Ned Tijdschr Klin Chem Labgeneesk 2016; 41: 6-16

Innovative haematological parameters in clinical practice

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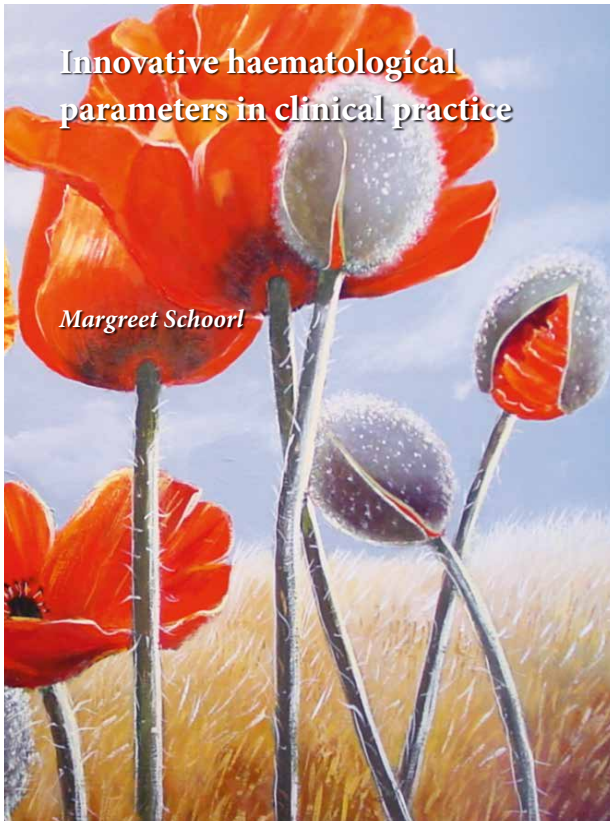
1. Introduction

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 (1). 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 (2).

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 (3-6). 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 (7).

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% (8).

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 (1).



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 µm 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 x10^9 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 (9).

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).

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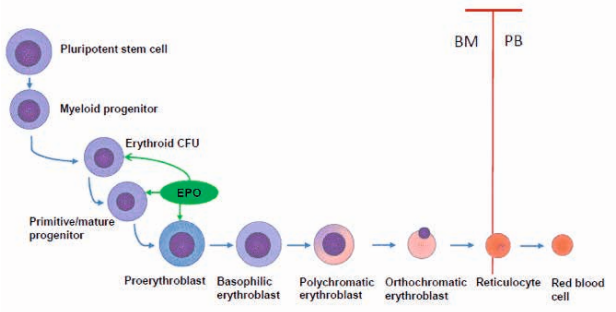


Figure 1. Maturation of red blood cells.
Abbreviations: BM = bone marrow; PB = peripheral blood; EPO = erythropoietin; CFU = colony forming unit

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).

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

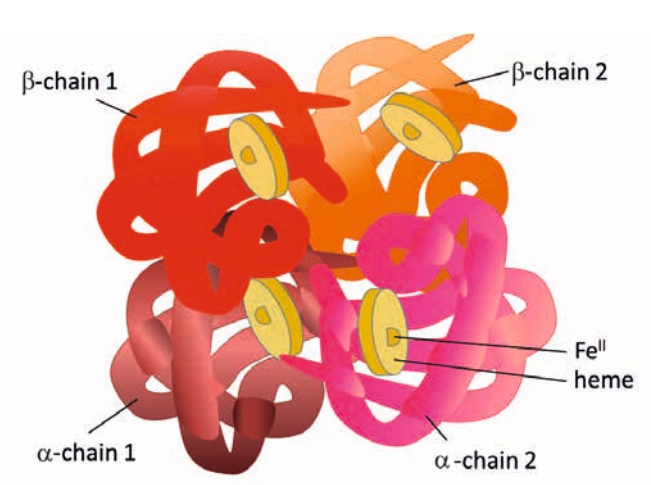
Normal adult haemoglobin is composed of four polypeptide chains: two α-chains and two β-chains, which together form a tetramer ($\alpha_2\beta_2$). Each chain is folded around a haem group, consisting of a porphyrin ring and an atom of iron (Figure 2). Iron is an essential component of haem, the iron-porphyrin 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_2 (2-3%) and HbF (0-1%) are also present. HbA_2 is a tetramer of two α-chains and two δ-chains ($\alpha_2\delta_2$). HbF is a tetramer of two α-chains and two γ-chains ($\alpha_2\gamma_2$) (12).

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

α-thalassaemias are the most prevalent disorders of haemoglobin synthesis (13). The α-globin genes α_1 and α_2 are situated in a linked cluster on the short arm of chromosome 16. Deletions or mutations in one to four of the α genes result in reduced or even absent globin chain production. The resulting aberrations involve various degrees of imbalance between α- and β-chain synthesis. Subjects can be classified according to haematological, biochemical and molecular criteria. Six genotypes are distinguished:



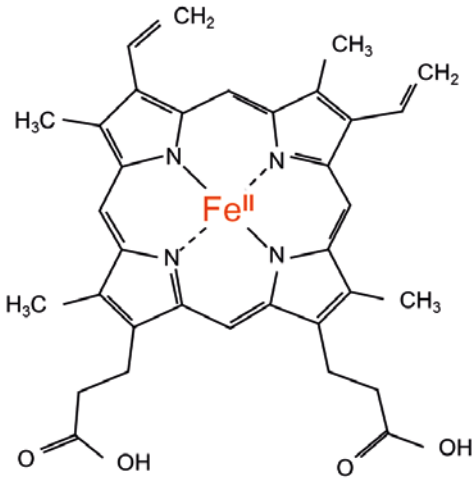


Figure 2. Normal haemoglobin molecule (haemoglobin A), showing two α and two β chains (left) and normal haemoglobin structure (right). Modified from the Internet Encyclopedia of Science.

1 Normal genotype, with four α-globin genes: αα/αα
2 α-thalassaemia 2 heterogosity, with three functional α-globin genes: αα/-α
3 α-thalassaemia 1 heterogosity, with two functional α-globin genes: αα/--
4 α-thalassaemia 2 homozygosity, with two functional α-globin genes: -α/-α
5 α-thalassaemia 1 and α-thalassaemia 2 double heterogosity, or Hb H-disease, with one functional α-globin gene: --/-α
6 α-thalassaemia 1 and homozygosity or Hb Bart's hydrops, with no functional α-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 α-thalassaemia 2 heterozygosity, (also called α-thalassaemia carrier state) are characterised by normal to moderately decreased mean cell volume (MCV) values with normal or slightly decreased haemoglobin concentrations (15, 16). Subjects with homozygous α-thalassaemia 2 and heterozygous α-thalassaemia 1 (both indicated as α-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 (17).

3.1.2 β*-thalassaemia*

The β-like globin genes are arranged in a cluster on the short arm of chromosome 11. Mutations causing β-thalassaemia result in a lack of β-globin production, which ranges from minimal (mild β^+-thalassaemia alleles) to a complete absence (β^0- alleles). β-Thalassaemia is an extremely heterogeneous condition. More than 40 different lesions of the β-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 β-thalassaemia (β-thalassaemia minor) are clinically asymptomatic. Almost all heterozygous conditions (except the so-called normal HbA_2-thalassaemias, e.g. δβ-thalassaemia), are characterised by an increased HbA_2 content (≥ 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 δ-chain synthesis.

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

Red blood cells have a shortened life-span due to precipitated α-chain inclusions. Haemoglobin in the red blood cells consists of HbF and a small fraction of HbA_2, varying from 1-3% in β^0- thalassaemia to 7% in β^+-thalassaemia (17).

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^{Punjab}, 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 β^A gene and one affected β gene, for instance β^S and β^C. HbS is the most common Hb variant, frequently indicated as HbS/C compound heterozygosity, HbS/β-thalassaemia 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 (14). 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 (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 (19).

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.

Table I. Estimated percentage of residents carrying a significant haemoglobin gene variant. Modified from Modell B. et al (18).

			% of population carrying a significant variant						
Region	Country	Residents 2003	AC	AS	AE	AD etc	beta-thal	alfa-thal	total%
Northern Europe	Norway / Sweden / Denmark	19.087.200	0.01	0.08	0.07	0.03	0.22	0.02	0.43
	England / Wales	53.125.596	0.13	0.46	0.06	0.18	0.45	0.07	1.35
Western Europe	Netherlands	15.810.000	0.10	0.47	0.07	0.03	0.42	0.01	1.10
	Belgium / Luxemburg	10.720.350	0.02	0.18	0.01	0.03	0.36	0.02	0.62
	France	59.768.000	0.10	0.60	0.15	0.03	0.42	0.02	1.32
	Germany	82.087.000	0.01	0.05	0.02	0.03	0.24	0.01	0.36
	Austria / Switzerland	15.093.081	0.01	0.08	0.03	0.03	0.24	0.02	0.41
Southern Europe	Italy	57.695.000	0.01	0.22	0.006	0.03	3.33	0.01	3.61
	Spain	40.847.371	0.03	0.09	0.002	0.03	0.25	0.01	0.41
	Portugal	10.356.100	0.01	0.57	0.002	0.03	0.45	0.003	1.07
	Greece	11.783.586	0.002	0.53	0.004	0.03	6.29	0.51	7.34
	Cyprus	721.000		1.00		0.03	15.00	1.00	17.03
Eastern Europe	Bulgaria	7.824.000				0.03	3.00		3.03
	Romania	21.734.000				0.03	1.00		1.03

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 (20).

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* (17).

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 (2). In the Netherlands, the incidence of iron-deficiency anaemia in the general practice is 4.3 per 1000 patients per year (21).

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 (22).

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 (23).

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 (23, 24).

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) (25, 26).

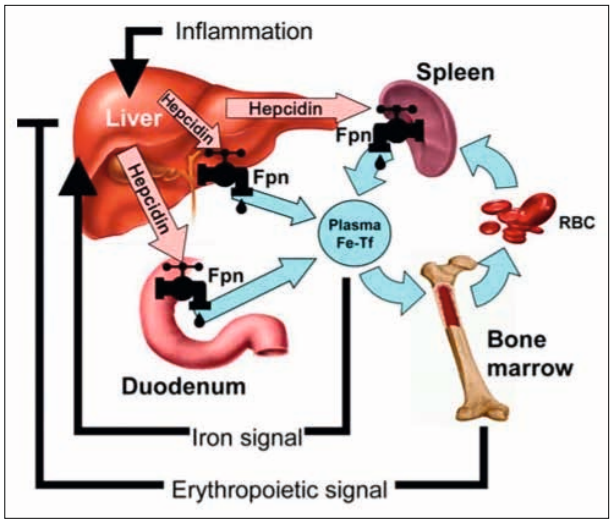


Figure 3. The role of hepcidin in iron metabolism.
Hepcidin - ferroportin interaction determines the flow of iron into plasma. The hepcidin concentration is in turn regulated by iron, erythropoietic activity and inflammation.
Abbreviations: RBC = red blood cells; Fpn = ferroportin; Fe-Tf = iron-transferrin
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 (11).

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 (19).
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 (21).

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 (13, 14).
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 (17).

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 (29-31). Normal and increased levels of transferrin saturation are more useful for excluding iron deficiency than decreased values are for identifying it (32).

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 µg/L are indicative of iron deficiency and of a lack of stainable iron in the bone marrow (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 (11, 19, 34). However, the sTfR concentration is increased by enhanced erythropoiesis in case of haemolytic anaemia and thalassaemia (19, 35, 36). The clinical utility of sTfR measurements is also restricted by the lack of standardization and availability of various antigens being used for the assay (37). 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 (11, 38, 39).

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 (20).
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 (40-43).
ZPP reflects a *long term* impression in accordance with the lifespan of red blood cells (44). 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 (45).

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 (45).

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) (45).

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 (45).

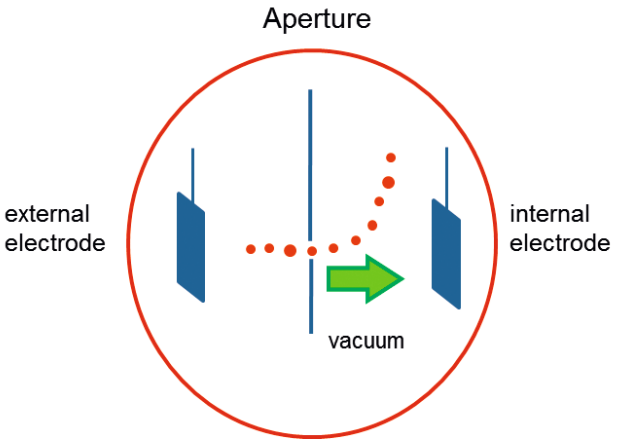


Figure 4. The Coulter principle for counting and sizing cells. Provided by Sysmex Europe, Hamburg, Germany

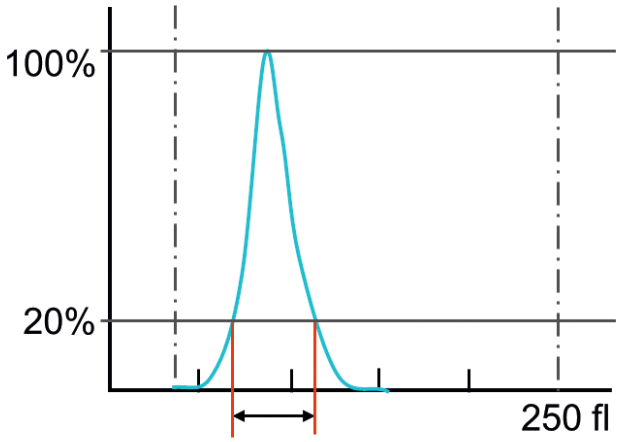


Figure 5. Red blood cell histogram.
Determination of the RDW-SD is an actual measurement of the width of the red blood cell distribution curve. The measurement is performed at a relative height of 20% above the baseline, indicated by the red lines. The narrower the curve is spread by red blood cells of different sizes, the lower the RDW-SD value will be.
Provided by Sysmex Europe, Hamburg, Germany.

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 (48).

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 (45).

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 (11).

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.

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.

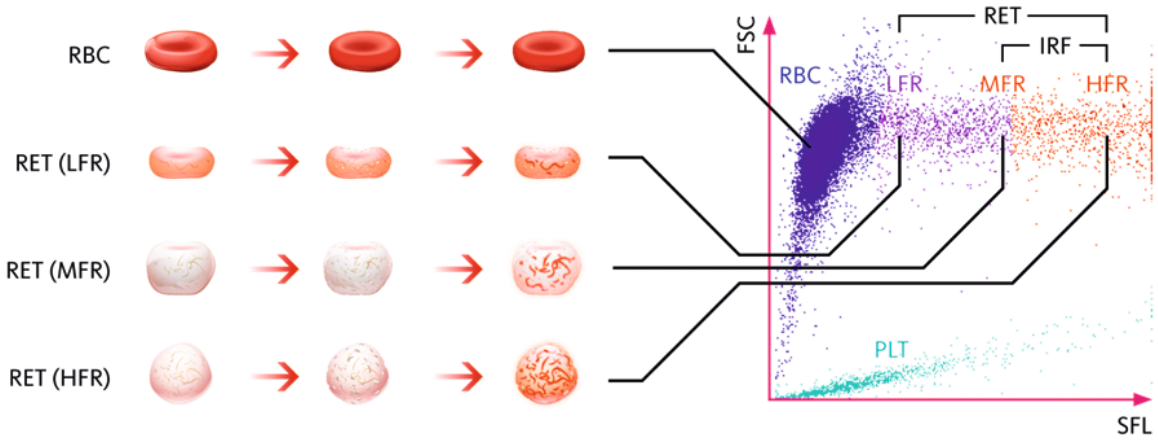


Figure 6. Indication of the location of the reticulocyte fractions (LFR, MFR, HFR) in the reticulocyte channel of the Sysmex XE-2100 Haematology analyser. IRF (MFR+HFR) yield higher fluorescent intensity.
Provided by Sysmex Europe, Hamburg, Germany.
Abbreviations: RBC = red blood cells; RET = reticulocytes; 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 (49-53). 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 (55). The difference between these two parameters is expressed as Delta-He (Figure 7).

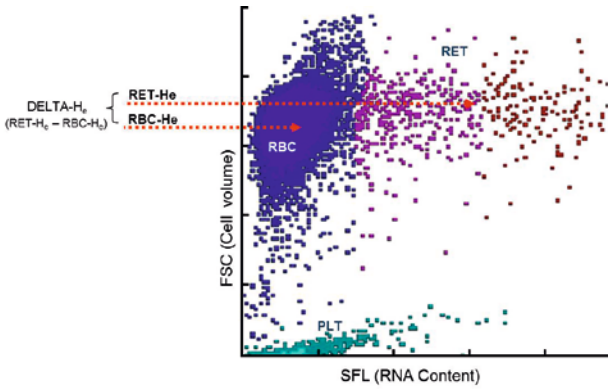


Figure 7. Indication of the location of RET-He and RBC-He in the reticulocyte channel of the Sysmex XE-2100 Haematology analyser. The FSC intensity in the reticulocyte channel reflects the volume and haemoglobin content of red blood cells and reticulocytes.
Provided by Sysmex Europe, Hamburg, Germany.
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, 58-60). 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, 61-66). Multivariant discriminant analysis with algorithms including MCV, MCH, RBC and RDW has proven to be useful for the differential diagnosis of α- or β-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 α- or β-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 pro-inflammatory 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 β-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 β-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.

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