ned Tijdschr Geneeskd 2006;150:1002-1006.
- 55. Guralnik JM, Eisensteadt RS, Ferruci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the United States; evidence for a high rate of unexplained anemia. Blood 2004;104:2263-2268.
- World Health Organisation (WHO). Nutritional anaemias: report of a WHO scientific group. Genève: WHO;1968.
- 57. Izaks GJ, Westendorp RGJ, Knook DL. The definition of anemia in older persons. JAMA 1999;281:1714-1717.
- 58. Ceylan C, Miskioglu M, Colak H, Kiliccioglu B, Ozdemir E. Evaluation of reticulocyte parameters in iron deficiency, vitamin B12 deficiency and β-thalassemia minor

- patients. Int Jnl Lab Hem 2007;29:327-334.
- 59. Saleh E, McClelland DBL, Hay A, Semple D, Walsh TS. Prevalence of anaemia before major joint arthroplasty and the potential impact of preoperative investigation and correction on perioperative blood transfusions. Br J Anaesth 2007;99:801-808.
- Goodnough LT, Shandor A, Spivak JL, Waters JH, Friedman AJ, Carson JL, Keating EM, Maddox T, Spence R. Detection, evaluation and management of anaemia in the elective surgical patient. Anesth Analg 2005;101:1858-1861.
- Carrascal Y, Maroto L, Rey J et al. Impact of preoperative anaemia on cardiac surgery in octogenarians. Interact Cardiovasc Thorac Surg 2010;10:249-255.
- Karski JM, Mathieu M, Cheng D, Carroll J, Scott GJ. Etiology of preoperative anaemia in patients undergoing scheduled cardiac surgery. Can J Anaesth 1999;46:979-982.
- Karkouti K, Wijeysundera DN, Beattie WS. Risk associated with preoperative anaemia in cardiac surgery. A multicenter cohort study. Circulation 2008;117:478-484.
- 64. Spahn DR. Anemia and patient blood management in hip and knee surgery; a systemetic review of the literature. Anesthesiology 2010;113:482-495.
- 65. Goodnough LT, Maniatis A, Earnshaw P, Benoni G, Beris P, Bisbe E, Fergusson DA, Gombotz H, Habler O, Monk TG, Ozier Y, Slappendel R, Szpaski M. Detection, evaluation, and management of preoperative anaemia in the elective orthopaedic surgical patient: NATA guidelines. Br J Anaesth 2011;106:13-22.
- 66. Gruson KI, Aharonoff GB, Egol KA, Zuckerman JD, Koval KJ. The relationship between admission hemoglobin level and outcome after hip fracture. J Orthop Trauma 2002;16:39-44.
- 67. Halm EA, Wang JJ, Boockvar K, Penrod J, Silberzweig SB, Magaziner J, Koval KJ, Siu AL. The effect of perioperative anaemia on

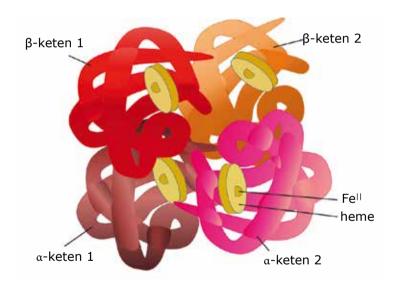
- clinical and functional outcomes in patients with hip fracture. J Orthop Trauma 2004:18:369-374.
- Conlon NP, Bale EP, Herbison GP, Mc-Carroll M. Postoperative anemia and quality of life after primary hip arthroplasty in patients over 65 years old. Anesth Analg 2008;106:1056-1061.
- Muñoz M, Garcia-Erce JA, Remacha AF. Disorders of iron metabolism. Part II: Iron deficiency and iron overload. J Clin Pathol 2011;64:287-296.
- van Haelst IM, Egberts AC, Doodeman HJ, van Solinge WW, Kalkman CJ, Bennis M, Traast HS, van Klei WA. Occurrence and determinants of poor response to short-term pre-operative erythropoietin treatment. Acta Anaesthesiol Scand 2013;57:350-357.
- 71. Andrews CM, Lane DW, Bradley JG. Ironpre-load for major joint replacement. Transfus Med 1997;7:281-286.
- 72. Muusze RG, Corbey AMH, Ulenkate HJLM. Protocol for transfusion-free major orthopaedic operations using Ret-He. Sysmex J Int 2009;19:11-18.
- Muñoz M, Garcia-Erce JA, Cuenca J, Bisbe E, Naveira E, AWGE (Spanish Anaemia Working Group). On the role of iron therapy for reducing allogeneic blood transfusion in orthopaedic surgery. Blood Transfus 2012;10:8-22.





Bloedarmoede of anemie komt overal in de wereld vaak voor. Volgens de Wereld Gezondheids-organisatie (WHO) hebben 1.62 miljard mensen bloedarmoede, wat overeenkomt met 25% van de wereldbevolking. Anemie is een toestand met een te laag gehalte aan hemoglobine (Hb) in het bloed. Dit kan het gevolg zijn van een verstoorde aanmaak, verhoogde afbraak of verlies van rode bloedcellen. Hemoglobine is het rode, zuurstof- en koolstofdioxide transporterend eiwit in bloed, dat zich bevindt in de rode bloedcellen.

Een hemoglobinemolecuul bestaat uit vier polypeptide ketens: twee  $\alpha$ -ketens en twee  $\beta$ -ketens. Elke keten ligt rondom een haem-groep, die bestaat uit een porfyrinering en een ijzeratoom (Figuur 1). IJzer is een essentiële component in het haem. Afwijkingen in de  $\alpha$ -keten of  $\beta$ -keten leiden tot de productie van afwijkend hemoglobine, ook wel hemoglobinopathie of thalassemie genoemd. Deze erfelijke afwijkingen zijn geassocieerd met milde vormen van anemie. Bij mensen afkomstig uit het Middellandse Zeegebied en Afrika komen hemoglobinopathieën veel frequenter voor dan bij mensen uit bijvoorbeeld West-Europa. Als gevolg van migratie komen hemoglobinopathieën echter ook in Noorden West-Europa steeds meer voor. In Nederland is de frequentie circa 1%.



**Figure 1.** Normaal hemoglobine molecuul.

Gemodificeerd naar een illustratie van Internet Encyclopedia of Science.

Hemoglobine bevat dus ijzer. Een tekort aan ijzer kan het directe gevolg zijn van te weinig ijzer in de voeding, maar het kan ook het gevolg zijn van onvermogen om het ijzer op te nemen of van een toegenomen verlies van ijzer. Met name bij jonge kinderen, vegetariërs en vrouwen in de vruchtbare levensfase met ernstig menstrueel bloedverlies of tijdens de zwangerschap komt ijzergebreksanemie frequent voor.

IJzertekort is echter niet de enige oorzaak van anemie. Tekorten in andere essentiële voedingsstoffen, waaronder vitamines A, B2, B12 en foliumzuur komen regelmatig voor.

Daarnaast speelt het hormoon erythropoïetine (EPO) een belangrijke rol in de aanmaak van rode bloedcellen. EPO wordt geproduceerd door de nieren. Bij patiënten met een nieraandoening komt een anemie door een tekort aan EPO vaak voor.

## Algemene diagnostiek van anemie

Wanneer er bij een patiënt anemie is vastgesteld op basis van een hemoglobine bepaling, wordt er aanvullende diagnostiek verricht om de oorzaak van de anemie vast te stellen (http://nl.wikipedia.org/wiki/bloedarmoede; geraadpleegd 10-02-2015). Allereerst wordt er onderscheid gemaakt tussen verschillende typen anemie. Het onderscheid wordt gemaakt door het meten van het mean corpuscular volume (MCV) van de rode bloedcellen:

- microcytaire anemie bij MCV < 80 fl
- normocytaire anemie bij MCV 80-100 fl
- macrocytaire anemie bij MCV > 100 fl

Het beeld van een microcytaire anemie wordt gevonden bij een tekort aan ijzer, bij erfelijk afwijkende vormen van hemoglobine, bijvoorbeeld thalassemie of sikkelcelanemie, of bij chronische ziekten. Om een ijzergebreksanemie aan te tonen wordt de concentratie van ferritine bepaald in serum, vaak in combinatie met serumijzer en transferrine. Is de ferritineconcentratie lager dan  $12~\mu g/l$  en is er geen sprake van een ontstekingsproces, dan is een tekort aan ijzer zo goed als zeker de oorzaak van de microcytaire anemie.

Normocytaire anemie kan ontstaan door beenmergziekte, nierafwijkingen of als gevolg van hemolyse, afbraak van rode bloedcellen.

Macrocytaire anemie kan ontstaan ten gevolge van een tekort aan vitamine B12 en foliumzuur, overmatig gebruik van alcohol, medicatie, hypothyreoïdie, leverfunctiestoornissen of in geval van het myelodysplastisch syndroom (MDS).

# Diagnostiek van ijzergebreksanemie en thalassemie

Omdat het onderscheid tussen ijzergebreksanemie en thalassemie belangrijke klinische implicaties heeft, is een betrouwbare diagnose van belang om onnodig laboratoriumonderzoek te beperken en een ongepaste behandeling te voorkomen. Een breed assortiment van laboratoriumbepalingen is beschikbaar om het onderscheid tussen ijzergebreksanemie en thalassemie te vergemakkelijken. Van oudsher worden algoritmen gebruikt met combinaties van verschillende rode bloedcel (RBC) parameters. De eerste algoritmen hadden een beperkt rendement, toepassing van de zogenaamde England & Fraser formule (MCV – RBC – 5xHb – 3,4) en Mentzer formule (MCV/RBC) resulteert slechts voor 30-40% van patiënten met  $\beta$ -thalassemie in een juiste classificatie.  $^{2,3,4}$ 

In het eind van de jaren '80 werd de bepaling van zinkprotoporfyrine (ZPP) in rode bloedcellen aanbevolen voor het maken van het onderscheid tussen microcytaire anemieën. ZPP is een biomarker voor de detectie van al langer bestaand ijzergebrek. Toepassing van het algoritme 2x RDW (fL) - 5x RBC (x10¹²/L) - 250x reticulocyten (x10¹²/L) + 30x ZPP (µmol/mol Hb), de zogenaamde MCA-index, resulteerde in een correcte classificatie bij 90% van patiënten met ijzergebrek,  $\alpha$ -thalassemie of  $\beta$ -thalassemie.  $^5$ 

Met de beschikbaarheid van nieuwe generaties hematologie apparatuur zijn innovatieve hematologische parameters geïntroduceerd, zoals de fractie immature of onrijpe reticu-

locyten (IRF), hemoglobine inhoud van reticulocyten (RET-He) en hemoglobine inhoud van volwassen RBC (RBC-He). De hemoglobine inhoud van reticulocyten (Ret-He) wordt beschouwd als een gevoelige indicator voor het monitoren van *korte termijn* veranderingen in de ijzer en vitamine beschikbaarheid voor de aanmaak van rode bloedcellen (erythropoïese). Bij patiënten met een verminderde ijzervoorraad neemt het Ret-He reeds enkele dagen na ijzersuppletie toe.

In dit proefschrift zijn resultaten van klinische studies beschreven inzake de toegevoegde waarde van innovatieve parameters voor hemoglobinisatie van rode bloedcellen en reticulocyten. Doelstelling van het onderzoek was verbetering van anemiediagnostiek door toepassing van geavanceerde discriminerende algoritmen voor screening en diagnose van ijzer-deficiënte erythropoïese en thalassemie.

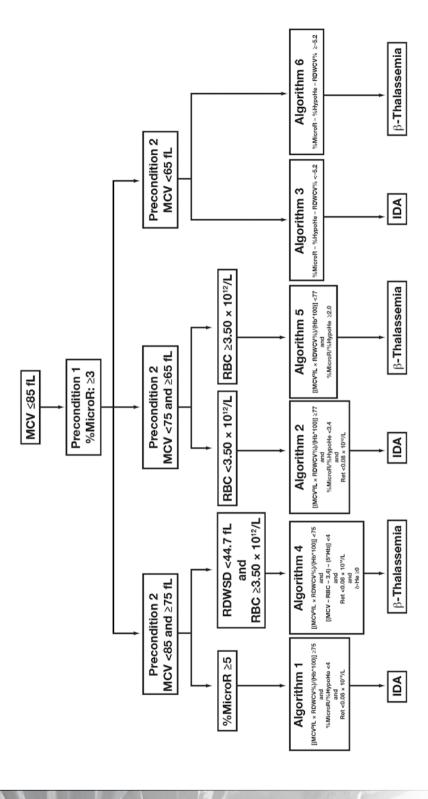
### 1. IJZERGEBREKSANEMIE EN THALASSEMIE

In **hoofdstuk 2** zijn veranderingen in de hemoglobinisatie van reticulocyten en volwassen RBC beschreven. Met behulp van conventionele en innovatieve hematologische parameters werd de beschikbaarheid van ijzer voor de erythropoïese bestudeerd bij patiënten met ijzergebreksanemie en bij patiënten met  $\alpha$ - of  $\beta$ -thalassemie. Resultaten van een groep gezonde proefpersonen (referentiegroep) werden gebruikt voor het vaststellen van referentieranges voor RBC-He, Ret-He en IRF. De Ret-He en RBC-He resultaten waren verlaagd bij de groepen patiënten met ijzergebreksanemie en  $\alpha$ - of  $\beta$ -thalassemie. De Ret-He/RBC-He ratio was duidelijk verlaagd bij de groep patiënten met ijzergebreksanemie (1,02  $\pm$  0,08, gemiddelde  $\pm$  SD) en in mindere mate bij de groep met  $\beta$ -thalassemie (1,06  $\pm$  0,04) in vergelijking met de referentiegroep (1,11  $\pm$  0,02) en de groep met  $\alpha$ -thalassemie (1,11  $\pm$  0,07). Als indicatoren voor ijzer-deficiënte erythropoïese worden cut-off waarden voor Ret-He van <1850 aMol en een Ret-He/RBC-He ratio van <1,02 geadviseerd.

Het gebruik van de combinatie MCV, Ret-He en Ret-He/RBC-He ratio is een geschikte methode voor het aantonen van ijzer-deficiënte erythropoïese of thalassemie. Inmiddels wordt deze methode al vele jaren in het Laboratorium voor Klinische Chemie, Hematologie en Immunologie van het Medisch Centrum Alkmaar gebruikt ten behoeve van de eerste lijns diagnostiek bij patiënten met microcytaire erythropoïese, zwangeren en follow-up van ijzersuppletie.

Recent werden methoden ontwikkeld voor het vaststellen van het percentage hypochrome en microcytaire RBC, aangeduid als respectievelijk %HypoHe en %MicroR. Het %HypoHe geeft het percentage hypochrome RBC aan met een hemoglobinegehalte < 17 pg (= 1062 aMol). Het %MicroR geeft het percentage microcytaire RBC aan met een volume < 60 fL. Bij patiënten met  $\beta$ -thalassemie ligt het %HypoHe onder het referentiegebied als gevolg van verminderde productie van hemoglobine. Resultaten van het %HypoHe verschillen niet van die bij patiënten met ijzergebreksanemie. Resultaten voor het %MicroR zijn verhoogd bij patiënten met  $\beta$ -thalassemie in vergelijking met de resultaten bij patiënten met ijzergebreksanemie.

In een Europese multicenter studie zijn ten behoeve van het maken van onderscheid tus-



Afkortingen: CV = variatiecoëfficiënt; Hb = hemoglobine; IDA = ijzergebreksanemie; MCV = mean cell volume; %MicroR = percentage microcytaire rode bloedcellen; %HypoHe = percentage hypochrome rode bloedcellen; RDW = red blood cell distribution width; RBC = rode bloedcellen; Flow diagram voor het maken van onderscheid tussen ijzergebreksanemie en  $\beta$ -thalassemie. RET = reticulocyten; SD = standaard deviatie. Figure 2.

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sen ijzergebreksanemie en  $\beta$ -thalassemie nieuwe algoritmen ontwikkeld op basis van de parameters Ret-He en Delta-He (RET-He minus RBC-He) en de percentages HypoHe en MicroR. (**hoofdstuk 8**) De studie omvatte het vaststellen van de sensitiviteit en specificiteit van de nieuwe algoritmen tussen deze groepen patiënten en een controlegroep van gezonde personen en het vergelijk van deze algoritmen met de toen al beschikbare algoritmen voor het maken van dat onderscheid. Voorselectiecriteria en algoritmen voor het onderscheid tussen ijzergebreksanemie en  $\beta$ -thalassemie zijn weergegeven in Figuur 2.

Toepassing van het flowdiagram voor het aantonen van de onderliggende diagnoses van ijzergebreksanemie resulteerde in een AUC van 0.88, een sensitiviteit van 79%, een specificiteit van 97%, en een positieve en negatieve voorspellende waarde van respectievelijk 74% en 98%. Voor de detectie van  $\beta$ -thalassemie werden vergelijkbare resultaten gevonden (respectievelijk 0.86, 74%, 98%, 75% en 99%). De diagnostische toepasbaarheid van de algoritmen in het flowdiagram is, in vergelijking met conventionele discriminerende algoritmen beschreven door Engeland en Fraser, Green & King en Mentzer, uitstekend. <sup>2,3,4</sup> Wij adviseren om de nieuwe algoritmen, met daarin een uitgebreid assortiment van RBC parameters, in de dagelijkse praktijk te integreren in laboratoriumapparatuur ten behoeve van diagnose van ijzergebreksanemie of thalassemie. In dit verband wordt vermeld dat de firma Sysmex binnenkort de analyse van Delta-He als officiële parameter in het testmenu opneemt.

### Gecombineerde ijzergebreksanemie en thalassemie

Voor het juist diagnosticeren van patiënten met een gecombineerde  $\beta$ -thalassemie en ijzergebreksanemie adviseren wij de toepassing van Ret-He en Ret-He/RBC-He ratio als extra parameters. In deze specifieke groep patiënten zijn de resultaten van Ret-He en Ret-He/RBC-He ratio duidelijk verlaagd (respectievelijk <1850 aMol en <1,02).

De M-H ratio is de verhouding tussen het percentage microcyten en het percentage hypochrome RBC. Bij patiënten met een gecombineerde  $\beta$ -thalassemie en ijzergebrek is een combinatie van het %Hypo-He en de M-H ratio veelbelovend. Bij deze groep patiënten komt een sterk verhoogd percentage Hypo-He (> 20) voor, in combinatie met een verlaagde M-H ratio (<11,5) (eigen observatie in een kleine groep patiënten, data niet gepubliceerd). Verder onderzoek is echter nodig om hier een meer gefundeerd oordeel over te kunnen geven.

# 2. ANEMIE BIJ CHRONISCHE ZIEKTE (ANEMIE BIJ ONTSTEKING)

In de **hoofdstukken 3, 6 en 7** zijn veranderingen in de erythropoïese activiteit, functioneel ijzergebrek en hemoglobinisatie van rode bloedcellen en reticulocyten onderzocht bij patiënten met chronische nierafwijkingen en bij patiënten met longontsteking (communityacquired pneumonie, CAP).

#### 2.1 Chronische nierziekte

Bloedarmoede komt frequent voor bij patiënten met een chronische nierziekte, vooral bij patiënten met eindstadium nierfalen, waarbij nierfunctievervangende therapie (hemo-

dialyse), noodzakelijk is. In deze groep patiënten komt uitputting van de ijzervoorraad vaak voor als gevolg van bloedverlies tijdens de hemodialyse behandeling en een slechte ijzerabsorptie. Bovendien neemt het risico op ijzergebrek toe door bloedafnames voor laboratoriumonderzoek (jaarlijks ongeveer 500 ml bloed) en een verminderde levensduur van de RBC.

De pathogenese van bloedarmoede bij patiënten met chronische nierziekte is multifactorieel, maar de belangrijkste factor is een lage productie van EPO door de nieren.

Bij hemodialysepatiënten wordt correctie van de anemie gestimuleerd door gecombineerde suppletie van EPO en intraveneus ijzer toediening. Na toediening van EPO wordt, onafhankelijk van de ijzerparameters, een toename van het aantal reticulocyten en immature reticulocyten gedetecteerd, in combinatie met een afname van Ret-He. Als gevolg van de versnelde erythropoïese door EPO-toediening neemt de behoefte aan ijzer toe. IJzersuppletie brengt echter risico's met zich mee, zoals orgaanschade als gevolg van ijzerstapeling. Herziene Europese richtlijnen voor de behandeling van anemie bij patiënten met chronisch nierfalen (EBPG, revised European Best Practice Guidelines) adviseren hematologische parameters, zoals de Ret-He en het percentage van hypochrome rode bloedcellen (%Hypo), voor de evaluatie van de beschikbaarheid van ijzer. Volgens de richtlijnen zijn een Ret-He van <1812 aMol of %Hypo >10% indicaties voor ijzersuppletie.<sup>7</sup>

In **Hoofdstuk 3** zijn de erythropoïese activiteit, functioneel ijzergebrek en hemoglobinisatie van RBC en reticulocyten bij patiënten met chronische nierziekte onderzocht.

Er werd aangetoond dat het aantal reticulocyten en de fractie onrijpe reticulocyten (IRF) duidelijk verhoogd zijn bij patiënten met uremie en hemodialyse. Tevens wordt, bij een bepaalde MCV-waarde, een verlaagde Ret-He en RBC-He waargenomen. De Ret-He/RBC-He ratio is duidelijk afgenomen bij patiënten met hemodialyse (1,05  $\pm$  0,05, gemiddelde  $\pm$  SD) en uremie (1,02  $\pm$  0,10) in vergelijking met de referentiegroep van gezonde personen (1,11  $\pm$  0,02).

Uit een gecombineerde interpretatie van een MCV waarde binnen het referentiegebied en verlaagde waarden voor Ret-He en Ret-He/RBC-He ratio respectievelijk, kan een verminderde mate van hemoglobinisatie worden afgeleid bij patiënten met hemodialyse of uremie. De verminderde hemoglobinisatie wordt ondersteund door verhoogde concentraties serum transferrine receptor (sTfR) en ZPP. De combinatie van de resultaten impliceren een verminderde beschikbaarheid van ijzer voor hemoglobine synthese.

Wij concluderen dat voor het monitoren van de functionele beschikbaarheid van ijzer voor erythropoïese naast biomarkers voor evaluatie van de ijzerstatus ook longitudinale follow-up van Ret-He resultaten noodzakelijk is. Bovendien ondersteunt de evaluatie van Ret-He de *korte termijn* correctie van intraveneuze ijzersuppletie.

### 2.2 Community-acquired pneumonie

Anemie als gevolg van een ontstekingsreactie is de meest voorkomende oorzaak van bloedarmoede bij patiënten met acute of chronische inflammatoire aandoeningen zoals infectie, kanker, reumatoïde artritis en chronische nierziekte. Anemie als gevolg van ontsteking ("inflammatie") gaat gepaard met activatie van witte bloedcellen en productie van cytokines die de erythropoïetine productie, de erythropoïëse, de levensduur van de rode bloedcellen verminderen en de ijzerhuishouding ontregelen.<sup>8</sup>

De ontdekking van hepcidine heeft nieuw licht geworpen op de ijzerhuishouding. Hepcidine is een regulerend eiwit dat de absorptie van ijzer uit het maag-darm kanaal en de distributie van ijzer uit de lichaamsdepots controleert. De synthese van hepcidine wordt gestimuleerd door interleukine-6. Wanneer functioneel ijzergebrek en ontsteking in combinatie voorkomen, zal een verhoogde hepcidine synthese de absorptie van ijzer beperken.

In de **hoofdstukken 6 en 7** is functioneel ijzergebrek onderzocht bij patiënten met een community-acquired pneumonie.

Veranderingen in Ret-He werden vastgesteld gelijktijdig met een toename van ontstekingsmerkers. Bij ziekenhuisopname waren de resultaten voor Ret-He laag-normaal. Tot dag 4 na ziekenhuisopname werd een gestage daling van 3-8% waargenomen. Tijdens de antibioticabehandeling nam het aantal reticulocyten toe vanaf dag 4 tot dag 14. In diezelfde periode herstelden de Ret-He resultaten naar normale waarden.

In het begin van een longontsteking treedt een verlaging van de Ret-He en Ret-He/RBC-He ratio op als gevolg van een acute hemoglobinisatie stoornis, die kan worden verklaard door functioneel ijzertekort (**hoofdstuk 6**).

Bij ziekenhuisopname waren de hepcidine concentraties hoog-normaal terwijl de concentraties C-reactief proteïne (CRP) en interleukine-6 verhoogd waren. Vanaf het moment van ziekenhuisopname tot dag 14 daalden de concentraties hepcidine, CRP en interleukine-6 naar waardes in het referentiegebied.

De tijdelijke verhoging van hepcidine, in combinatie met de toename van interleukine-6 en CRP, ondersteunt de hypothese dat hepcidine een tijdelijke, ontstekingsafhankelijke afname van Ret-He induceert in patiënten longontsteking (**hoofdstuk 7**).

### 3. ZWANGERSCHAP

Bij zwangere vrouwen varieert de prevalentie van bloedarmoede ten gevolge van ijzergebrek van 6 tot 30%. De hoogste frequenties worden waargenomen in landen waar routinematige ijzersuppletie tijdens de zwangerschap niet gebruikelijk is. <sup>10</sup> Bloedarmoede tijdens de zwangerschap is o.a. te wijten aan fysiologische hemodilutie en onvoldoende beschikbaarheid van essentiële voedingsstoffen voor hemoglobinesynthese en productie van rode bloedcellen, zoals ijzer, foliumzuur en vitamine B12. Omdat de mate van hemodilutie een brede inter-individuele variatie kent, laten hemoglobine concentraties overeenkomstige variaties zien. Daarom is het moeilijk vast te stellen of een verlaagde hemoglobine concentratie een fysiologische conditie betreft dan wel het gevolg is van ijzer-deficiënte erythropoïese.

Tijdens de zwangerschap is ongeveer 1000 mg extra ijzer nodig (2-3x zoveel als normaal) voor de toegenomen aanmaak van de erythrocytenmassa van moeder en foetus. <sup>10,11</sup> De ijzervoorraad van de moeder en de opname van ijzer via de voeding zijn dan gemakkelijk onvoldoende voor een adequate erythropoïese. De toegenomen ijzerbehoefte tijdens de zwangerschap maakt bloedarmoede als gevolg van ijzergebrek een veel voorkomende aandoening.

Verlaagde hemoglobine concentraties, als gevolg van functionele ijzerdeficiëntie in het laatste trimester van de zwangerschap, kunnen worden veroorzaakt door complicaties zo-

als infecties, laag geboortegewicht en vroeggeboorte. Positieve effecten van ijzersuppletie bij zwangeren zijn betere fysieke conditie, preventie van postpartum ijzergebrek door bloedverlies bij de bevalling, en verbeterde ijzerreserves bij de pasgeborene om ijzergebrek in de eerste jaren van het leven te voorkomen.<sup>12,13</sup>

Zwangerschap gaat gepaard met een toename van inflammatoire biomarkers, vooral in het eerste en derde trimester. Tijdens een zwangerschap kunnen functionele en absolute ijzer-deficiënte erythropoïese naast elkaar voorkomen. Als gevolg van een acute fase respons tijdens ontsteking neemt de serum ferritine concentratie toe, hetgeen kan resulteren in een overschatting van de ijzervoorraden indien de resultaten van deze test onjuist worden geïnterpreteerd. Sien in de van de van

In de verloskundige praktijk worden verschillende richtlijnen voor de diagnostiek toegepast. De Koninklijke Nederlandse Organisatie voor Verloskundigen (KNOV) en de Wereld Gezondheidsorganisatie (WHO) hanteren hemoglobine concentraties van 6,3 respectievelijk 6,8 mmol/l voor het vaststellen van bloedarmoede en als indicatie voor het starten van ijzersuppletie. Hieruit kan worden geconcludeerd dat de anemiediagnostiek tijdens de zwangerschap nog kan worden verbeterd.

In **hoofdstuk 4** worden veranderingen in de hemoglobinisatie van rode bloedcellen tijdens de zwangerschap beschreven. Het doel van het onderzoek was om inzicht te verkrijgen in de toegevoegde waarde van de fractie onrijpe reticulocyten (IRF) en Ret-He om veranderingen in hemoglobinisatie vast te stellen ten behoeve van een juiste discriminatie en indicatie voor ijzersuppletie.

Resultaten toonden aan dat een hemoglobine meting in veel gevallen niet volstaat voor anemie screening tijdens de zwangerschap. Uit bevindingen van Ervasti en Wheeler blijkt dat Ret-He een nuttig hulpmiddel is bij screening en monitoring van de beschikbaarheid van ijzer voor erythropoïese. 14,17

Als hulpmiddel voor het screenen van de beschikbaarheid van ijzer tijdens het derde trimester van de zwangerschap bevelen wij een Ret-He aan met een cut-off waarde van <1850 amol . Een verlaagde Ret-He wordt beschouwd als indicatie voor onvoldoende hemoglobinisatie van RBC, met name in geval van een hemoglobine concentratie Hb  $\leq$  6.8 mmol/l en een MCV 80-100 fl.

In **hoofdstuk 5** zijn effecten van ijzersuppletie op RBC-He en Ret-He gedurende de zwangerschap onderzocht. Het doel van de studie was om het effect van ijzersuppletie te evalueren op Ret-He en RBC-He bij zwangeren met hemoglobine concentraties in de range van 6,3 tot 6,8 mmol/l in het derde trimester van de zwangerschap. Criteria voor verdenking op ijzer-deficiënte erythropoïese zijn Hb  $\leq$ 6,8 mmol/l, Ret-He <1850 amol en ZPP >75  $\mu$ mol/mol heam.

Na 4 weken ijzersuppletie zijn de resultaten van Ret-He en Ret-He/RBC-He-ratio toegenomen tot de ondergrens van de range voor referentiewaarden. De normalisering van Ret-He en Ret-He/RBC-He ratio reflecteert de *korte termijn* veranderingen met betrekking tot de 'kwaliteit' van de erythropoïese. Tevens zijn de hemoglobine concentratie en

het absolute aantal reticulocyten na ijzersuppletie toegenomen. In eerdere studies is aangetoond dat voedingssupplementen geen significante veranderingen in RBC-He en ZPP opleveren. Het ontbreken van een effect kan worden verklaard door het feit dat zowel RBC-He als ZPP beide de *lange termijn* veranderingen in hemoglobinisatie weerspiegelen overeenkomstig de levensduur van volwassen RBC (circa 100 - 120 dagen). 19

Wij concluderen dat aanpassingen noodzakelijk zijn in de herziene richtlijn 'Anemie in de verloskundige praktijk'<sup>20,21</sup>, die MCV als beslissende merker voor ijzersuppletie in het derde trimester van de zwangerschap vermeldt bij zwangeren met een hemoglobine concentratie <6,5 mmol/L.

Wij adviseren dat Ret-He en Ret-He/RBC-He ratio worden geïntegreerd in het protocol voor screening en monitoring van anemie, met name bij een hemoglobine concentratie in de controversiële range van 6,3-6,8 mmol/l.

### 4. TOEKOMSTPERSPECTIEVEN

Anno 2015 is het ijzermetabolisme nog steeds actueel voor de screening en het onderscheiden van onderliggende oorzaken van bloedarmoede.

Het thema van dit proefschrift beschrijft de toegevoegde waarde van innovatieve hematologische parameters voor hemoglobinisatie van RBC (RBC-He) en reticulocyten (Ret-He). Wij adviseren om het nieuwe flowdiagram voor screening op bloedarmoede te implementeren om daarmee onnodig laboratoriumonderzoek te beperken en de onderliggende oorzaak van de anemie juist te beoordelen.

Diagnostische richtlijnen worden gebruikt voor de het stellen van verschillende diagnoses en de follow-up van bloedarmoede. Het Nederlands Huisartsen Genootschap en de Nederlandse Vereniging voor Klinische Chemie en Laboratoriumgeneeskunde (NHG-Standaard Anemie en NVKC Reflex diagnostiek Anemie) propageren hemoglobine concentraties onder de referentiewaarden voor discriminatie van bloedarmoede. 22,23 MCV en ferritine concentraties worden aanbevolen als reflex-testen en evaluatie van de ijzerstatus. Echter, het verband tussen serum-ferritine en ijzervoorraden wordt beïnvloed door acute en chronische infecties en inflammatoire aandoeningen, leverziekte en maligniteit.

Naar onze mening vereisen de hierboven genoemde richtlijnen een state-of-the-art update.

Wij adviseren fabrikanten van hematologie apparatuur om innovatieve software toepassingen te ontwikkelen en te implementeren, waaronder de nieuwe algoritmen ten behoeve van het stellen van verschillende diagnoses als oorzaken van bloedarmoede en monitoring van therapie. Diverse leveranciers bieden inmiddels de mogelijkheid voor analyse van de nieuwe parameters voor hemoglobinisatie van RBC en reticulocyten. De onderlinge vergelijkbaarheid van de betreffende parameters is echter een punt van zorg. Vooral in multilocatie settings is kalibratie en afstelling van de meetkanalen een belangrijk aandachtspunt. In geval van multicenter evaluatiestudies, waarbij gebruik gemaakt wordt

van hematologie apparatuur van verschillende leveranciers, is onderlinge afstemming c.q. standaardisatie van de parameters van essentieel belang.

De incidentie van anemie neemt toe met de leeftijd. 24,25 Volgens de WHO-criteria (man: Hb <8,1 mmol/L, vrouw: Hb <7,5 mmol/L) heeft ongeveer 16% van de mannen en 10% van de vrouwen in de leeftijd van 75-85 jaar bloedarmoede.<sup>26,27</sup> Onder de mensen ouder dan 85 jaar neemt de prevalentie van anemie nog verder toe. Resultaten van de 'Leiden 85plus studie' hebben aangetoond dat de mortaliteit toeneemt bij een daling in de concentratie hemoglobine. 25,28 Bij ouderen is bloedarmoede vaak het gevolg van een verminderde nierfunctie al dan niet in combinatie met een chronische ziekte. Waar nierfalen wordt geassocieerd met bloedarmoede als gevolg van verminderde EPO productie, wordt anemie als gevolg van een chronische ziekte gekenmerkt door ijzer-deficiënte erythropoïese. Bij ouderen komen ook regelmatig tekorten voor van andere nutriënten die nodig zijn voor de erythropoïese. Een bekend voorbeeld hiervan is een tekort aan foliumzuur of vitamine B12. Het tekort kan het gevolg zijn van onvoldoende opname via de voeding of het kan een gevolg zijn van malabsorptie. In geval van een vitamine B12 of foliumzuur deficiëntie neemt de Ret-He toe, als gevolg van een toename van het volume van de RBC.29 Ten behoeve van monitoring van de nutriënten status bij ouderen in de routine adviseren wij om de parameters RBC-He, Ret-He, Ret-He/RBC-He ratio of delta-He in een flowdiagram op te nemen.

Er is een toenemende belangstelling voor diagnose en behandeling van anemie ter voorbereiding van een electieve chirurgische ingreep teneinde het aantal bloedtransfusies te minimaliseren. De prevalentie van preoperatieve anemie loopt sterk uiteen (10-40%). Signification die groep overwegend oudere patiënten, is ijzertekort als gevolg van een verminderde nierfunctie of chronische ziekte de meest voorkomende oorzaak. Bij ouderen is de concentratie hemoglobine bij opname van invloed op het functionele herstel van de heupfractuur en de kwaliteit van leven na de operatie. Signification die proposition di

Vroege opsporing en behandeling van pre-operatieve anemie is nodig vanwege de associatie met ongunstig post-operatief herstel.<sup>32,37</sup> Implementeren van bloed management strategieën, inclusief preoperatieve toediening van erythropoietein (EPO), vermindert niet alleen de noodzaak van bloedtransfusie, maar verbetert tevens het postoperatieve herstel.<sup>38,39</sup>

Als gevolg van suppletie met EPO wordt de productie van RBCs gestimuleerd waardoor de ijzerbehoefte van het erythron toeneemt. Om aan de verhoogde ijzerbehoefte te voldoen wordt EPO therapie meestal gecombineerd met oraal ijzersuppletie. In veel gevallen is de ijzeropname verminderd, waardoor een ijzerdeficiënte erythropoïese kan ontstaan. Verminderde ijzeropname komt onder andere voor bij darmziekten, infectie met Helicobacter pylori of gebruik van bepaalde medicijnen zoals antibiotica, protonpompremmers en vitamine K-antagonisten. Vanwege de beperkingen van orale ijzertherapie in de groep patiënten met electieve chirurgie heeft suppletie met intraveneus ijzer naar onze mening de voorkeur. Val. 43,43,444

Wij adviseren om RBC-He, Ret-He, Ret-He/RBC-He ratio of delta-He in protocollen voor de pre-operatieve anemiescreening en monitoring op te nemen met als doel verbetering van de effectiviteit van pre-operatieve EPO- en ijzersuppletie therapie.

Samenvattend, wordt in dit proefschrift de basis gelegd voor een verbeterde diagnostiek van anemie met behulp van specifieke parameters en geavanceerde algoritmen, die worden geformuleerd ten behoeve van groepen betrokken patiënten. Het incorporeren van dergelijke strategieën in een gemakkelijk toegankelijk software programma gekoppeld aan de hematologie apparatuur, bij voorkeur geleverd door de fabrikant van de apparatuur om deze interactie te vergemakkelijken, zal de patiëntenzorg aanzienlijk verbeteren.



### LITERATUUR REFERENTIES

- Modell B, Darlison M, Birgens H, Cario H, Faustino P, Giordano PC, Gulbis B, Hopmeier P, Lena-Russo D, Romao L, Theodorsson E. Epidemiology of haemoglobin disorders in Europe; an overwiew. Scand J Clin Lab Invest 2007;67:39-70.
- 2. England JM, Fraser PM: Differentiation of iron deficiency from thalassaemia trait by routine blood count. Lancet 1973; i:449-452.
- Mentzer WC: Differentiation of iron deficiency from thalassaemia trait. Lancet 1973;i:882.
- Green R, King R: Red cell discriminant function, β-thalassaemia minor versus iron deficiency. Blood cells 1989;15:481-495.
- Bartels PCM, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocyte count and red cell zinc protoporphyrin for appropriate classification of microcytic anemia. Sysmex J Int 2003;13:78-82.
- d'Onofrio G, Zini G, Ricerca BM, Mancini S, Mango G. Automated measurement of red blood cell microcytosis and hypochromia in iron deficiency and betathalassemia trait. Arch Pathol Lab Med 1992;116:84-89.
- Locatelli F, Aljama P, Bárány P, Canaud B, Carrera F, Eckhardt KU, Hörl WH, Macdougal IC, Macleod A, Wiecek A, Cameron S. Revised European best practice guidelines for management of anaemia in patients with chronic renal failure. Nephrol Dial Transplant 2004;19(suppl 2):ii1ii47.
- 8. Weiss G. Goodnough LT. Anemia of chronic disease. N Engl J Med 2005;352:1011-
- 9. Ganz T. Hepcidin and iron regulation, 10 years later. Blood 2011;117:4425-4433.
- 10. Hercberg S, Preziosi P, Galan P. Iron de-

- ficiency in Europe. Public Health Nutr 2001;4:537-545.
- Bentley DP. Iron metabolism and anaemia in pregnancy. In: Letsky EA, guest ed. Haematological disorders in pregnancy (Clinics in Haematology vol.14).
   London. W.B. Saunders Comp 1985;613-628.
- 12. Milman N, Bergholt T, Byg K, Eriksen L, Graudal N. Iron status and iron imbalance during pregnancy. A critical reappraisal of iron supplementation. Acta Obstet Gynecol Scand 1999;78:749-57.
- 13. Wiegerinck MM, Mol BWJ. Insufficient evidence supporting iron supplementation in anaemia during pregnancy. Ned Tijdschr Geneeskd 2012;156:A4293.
- 14. Wheeler S. Assessment and interpretation of micronutrient status during pregnancy. Proc Nutr Soc 2008;67:437-450.
- Rogers JT, Bridges KR, Durnowicz GP, Glass J, Auron PE, Munro HN. Translational control during the acute phase response. Ferritin synthesis in response to interleukin-1. J Biol Chem 1990;265:14572-14578.
- Milman N, Bergholt T, Byg K, Eriksen L, Hvas A. Reference intervals for haematological variables during normal pregnancy and postpartum in 434 healthy Danish women. Eur J Hematol 2007;79: 39-46.
- 17. Ervasti M, Kotisaari S, Heinonen S, Punnonen K. Use of advanced red blood cell and reticulocyte indices improves the accuracy in diagnosing iron deficiency in pregnant women at term. Eur J Hematol 2007;79:539-45.
- 18. Harthoorn EJ, Lindemans J, Langenhuijsen MMAC. Erythrocyte zinc protoporphyrin testing in pregnancy. Acta Obstet Gynecol Scand 2000;79:660-66.
- 19. Thomas C, Thomas L. Biochemical markers and hematologic indices in the diag-

- nosis of functional iron deficiency. Clin Chem 2002;48:1066-1076.
- KNOV Anemie in de verloskundige praktijk. Praktijkkaart behorende bij de gelijknamige KNOV-standaard 2010. Utrecht: Koninklijke Nederlandse Organisatie voor Verloskundigen.
- Beentjes M, Jans S. Revised practice Guideline 'Anaemia in midwifery practice'. Ned Tijdschr Geneeskd 2012;156: A3711.
- Verboeket-van de Venne WPHG, Oosterhuis WP, Keuren JFW, Ulenkate HJLM, Leers MPG. Richtlijn NVKC Reflexdiagnostiek bij anemie. NVKC 2012.
- 23. NHG werkgroep Anemie, oktober 2014. NHG Richtlijn Anemie. www.nhg.org/ standaarden/volledig/nhg-standaardanemie
- 24. Beghe C, Wilson A, Ershler W. Prevalence and outcomes of anaemia in geriatrics: a systematic review of the literature. Am J Med 2004;116:3S-10S.
- Izaks GJ, Westendorp RGJ. Grenswaarden voor anemie bij oude mensen. Ned Tijdschr Geneeskd 2006;150:1002-1006.
- Guralnik JM, Eisensteadt RS, Ferruci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the United States; evidence for a high rate of unexplained anemia. Blood 2004;104:2263-2268.
- World Health Organisation (WHO). Nutritional anaemias: report of a WHO scientific group. Genève: WHO;1968.
- Izaks GJ, Westendorp RGJ, Knook DL. The definition of anemia in older persons. JAMA 1999;281:1714-1717.
- Ceylan C, Miskioglu M, Colak H, Kiliccioglu B, Ozdemir E. Evaluation of reticulocyte parameters in iron deficiency, vitamin B12 deficiency and β-thalassemia minor patients. Int J Lab Hem 2007;29:327-334.
- 30. Saleh E, McClelland DBL, Hay A, Semple D, Walsh TS. Prevalence of anaemia befo-

- re major joint arthroplasty and the potential impact of preoperative investigation and correction on perioperative blood transfusions. Br J Anaesth 2007;99:801-808
- Goodnough LT, Shandor A, Spivak JL, Waters JH, Friedman AJ, Carson JL, Keating EM, Maddox T, Spence R. Detection, evaluation and management of anaemia in the elective surgical patient. Anesth Analg 2005;101:1858-1861.
- 32. Carrascal Y, Maroto L, Rey J et al. Impact of preoperative anaemia on cardiac surgery in octogenarians. Interact Cardiovasc Thorac Surg 2010;10:249-255.
- Karski JM, Mathieu M, Cheng D, Carroll J, Scott GJ. Etiology of preoperative anaemia in patients undergoing scheduled cardiac surgery. Can J Anaesth 1999;46:979-982.
- Gruson KI, Aharonoff GB, Egol KA, Zuckerman JD, Koval KJ. The relationship between admission hemoglobin level and outcome after hip fracture. J Orthop Trauma 2002:16:39-44.
- 35. Halm EA, Wang JJ, Boockvar K, Penrod J, Silberzweig SB, Magaziner J, Koval KJ, Siu AL. The effect of perioperative anaemia on clinical and functional outcomes in patients with hip fracture. J Orthop Trauma 2004;18:369-374.
- Conlon NP, Bale EP, Herbison GP, McCarroll M. Postoperative anemia and quality of life after primary hip arthroplasty in patients over 65 years old. Anesth Analg 2008;106:1056-1061.
- Karkouti K, Wijeysundera DN, Beattie WS. Risk associated with preoperative anaemia in cardiac surgery. A multicenter cohort study. Circulation 2008;117:478-484.
- 38. Spahn DR. Anemia and patient blood management in hip and knee surgery; a systemetic review of the literature. Anesthesiology 2010;113:482-495.

- 39. Goodnough LT, Maniatis A, Earnshaw P, Benoni G, Beris P, Bisbe E, Fergusson DA, Gombotz H, Habler O, Monk TG, Ozier Y, Slappendel R, Szpaski M. Detection, evaluation, and management of preoperative anaemia in the elective orthopaedic surgical patient: NATA guidelines. Br J Anaesth 2011;106:13-22.
- Muñoz M, Garcia-Erce JA, Remacha AF. Disorders of iron metabolism. Part II: Iron deficiency and iron overload. J Clin Pathol 2011;64:287-296.
- 41. van Haelst IM, Egberts AC, Doodeman HJ, van Solinge WW, Kalkman CJ, Bennis M, Traast HS, van Klei WA. Occurrence and determinants of poor response

- to short-term pre-operative erythropoietin treatment. Acta Anaesthesiol Scand 2013;57:350-357.
- 42. Andrews CM, Lane DW, Bradley JG. Iron-pre-load for major joint replacement. Transfus Med 1997;7:281-286.
- 43. Muusze RG, Corbey AMH, Ulenkate HJLM. Protocol for transfusion-free major orthopaedic operations using Ret-He. Sysmex J Int 2009;19:11-18.
- 44. Muñoz M, Garcia-Erce JA, Cuenca J, Bisbe E, Naveira E, AWGE (Spanish Anaemia Working Group). On the role of iron therapy for reducing allogeneic blood transfusion in orthopaedic surgery. Blood Transfus 2012;10:8-22.



# 1 Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia.

Authors: Piet C.M. Bartels, Margreet Schoorl, Marianne Schoorl

*Piet C.M. Bartels* participated in the study design and interpretation of data, provided intellectual content of haemoglobinization of red blood cells and iron-deficient erythropoiesis, has drafted the manuscript and gave final approval.

*Margreet Schoorl* participated in the design of the study, was responsible for the correct analysis and interpretation of haemocytometric parameters, participated in the interpretation of the data, reviewed the draft and made comments which were incorporated to form the final draft.

*Marianne Schoorl* was responsible for the statistical evaluation of data, reviewed the draft and made comments which were incorporated to form the final draft.

# 2 Erythropoiesis activity, iron availability and reticulocyte hemoglobinization during treatment with hemodialysis and in subjects with uremia.

Authors: Marianne Schoorl, Margreet Schoorl, Menso J Nubé, Piet C.M. Bartels

*Marianne Schoorl* participated in the study design and interpretation of data, was responsible for the statistical evaluation of data, reviewed the draft and made comments which were incorporated to form the final draft.

*Margreet Schoorl* participated in the design of the study, was responsible for the correct analysis and interpretation of haemocytometric parameters, participated in the interpretation of the data, reviewed the draft and made comments which were incorporated to form the final draft.

*Menso J. Nubé* participated in the design and interpretation of data, provided medical history concerning haemodialysis and uraemia and made comments which were incorporated to form the draft.

*Piet C.M. Bartels* participated in the study design and interpretation of data, provided intellectual content of haemoglobinization of red blood cells and iron-deficient erythropoiesis, has drafted the manuscript and gave final approval.

# 3 Changes in red blood cell hemoglobinization during pregnancy. Authors: Margreet Schoorl, Derek van der Gaag, Marianne Schoorl, Piet C.M. Bartels

*Margreet Schoorl* participated in the design of the study, was responsible for correct analysis and interpretation of hemocytometric parameters, participated in the interpretation of the data, and has drafted and revised the article to form the final draft.

*Derek van der Gaag* participated in the design of the study and was responsible for statistical evaluation of analytical results.

*Marianne Schoorl* participated in the design of the study and was responsible for statistical evaluation, reviewed the draft and made comments which were incorporated to form the final draft.

Piet C.M. Bartels participated in the study design and interpretation of data, provided



intellectual insight into hemoglobin content of red blood cells during pregnancy and the importance of the work, drafted the manuscript and gave final approval of the version to be published.

4 Effects of iron supplementation on red blood cell hemoglobin content in pregnancy. Authors: Margreet Schoorl, Marianne Schoorl, Derek van der Gaag, Piet C.M. Bartels

*Margreet Schoorl* participated in the design of the study, was responsible for correct analysis and interpretation of hemocytometric parameters, participated in the interpretation of the data, and has drafted and revised the article.

*Marianne Schoorl* participated in the design of the study and was responsible for statistical evaluation.

*Derek van der Gaag* participated in the design of the study and was responsible for statistical evaluation of analytical results.

*Piet C.M.Bartels* participated in the study design and interpretation of data, provided intellectual insight into hemoglobin content of red blood cells during pregnancy and the importance of the work, drafted the manuscript and gave final approval of the version to be published.

5 Temporary impairment of reticulocyte haemoglobin content in subjects with community-acquired pneumonia.

Authors: Margreet Schoorl, Dominic Snijders, Marianne Schoorl, Wim G. Boersma, Piet C.M. Bartels

*Margreet Schoorl* participated in the design of the study, was responsible for the correct analysis and interpretation of haemocytometric parameters, participated in the interpretation of the data and has drafted and revised the article.

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*Marianne Schoorl* was responsible for the correct analysis and interpretation of inflammation parameters, and statistical evaluation.

*Wim G. Boersma* participated in the design and interpretation of data, provided medical history concerning inflammation and pneumonia and has drafted the manuscript.

*Piet C.M. Bartels* participated in the study design and interpretation of data, provided intellectual content of haemoglobinization of red blood cells during inflammation and importance of the work, has drafted the manuscript and gave final approval.

6 Transient impairment of reticulocyte hemoglobin content and hepcidin-25 induction in patients with community-acquired pneumonia.

Authors: Margreet Schoorl, Dominic Snijders, Marianne Schoorl, Wim G. Boersma, Piet C.M. Bartels

*Margreet Schoorl* participated in the design of the study, was responsible for correct analysis and interpretation of hemocytometric parameters, participated in the interpretation of the data and drafted and revised the article.

*Dominic Snijders* participated in the design and interpretation of data and provided content of clinical treatment of inflammation and pneumonia.

*Marianne Schoorl* was responsible for correct analysis and interpretation of inflammation biomarkers and statistical evaluation of analytical results.

*Wim G Boersma* participated in the design and interpretation of data, provided content of inflammation and pneumonia and drafted the manuscript.

*Piet C.M. Bartels* participated in the design and interpretation of data, provided intellectual content of hemoglobinization of red blood cells during inflammation and importance of the work, drafted the manuscript and provided final approval of the manuscript to be published.

7 Efficacy of advanced discriminating algorithms for screening on iron-deficiency anemia and β-thalassemia trait. A multicenter evaluation.

Authors: Margreet Schoorl, Marianne Schoorl, Jo Linssen, Miriam Martinez Villanueva, José A Velasco Noguera, Pedro Hernandez Martinez, Piet C.M. Bartels

*Margreet Schoorl* participated in the design of the study, was responsible for correct analysis and interpretation of hemocytometric parameters, participated in the interpretation of the data and has drafted and revised the article.

*Marianne Schoorl* participated in the design of the study and was responsible for statistical evaluation of analytical results.

*Jo Linssen* participated in the design and interpretation of data and statistical evaluation of analytical results.

*Miriam Martinez Villanueva* participated in correct analysis and interpretation of hemocytometric parameters and the interpretation of the data.

José A Velasco Noguera was responsible for correct analysis and interpretation of hemocytometric parameters and participated in the interpretation of the data.

*Pedro Hernandez Martinez* was responsible for correct analysis of hemoglobin electrophoresis.

*Piet C.M. Bartels* participated in the design and interpretation of data, provided intellectual content of hemoglobinization of red blood cells during inflammation and importance of the work, has drafted the manuscript and gave final approval of the version to be published.

8 Application of innovative haemocytometric parameters and algorithms for improvement of microcytic anaemia discrimination. *Minireview* 

Authors: Margreet Schoorl, Marianne Schoorl, Johannes van Pelt, Piet C.M. Bartels

*Margreet Schoorl* participated in het design of the study, provided intellectual insight into hemoglobin content of red blood cells and the importance of the work for screening anaemia and has drafted and revised the article.

*Marianne Schoorl* participated in the design of the study and provided intellectual insight into hemoglobin content of red blood cells and made comments which were incorporated to form the final draft.

*Johannes van Pelt* reviewed the draft and made comments which were incorporated to form the final draft.



*Piet C.M. Bartels* participated in the design of the study and provided intellectual insight into hemoglobin content of red blood cells and the importance of the work, drafted the manuscript and made comments which were incorporated to form the final draft and gave final approval of the version to be published.

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All (co)-authors declared no potential conflict of interest. None of the authors has accepted any funding or support from an organization. Dankwoord

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*De promotiecommissie*, bestaande uit Dr. J.H. Klinkspoor, Prof. Dr. M.H.J. van Oers, Prof. Dr. J.A.M. van der Post, Prof. Dr. C.E. van der Schoot, Prof. Dr. W.W. van Solinge, Prof. Dr. D.W. Swinkels, Dr. S.S. Zeerleder, voor het kritisch lezen van het manuscript.

Het doen van onderzoek is in een perifeer ziekenhuis, naast de gewone werkzaamheden, alleen mogelijk in een team van enthousiaste collega's en medewerkers. De medewerkers van het laboratorium voor KCHI wil ik bedanken voor de hulp bij het verrichten van celtellingen m.b.v. de Sysmex XE-2100 hematologie analyzer, het verzamelen van gegevens en het printen van 'plotjes'. Ook Trijntje en Els hebben hun steentje bijgedragen met het invoeren van vele resultaten in Excel. Dank daarvoor!

Mooie voorbeelden van 'innovatie netwerken' en 'customer intimacy'

Verschillende leveranciers hebben een bijdrage geleverd middels kennisdeling en innovatie. In het bijzonder wil ik Sysmex Nederland en Sysmex Europe bedanken. Jan-Willem, van jou leerde ik de eerste beginselen van het beoordelen van de Sysmex NE-8000 scattergrammen. Door de combinatie van hemocytometrie en microscopie werd de kwaliteit van het beoordelen van microscopische differentiaties verbeterd. Later, als directeur van Goffin Meyvis en Sysmex NL, heb je ons laboratorium altijd met de nieuwste mogelijkheden en software applicaties gefaciliteerd hetgeen menig keer resulteerde in een mooie publicatie. Ook aan de waardevolle inbreng van kennis en de prettige samenwerking met Jo (Sysmex Europe) bewaar ik fijne herinneringen.

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Voor het vormgeven van mijn proefschrift wil ik graag Douwe en Imre bedanken. Douwe, jij hebt er voor gezorgd dat de lay-out van de manuscripten, tabellen en figuren er prima uit zijn komen te zien en alle  $\alpha$ -tjes en  $\beta$ -tjes op de juiste plaats staan. Het proefschrift ziet er prachtig uit!

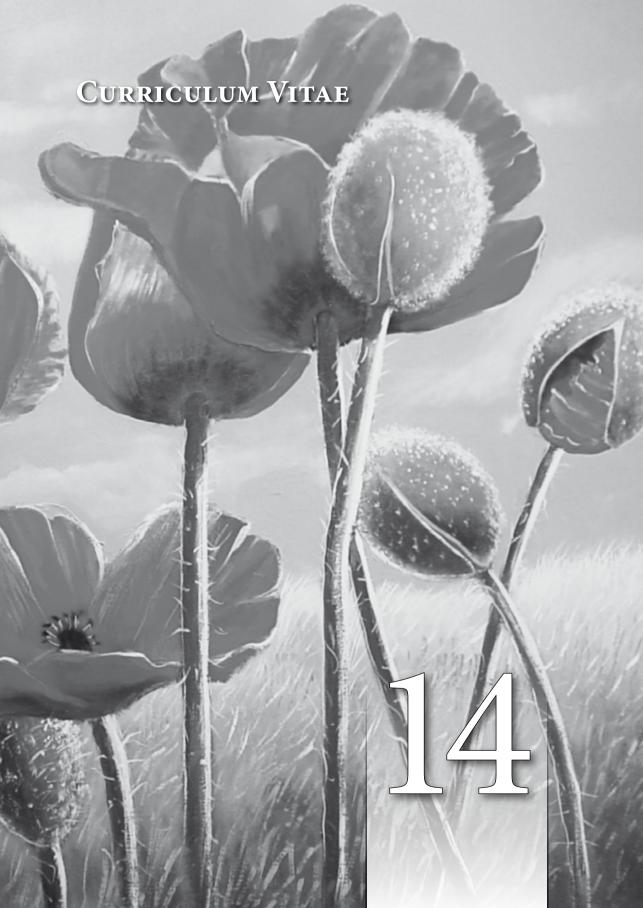
Rode bloedcellen zorgen voor het zuurstoftransport in het lichaam en zijn gevuld met hemoglobine, ook wel 'de rode bloedkleurstof' genoemd. Bij bloedarmoede (anemie) is de hemoglobine concentratie in de rode bloedcellen verminderd. Microscopisch gezien zijn de minder goed gevulde rode bloedcellen lichter van kleur ('hypochroom').

Duinpapavers zijn er in vele soorten en kleuren, soms donkerrood soms bleekrood. Het schilderij *Duin papavers* heeft voor mij een associatie met de uitrijping van rode bloedcellen. Eerst een knop met nog weinig rode kleur (jonge rode bloedcel / reticulocyt), een paar dagen later een mooie rode bloem (volwassen rode bloedcel / erythrocyt).

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Margreet Schoorl is geboren op 17 juni 1955 te Zuid-Scharwoude (gemeente Langedijk). Na de opleiding aan de Openbare MULO te Langedijk, waar zij het MULO-B diploma behaalde in 1971, volgde zij de Hogere Beroepsopleiding aan het Bakhuis Roozeboom Instituut te Beverwijk. Zij behaalde het diploma in de richting Klinische Chemie A in juni 1974. Daarna aanvaardde zij een functie als analist in het klinische chemisch laboratorium van het Medisch Centrum Alkmaar. Zij ontwikkelde zich voortvarend en werd binnen 5 jaar benoemd tot hoofd van de afdeling Hematologie.

De expertise in het klinisch chemisch laboratorium verdiepte en verbreedde zich vanaf 1989, met de komst van een medisch immunoloog, tot het Laboratorium voor Klinische Chemie, Hematologie en Immunologie. Diverse cursussen op het gebied van flowcytometrie werden gevolgd, om daarmee immunocytologisch onderzoek, onder andere ten behoeve van leukemie diagnostiek, op te zetten. In het jaar 2000 werden moleculair biologische technieken in het laboratorium geïntroduceerd. Ook hiervoor werden diverse cursussen gevolgd, onder andere aan de Hoge School West Brabant en de Hoge School Alkmaar. De functie van hoofd van de afdeling Hematologie ontwikkelde zich tot hoofd van de afdeling Hematologie, Immunocytologie & Moleculaire Biologie.

Margreet heeft zich gespecialiseerd in het genoemde vakgebied en heeft daarnaast toegepast wetenschappelijk onderzoek verricht op uiteenlopende terreinen o.a. kwaliteit en validatie, evaluatie en optimalisering van workflow van hematologie apparatuur, leukocytenactivatie bij hemodialyse, ontstekingsparameters en nutriënten in combinatie met stimulering van de erytropoïese activiteit. Diverse stageprojecten werden begeleid op het niveau van MBO-, HBO, Bachelor en Master opleidingen. Vanwege haar expertise wordt Margreet regelmatig gevraagd als spreker bij symposia en nascholingsactiviteiten.

Een eerste link met professionalisering van management in de gezondheidszorg werd in 1984 gelegd door het volgen van de opleiding Management voor non-profit organisaties aan het Instituut voor Sociale Wetenschappen (ISW). In de jaren daarna werd aandacht besteed aan de implementatie van competentiemanagement, resultaat & ontwikkelingsgesprekken en coaching van medewerkers.

Concurrentie tussen laboratoria werd vanaf 2005 in toenemende mate merkbaar. Gezien de marktontwikkelingen stond het laboratorium voor de strategische uitdaging om naast klantgericht meer marktgericht te opereren. In 2008 en 2009 werden aan de Nyenrode Business University de collegereeks Business Development & Blue Ocean Strategy en de collegereeks Management in de zorg gevolgd. Margreet was mede-initiator van de werkconferenties *Buiten winnen is binnen beginnen en Een goede relatie met de klant*. Publicaties zijn verschenen op het gebied van klanttevredenheid, kostenbewustzijn, cliëntgericht werken, marketing en ondernemerschap in het laboratorium.

In 2010 is het laboratorium geïntegreerd met het laboratorium van het Gemini Ziekenhuis in Den Helder. Optimalisering en harmonisatie van werkprocessen, verbetering van logistiek en monsterstromen waren belangrijk voor efficiënt en effectief werken op de verschillende locaties van het laboratorium voor Klinische Chemie, Hematologie & Immunologie. In 2011 werd daartoe de leergang Procesmanagement afgerond (Schouten & Nelissen).

Vanaf 2010 werden plannen geconcretiseerd voor de bewerking van een proefschrift. De voltooiing van dit proefschrift biedt mogelijkheden om de verworven kennis met betrekking tot de toegevoegde waarde van nieuwe diagnostische hematologische parameters te delen met medische professionals en zich verder te ontplooien in haar functie van locatiemanager en onderzoeker in het Laboratorium voor Klinische Chemie, Hematologie & Immunologie van het Medisch Centrum Alkmaar.



- Bartels PCM, van Houte AJ, Schoorl M. Discrepancies in current automated enumeration methods for reticulocytes. Sysmex J Intern 1993;3:75-79.
- van Houte AJ, Bartels PCM, Schoorl M, Mulder C. Methodology-dependent variations in reticulocyte counts using a manual and two different flowcytometric procedures. Eur J Clin Chem Clin Biochem 1994;32:859-863.
- Grooteman MPC, Nubé MJ, van Houte AJ, Schoorl M, van Limbeek J. Granulocytes sequestration in dialyzers: a comparative elution of three different membranes. Nephrol Dial Transplant 1995;10:1859-1864.
- 4. Bartels PCM, **Schoorl M**. Evaluation of the Sysmex SF-3000 automated haematology analyser. Sysmex J Int 1996;6:136-142.
- Bartels PCM, Schoorl M. Influence of storage conditions on results of monocyte counting on Sysmex NE-8000 and SE-9000 automated haematology analysers. Sysmex J Int 1996;6:143-146.
- Bartels PCM, Schoorl M. Mean Corpuscular Volume-dependent deviations in Red blood cell Distribution Width between Sysmex NE-8000 and SF-3000. Eur J Clin Chem Clin Biochem 1997;35:549-550.
- 7. Bartels PCM, **Schoorl M**, Willekens FLA. Evaluation of Sysmex SF-3000 performance concerning interpretive morphology flagging of the leucocyte differential count. Clin Lab Haem 1997;19:187-190.

- Bartels PCM, Schoorl M, Lombarts AJPF. Screening for EDTA-dependent deviations in platelet counts and abnormalities in platelet distribution histograms in pseudothrombocytopenia. Scand J Clin Lab Invest 1997;57:629-636.
- Grooteman MPC, Bos JC, Nubé MJ, van Limbeek J, Schoorl M, van Houte AJ. Mechanisms of intra-dialyser granulocyte sequestration: a sequential elution study. Nephrol Dial Transplant 1997;12:492-499.
- 10. Grooteman MPC, Nubé MJ, Daha MR, van Limbeek J, van Deuren M, Bet P, Schoorl M, van Houte AJ. Cytokine profiles during clinical high-flux dialysis: no evidence for cytokine generation by circulating monocytes. J Am Soc Nephrol 1997;8:1745-1754.
- 11. Bos JC, Grooteman MPC, van Houte AJ, **Schoorl M**, van Limbeek J, Nubé MJ. Low polymorphonuclear cell degranulation during citrate anticoagulation: a comparison between citrate and heparin dialysis. Nephrol Dial Transplant 1997;12:1387-1394.
- 12. Bartels PCM, **Schoorl M**. Time dependent increase of differential monocyte count on the Sysmex NE-8000. Clin Lab Haem 1998;20:165-168.
- 13. Bartels PCM, Schoorl M, **Schoorl M**, Wiering JGA, Nubé MJ. Activation of coagulation during treatment with haemodialysis. Scand J Clin Lab Invest 2000;60:283-290.
- 14. Grooteman MPC, Tellingen A, van Houte AJ, Bos JC, **Schoorl M**, van Limbeek J, Nubé MJ. Hemodialysis-in-



- duced degranulation op polymorphonuclear cells: no correlation between membrane markers and degranulation products. Nephron 2000;85:267-272.
- Schouten WEM, Grooteman MPC, van Houte AJ, Schoorl M, van Limbeek J, Nubé MJ. Effects of dialyzer and dialysate on the acute phase reaction in clinical bicarbonate dialysis. Nephrol Dial Transplant 2000;15;379-387.
- 16. Bartels PCM, **Schoorl M**. Evaluation of M6 leukemia progression with the Sysmex XE-2100. Sysmex J Int 2001;11:74-76.
- 17. Bartels PCM, Oomes J, **Schoorl M**, Schoorl M. Besturing en innovatie in een laboratoriumorganisatie met behulp van de Balanced Score Card methode. Ned Tijdschr Klin Chem 2002;27:141-144.
- 18. Van Tellingen A, Grooteman MPC, Schoorl M, Bartels PCM, Schoorl M, Van der Ploeg T, Ter Wee PM, Nubé MJ. Intercurrent clinical events are predictive of plasma C-reactive protein levels in hemodialysis patients. Kidney Int 2002;62:632-638.
- 19. Schouten WEM, Grooteman MPC, Schoorl M, van Houte AJ, Nubé MJ. Monocyte activation in peripheral blood and dialyzer eluates: phenotypic profile and cytokine release. Nephron 2002;91:646-653.
- 20. Bartels PCM, Schoorl M, **Schoorl M**, Nubé MJ. Deviations in coagulation activation due to treatment with different haemodialysis membranes. Scand J Clin Lab Invest 2003;63:417-424.

- Bartels PCM, Schoorl M, Schoorl M. Application of haemocytometry in combination with reticulocytes count and red cell zinc protoporphyrin for appropriate classification of microcytic anaemia. Sysmex J Int 2003;13:78-82.
- 22. Schoorl M, Bartels PCM, **Schoorl M**. Competentiemanagement als basis voor persoonlijke ontwikkelingsprofielen. Ned Tijdschr Klin Chem Labgeneesk 2004;29:293-294.
- 23. Bartels PCM, Schoorl M, **Schoorl M**. Activatie van stolling tijdens hemodialyse is afhankelijk van de wijze van antistolling. Ned Tijdschr Klin Chem Labgeneesk 2005;30:282-284.
- 24. Schoorl M, Schoorl M, Bartels PCM. Platelet activation and serotonin release during treatment with haemodialysis. Ned Tijdschr Klin Chem Labgeneesk 2006;31:236-238.
- Ven van de J, Schoorl M, Bartels PCM. Automated result interpretation in anemia testing using artificial neural network. Ned Tijdschr Klin Chem Labgeneesk 2006;31:230-231.
- Bartels PCM, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. Clin Lab 2006;52:107-114.
- 27. Schoorl, M, **Schoorl M**, Nubé MJ, Bartels PCM. Erythropoiesis activity, iron availability and reticulocyte hemoglobinization during treatment with hemodialysis and in subjects with uremia. Clin Lab 2006;52:621-629.

- 28. Gritters M, Grooteman MPC, Schoorl M, **Schoorl M**, Bartels PCM, Scheffer PG, Teerlink T, Schalkwijk CG, Spreeuwenberg M, Nubé MJ. Citrate anticoagulation abolishes degranulation of polymorphonuclear cells and platelets and reduces oxidative stress during haemodialysis. Nephrol Dial Transplant 2006;21:153-159.
- 29. Gritters M, Borgdorff P, Grooteman MPC, Schoorl M, Schoorl M, Bartels PCM, Tangelder GJ, Nubé MJ. Reduction in platelet activation by citrate anticoagulation does not prevent intradialytic hemodynamic instability. Nephron Clin Pract 2007;106:c9-16.
- 30. Schoorl M, **Schoorl M**, Bartels PCM. Changes in platelet volume, morphology and RNA content in subjects treated with haemodialysis. Scand J Clin Lab Invest 2008;68:335-342.
- 31. Gritters M, Borgdorff P, Grooteman MPC, Schoorl M, **Schoorl M**, Bartels PCM, Tangelder GJ, Nubé MJ. Platelet activation in clinical haemodialysis: LMWH as a major contributor to bioincompatibility? Nephrol Dial Transplant 2008;23:2911-2917.
- 32. **Schoorl M**, Schoorl M, Bartels PCM. Buiten winnen is binnen beginnen. Ned Tijdschr Klin Chem Labgeneesk 2008;33:204-205.
- 33. Gritters-van den Oever, Schoorl M, Schoorl M, Bartels PCM, Grooteman MPC, Nubé MJ. The role of the extracorporeal circuit in the trapping and degranulation of platelets. Blood Purif 2009;28:253-259.

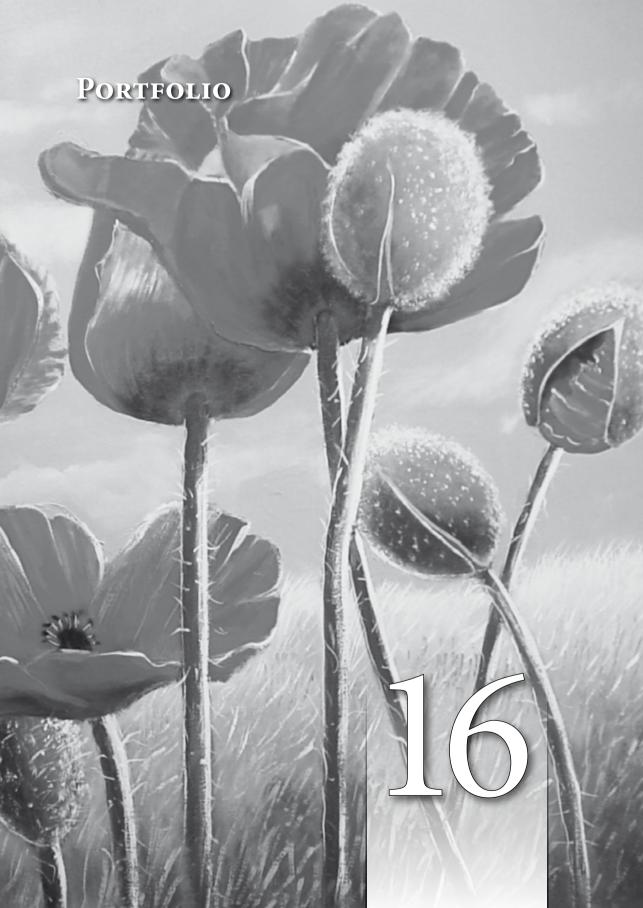
- 34. Gritters-van den Oever, Grooteman MPC, Bartels PCM, Blankestijn PJ, Bots ML, van den Dorpel MA, Schoorl M, Schoorl M, ter Wee PM, Nubé MJ. Post-dilution haemodiafiltration and low-flux haemodialysis have dissimilar effects on platelets: a side study of CONTRAST. Nephrol Dial Transplant 2009:24:3461-3468.
- 35. Schoorl M, van der Gaag D, Schoorl M, Bartels PCM. Changes in red blood cell hemoglobinization during pregnancy. Ned Tijdschr Klin Chem Labgeneesk 2010;35:206-208.
- Hooijberg JH, Schoorl M, Derkx S, van der Veer R, Bartels PCM. Een marketingplan als nieuwe dimensie voor laboratoriumgericht ondernemerschap. Ned Tijdschr Klin Chem Labgeneesk 2010;35:184-185.
- Hooijberg JH, Tegelaers FPW, IJzendoorn-Borst T van, Schoorl M, Goudriaan-Barendrecht J, Slaman-Schilders I, Bartels PCM. Validation-at-thepoint-of-care' bij vier hematologische POCT-methoden. Ned Tijdschr Klin Labgeneesk 2011;36:32-35.
- Hooijberg JH, Visser HPJ, Loonen AH, Schoorl M, Chevallier M, Ossenkoppele, Bartels PCM. Sea-blue histiocyten bij een patiënt met de ziekte van Kikuchi-Fujimoto. Ned Tijdschr Hematol 2011;8:86-90.
- 39. Schoorl M, **Schoorl M**, Nubé MJ, Bartels PCM. Platelet depletion, platelet activation and coagulation during treatment with hemodialysis. Scan J Clin Lab Invest 2011;71:240-247.



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- 40. Schoorl M, Schoorl M, Linssen J, Martinez Villanueva MM, Velasco NoGuera JA, Hernandez Martinez P, Bartels PCM. Efficacy of advanced discriminating algorithms for screening on irondeficiency anemia and β-thalassemia trait. Am J Clin Pathol 2012;138:300-304.
- 41. Snijders D, Schoorl M, Schoorl M, Bartels PCM, van der Werff TS, Boersma WG. D-dimer levels in assessing severity and clinical outcome in patients with community-acquired pneumonia. A secondary analysis of a randomised clinical trial. Eur J Intern Med 2012;23:436-441.
- 42. **Schoorl M**, Snijders D, Schoorl M, Boersma WG, Bartels PCM. Temporary impairment of reticulocyte haemoglobin content in subjects with community-acquired pneumonia. Int Jnl Lab Hem 2012;34:390-395.
- 43. **Schoorl M**, Schoorl M, van der Gaag D, Bartels PCM. Effects of iron supplementation on red blood cell hemoglobin content in pregnancy. Hematology Reports 2012;4:e24.
- 44. **Schoorl M**, Snijders D, Schoorl M, Boersma WG, Bartels PCM. Transient impairment of reticulocyte haemoglo-

- bin content and hepcidin-25 induction in patients with community-acquired pneumonia. Scan J Clin & Lab Invest 2013;73:54-60.
- 45. Korterink JJ, Boersma B, **Schoorl M**, Porcelijn L, Bartels PCM. Pseudothrombocytopenia in a neonate due to mother? Eur J Pediatr 2013;172:987-989.
- 46. **Schoorl M**. Author reply on comment Burdick C. Combined Iron deficiency and Thalassemia Minor. Am J Clin Pathol 2013;139:259-262.
- 47. Schoorl M, **Schoorl M**, Nubé MJ, Bartels PCM. Coagulation activation, depletion of platelet granules and endothelial integrity in case of uraemia and haemodialysis treatment. BMC Nephrology 2013:14:72.
- 48. **Schoorl M**, Schoorl M, Oomes J, van Pelt J. Evaluation of a new method for platelet counting (PLT-F) on the Sysmex XN2000 hematology analyser. Am J Clin Pathol 2013:140:495-499.
- 49. **Schoorl M**, Schoorl M, van Pelt J. Evaluation of het new StaRRsed Inversa 24M analyser. Ned Tijdschr Klin Chem Labgeneesk 2013;38:149-150.



# **PORTFOLIO**

Name: Margreet Schoorl

Period: January 2005 - January 2015

Promotor: Prof.Dr. A. Sturk

Copromotores: Dr. P.C.M. Bartels and Dr. J. van Pelt

### **TRAINING**

Activity	City	Year	Workload (ECTS)
Applied Biosystems: Basic Real Time PCR Training Course.	Alkmaar	2005	0,2
Dade Behring Trombose & hemostase training: Trombose.	Amersfoort	2007	0,2
Dade Behring Trombose & hemostase training: Bloeding.	Amersfoort	2007	0,3
NVC nascholing: The Almelo Conference.	Amsterdam	2010	0,1
NVC Workshop: Flowcytometrie in MDS.	Amsterdam	2011	0,1
Sysmex Expertclass hematologie.	Etten-Leur	2013	0,3
Sysmex Advanced training Sysmex XN serie:	Etten-Leur	2013	0,4
- meet- en detectieprincipes en flaggings.			
Sysmex Expert Class Bodyfluids.	Etten-Leur	2014	0,2
Nyenrode Business University:	Breukelen	2008	0,5
- Business Development & Blue Ocean Strategy: Collegereeks			
Nyenrode Business University:	Breukelen	2009	0,8
- Management in de zorg: Collegereeks			
Schouten & Nelissen: Leergang Procesmanagement:	Utrecht	2011	2,3
- Inzicht in processen; Procesanalyse en -optimalisatie;			
Resultaten bereiken met uw team			
Foreest Medical School: GCP-training	Alkmaar	2011	0,3
Foreest Medical School: Wetenschappelijk Engels schrijven	Alkmaar	2012	0,6

# **CONGRESSES**

Activity	City	Year (ECTS)	Workload
ISLH Congress: - Technical Innovations in Laboratory Hematology.	Amsterdam	2006	0,7
Sysmex European Congress.	Lissabon	2007	0,6
Sysmex Congress:	Lund	2008	0,5
<ul> <li>Intelligent image analysis, tele-haematology and automatic dataflow in a modern haematology laboratory.</li> </ul>			•
NVKC Voorjaarssymposium.	Veldhoven	2008	0,3
VHL symposium:	Zwolle	2009	0,2
- Hemolyse			
Sysmex European Haematology Congress.	Istanbul	2009	0,4
ISLH Hematology Congress.	Brighton	2010	0,9
NVML congres:	Amersfoort	2010	0,2
- 'De Juiste Zet' het leveren van kwaliteit in de zorg.			
NVC congres:	Zwolle	2011	0,2
- Cytometrie en Kwaliteit (SIHON 25 jaar).			
IFCC Euromedlab Congress.	Berlijn	2011	0,6
NVC congres:	Zwolle	2012	0,3
- Kwaliteit en ontwikkeling in de flowcytometrie.			
16e Czech en Slovak Congress of Hematology and Medicine.	Brno	2012	0,3
ISLH Congress Hematologie Congres.	Nice	2012	0,9
Sysmex Scientific Seminar.	Dubai	2013	0,6
Emirates Hematology Congress.	Dubai	2013	0,4
SKML congres:	Ede	2013	0,3
- De juiste score.			
INK-najaarssymposium: de wisseling der seizoenen.	Sint- Michelsgeste	2014 el	0,3
NVC congres:	Zwolle	2014	0,3
- Kwaliteit en ontwikkeling in de flowcytometrie.			
ISLH Congress Hematologie Congres.	Den Haag	2014	0,6

# **CONFERENCES & MEETINGS**

Activity	City		Workload (ECTS)
SIHON najaarsvergadering.	Woerden	2005	0,1
Sysmex Integrated Laboratory Solutions.	Brussel	2005	0,3
SIHON voorjaarsvergadering.	Woerden	2005	0,1
Sysmex XE-2100 gebruikersdag.	Vught	2005	0,3
SIHON najaarsvergadering.	Woerden	2006	0,1
NVC nascholing: The Almelo conference.	Amsterdam	2006	0,1
Sysmex Haematology Quality management and	Utrecht	2006	0,2
new laboratory parameters.			
SIHON voorjaarsvergadering.	Woerden	2006	0,1
Instrumentation Laboratory:	Utrecht	2007	0,2
- ACL TOP gebruikersdag.			
Werkconferentie: een goede relatie met de klant.	Heerhugo- waard	2007	0.2
Werkconferentie: buiten winnen is binnen beginnen.	Alkmaar	2007	0,2
Sysmex X-Class gebruikersdag.	Leiden	2008	0,2
ECAT Foundation: 6th ECAT participants meeting.	Oegstgeest	2008	0,4
SIHON najaarsvergadering	Woerden	2008	0,2
NVC nascholing: The Almelo conference.	Amsterdam	2008	0,1
Beckman Coulter:	Nijmegen	2008	0,2
<ul> <li>Workshop Flowcytometry Microparticles.</li> </ul>			
Instrumenation Laboratory:	Utrecht	2008	0,3
- Wetenschappelijke dag Haemostasis.			
Instrumentation Laboratory: ACL TOP gebruikersdag.	Utrecht	2009	0,2
VUMC Cancer Center Amsterdam:	Warmen-	2009	0,3
- Symposium Nutrition and Cancer.	huizen		
Werkconferentie: marketing & ondernemerschap,	Heerhugo-	2009	0,1
kansen zienkansen pakken	waard		
NVKC Voorjaarssymposium.	Veldhoven	2009	0,2
Beckman Coulter:	Rotterdam	2010	0,2
- flowcytometriedag.			
SIHON voorjaarsvergadering.	Woerden	2011	0,2
Sysmex Hematologie gebruikersdag.	Utrecht	2011	0,3
Instumentation Laboratory ACL TOP gebruikersdag.	Utrecht	2011	0,2
Instrumentation Laboratory ACL TOP Stollings Seminar.		2012	0,2
Pathfast:	Milaan	2013	0,1
- workshop Presepsin.			
ECAT Foundation:	Oegstgeest	2014	0,3
- 9th ECAT participants meeting.			
SIHON voorjaarsvergadering.	Woerden	2014	0,2
Sysmex Hematologie gebruikersdag.	Hilversum	2015	0,2



# **ORAL PRESENTATIONS**

Activity	City	Year	Workload (ECTS)
Sysmex gebruikersdag. Beslisbomen: Facts or Feelings?	Vught	2005	0,5
Sysmex seminar. Klaar voor de start?	Utrecht	2006	0,5
Medisch Centrum Alkmaar.	Alkmaar	2006	0,5
- De professional van de toekomst: een leven lang leren en ontwikkelen!			
Instrumentation Laboratory ACL-TOP gebruikersdag.	Utrecht	2007	0,5
- Klaar voor de start?			
Sysmex X-Class gebruikersdag.	Leiden	2008	0,5
- Ret-He: wat kun je ermee?			
NVML Nascholing Hematologie. Anemie diagnostiek.	Nederland, 2x	2009	1,0
Sysmex Hematologie gebruikersdag.	Utrecht	2011	0,5
- Trainingsschema voor beginners: validatie van apparatuur.			
Regionale opleidingsdag voor KC-io.	Alkmaar	2011	0,5
- Pre-analytische aspecten bij stollingsonderzoek.			
16e Czech and Slovak Congress of Haematology	Brno	2012	0,5
and Medicine.			
- Evaluation of the new XN haematology analyser.			
Sysmex International Seminar.	Dubai	2013	0,5
- Evaluation of a new method for low platelet			
counting (PLT-F) on the Sysmex XN2000			
haematology analyser.			
Sysmex Hematologie Gebruikersdag.	Hilversum	2015	0,5
- Scattergrammen in beeld; casuïstiek voor			
X-class en XN-analyzers.			

# POSTER PRESENTATIONS

Activity	City	Year	Workload (ECTS)
NVKC Voorjaarscongres. Van de Ven J, <b>Schoorl M</b> , Bartels PCM.: Automated result interpretation in anemia testing using artificial neural networks.	Lunteren	2006	0,5
NVKC Voorjaarscongres. Schoorl M, Bartels PCM, <b>Schoorl M</b> .: Platelet activation and serotin release during treatment with haemodialysis.	Lunteren	2006	0,5
NVKC Voorjaarscongres. Smets EML, Oomes J, Chevallier M, <b>Schoorl M.</b> : Detectie van het HLA-B27 allel met het Applied Biosystems 7500 Real-time PCR System.	Lunteren	2006	0,5
ATS International Conference.  Schoorl M, Schoorl M, Snijders D, Boersma WG, Bartels PCM.: Acute impairment of reticulocyte haemoglobinization in subjects with community-acquired pneumonia.	San Francisco	2007	0,5
Sysmex European Congress. Schoorl M, <b>Schoorl M</b> , Bartels PCM.: Changes of platelets in morphology and RNA content during treatment with haemodialysis.	Lissabon	2007	0,5
NVKC Voorjaarscongres.  Schoorl M, Schoorl M, Bartels PCM.: Acute impairment of red blood cell haemoglobinisation in subjects with community-acquired pneumonia.	Lunteren	2008	0,5
NVKC Voorjaarscongres. Schoorl M, Koppes MA, <b>Schoorl M</b> , Snijders D, Boersma WG, Bartels PCM.: <i>Analytical evaluation of</i> <i>Vidas Procalcitonin versus Kryptor</i> <i>Procalcitonin sensitive assay.</i>	Lunteren	2008	0,5
NVKC Voorjaarscongres. <b>Schoorl M</b> , Schoorl M, Bartels PCM.: <i>Buiten winnen is binnen beginnen</i> .	Lunteren	2008	0,5



NVKC Voorjaarscongres. <b>Schoorl M</b> , Gaag D van der, Bartels PCM.: Changes in red blood cell haemoglobinization during pregnancy.	Lunteren	2010	0,5
NVKC Voorjaarscongres.  Schoorl M, Schoorl M, Bartels PCM.: Analytical validation of Biophen Heparin 3 versus HemosIL® Liquid Heparin assay on the ACL-TOP® analyser.	Lunteren	2010	0,5
NVKC Voorjaarscongres. Hooijberg JH, <b>Schoorl M</b> , Derkx S, van der Veer R, Bartels PCM.: <i>Een marketingplan als nieuwe dimensie</i> voor laboratoriumgericht ondernemerschap.	Lunteren	2010	0,5
Symposium Nederlandse Vereniging voor bloedtransfusie. Korterink JJ, Boersma B, <b>Schoorl M</b> , Porcelijn, Bartels PCM.: Het EDTA-fenomeen bij moeder en kind; een zeldzame verklaring voor passagère pseudo-trombocytopenie bij de pasgeborene.	Ede	2011	0,5
<ul> <li>NVKC Wetenschapsdag.</li> <li>ISLH Congress.</li> <li>Nordic Congress in Clinical Chemistry.</li> <li>Hematology Conference.</li> <li>Schoorl M, Chevallier M, Schoorl M, van Pelt J.:</li> <li>Evaluation of the new Sysmex XN2000 haematology analy</li> </ul>	Amersfoort Nice Reykjavic Dubai	2012 2012 2012 2013	0,5
<ul> <li>NVKC Wetenschapsdag.</li> <li>ISLH Congress.</li> <li>Nordic Congress in Clinical Chemistry.</li> <li>Hematology Conference.</li> <li>Schoorl M, Oomes J, Schoorl M, van Pelt J.:</li> <li>Low platelet count evaluation on the new Sysmex XN2000 haematology analyser.</li> </ul>	Amersfoort Nice Reykjavic Dubai	2012 2012 2012 2012 2013	0,5
NVKC Voorjaarscongres. <b>Schoorl M</b> , Schoorl M, van Pelt J.: <i>Evaluation of het new StaRRsed Inversa 24M analyser</i> .	Velthoven	2013	0,5
6th European Haematology Symposium.  Martinez-López de Castro A, Martinez-Villenueva M, Noguera-Vélasco JA, <b>Schoorl M</b> , De Miquel-Elizaga I, Cebreiros-López I, Ramirez-Ruiz C, Martinez-Hernánde Clinical utility of Case Manager software provided by the Sysmex XE-5000 analyzer for the screening of microcytic a		2013	0,5

# **TEACHING**

Mentor Biomedical Sciences	Student	Year	Workload (ECTS)
Evaluatie research-parameter voor trombocyten (PLT-X) bij MDS.	HLO	2014	3,5
Novel haematological parameters in the evaluation of anaemia in order to predict dysplasia in bone marrow.	BSc	2014	8,5
Hemocytometrie.	HLO	2013	6,0
Flagging performance van de leukocyten differentiatie van de nieuwe Sysmex XN2000 hematologie analyzer.	BSc	2012	5,0
Evaluatie van de Sysmex XN2000 hematologie analyzer.	HLO	2012	4,0
Evaluatie van de StaRRsed Inversa 24M bezinkingsanalyzer.	HLO	2012	3,0
Bruikbaarheid van nieuwe hematologische parameters voor anemie screening bij zwangeren.	MLO	2011	4,0
Kleihauertest.	MLO	2011	3,5
Anemie bij zwangeren.	BSc	2010	5,0
Onderlinge vergelijkbaarheid van D-dimeren m.b.v. ACL-TOP analyzer en mini-Vidas.	HLO	2009	3,5
IJzerutilisatie bij chronische nierinsufficiëntie.	HLO	2008	4,0
Transfix-reagens t.b.v. flowcytometrische stabiliteit van PLT-activatiemarkers.	HLO	2006	3,5

