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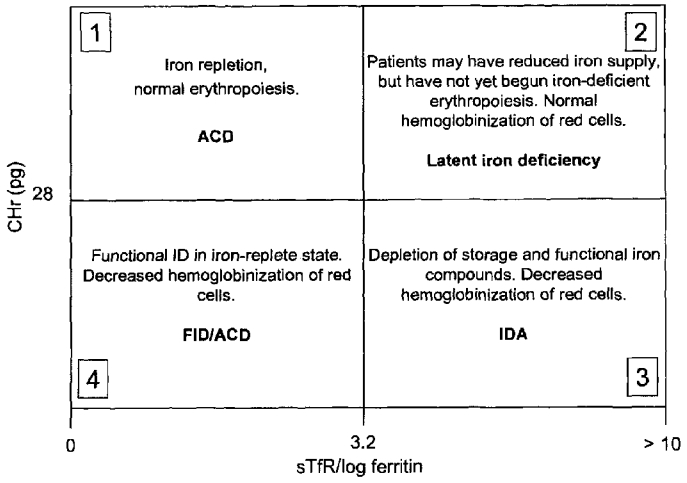
(54) **Title**: DIFFERENTIAL DIAGNOSIS OF IRON DEFICIENCIES BASED ON HEPCIDIN AND MEAN HEMOGLOBIN CONTENT PER RETICULOCYTE

Figure 1/4



(57) **Abstract**: The present invention relates to the technical field of the diagnosis of disorders of the iron metabolism. The invention concerns a method for diagnosing or distinguishing, specific stages in the evolution of iron deficiency ranging from excessive storage iron to advanced iron deficiency in an anemia patient by means of two parameters, preferably by a) determining the amount of hepcidin or a variant thereof in a sample from the patient; b) determining the mean hemoglobin content per reticulocyte in a sample from the patient; c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount; d) comparing the value determined in step b) to a reference value; and e) diagnosing or distinguishing if the patient suffers from ACD, from IDA or from FID/ ACD or optionally from latent iron deficiency.

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DIFFERENTIAL DIAGNOSIS OF IRON DEFICIENCIES BASED ON HEPCIDIN AND MEAN HEMOGLOBIN CONTENT PER RETICULOCYTE

The present invention relates to the technical field of the diagnosis of disorders of the iron metabolism. The invention concerns a method for diagnosing or distinguishing, specific stages in the evolution of iron deficiency ranging from excessive storage iron to advanced iron deficiency in an anemia patient by means of two parameters. The differential diagnosis can be used to for deciding on a suitable treatment for the stratified patients. The patients may suffer from anemia of chronic disease (ACD), from iron deficiency anemia (IDA) or from anemia of chronic disease with functional iron deficiency (FID/ACD) or optionally from latent iron deficiency. Patients with ACD are in iron-replete state, patients with IDA are in iron-depleted state and patients with FID/ACD have iron-deficient erythropoiesis in iron-replete state. In functional iron deficiency (FID) there is a failure to provide the bone marrow with iron despite replete iron stores. In the stage of latent iron deficiency iron stores were depleted recently but the bone marrow has not yet begun iron-deficient erythropoiesis.

Iron metabolism is a set of chemical reactions maintaining the homeostasis of iron. In the human body, iron is present in virtually all cells and is involved in numerous vital functions. E.g. it serves as a carrier of oxygen to the tissues from the lungs in the form of hemoglobin, as a transport medium for electrons within the cells in the form of cytochromes, and as an integral part of enzyme reactions in various tissues. Hence the regulation of iron is an important part of many aspects of human health.

Disturbances of the iron metabolism can lead to different diseases depending on whether they are caused by iron deficiency or iron overload. Iron deficiency ranges from latent iron depletion, which yields little physiological damage, to IDA, which can affect the function of numerous organ systems.

IDA occurs when the dietary intake or absorption of iron is insufficient, and hemoglobin, which contains iron, cannot be produced. IDA is the most common type of anemia.

Yet another anemia is ACD, which is a form of anemia seen in chronic illness, e.g. chronic infection, autoimmune diseases with inflammation, malignancy or end stage renal failure.

Another form is FID/ACD, which is a combined state of iron-restricted erythropoiesis with the ACD. The anemia is generally mild to moderate [preferably a hemoglobin (Hb) of about 100 g/L] (Means RT Jr, et al. Blood 1992; 80: 1639-47) but may deteriorate through independent processes like bleeding and cause iron deficiency and iron-restricted erythropoiesis, which is also named FID. According to Cavill (Blood 1993; 82: 1377) FID is the result of a failure of rate of delivery of iron through the plasma transferrin pool to the proliferating erythroblast. In the combined state of FID and ACD (FID/ACD) there is a failure to provide iron to erythroblasts because of reduction of the plasma transferrin pool and reduced release of iron from replete iron stores. (Thomas C, et al. Lab Hematol 2005; 11: 14-23). During inflammation hepcidin production leads to reduced iron release from the stores.

So far, it has been difficult to diagnose or distinguish the following iron metabolism related diseases and conditions in anemia patients from each other: a) ACD, b) IDA, and c) FID/ACD, and optionally also d) latent iron deficiency. So far, differential diagnosis was difficult to reach and required elaborate and diligent examination by an experienced medical practitioner. The methods available in the prior art for determining parameters involved in the iron metabolism merely served as a support of the practitioner for the diagnosis.

The so called sTfR/log ferritin index (sTfR-F index) is a well established measure of the depletion of the iron stores and of the body's functional iron compartments (Punnonen K, et al. Blood 1997; 89: 1052-7). Ferritin is an intracellular protein for storing iron within the cell. Iron is transported in the plasma by transferrin, which donates iron into the cells through its action with the transferrin receptor (TfR), a specific membrane receptor. A soluble form of the receptor (sTfR) circulates in the plasma in the form of a complex of transferrin and its receptor. The plasma/serum concentration of the sTfR is dependent on the marrow erythropoietic activity. sTfR levels are increased when erythropoiesis is hyper regenerative and decreased in situations characterized by diminished erythropoietic activity (Thomas. Clinical Laboratory Diagnostics, Textbook, First edition (1998)). sTfR levels are also influenced by the iron status (Baynes R. Clin Biochem 1996; 29: 209-15).

On the basis of the sTfR assay, a sTfR/log ferritin index of less than 1 suggests ACD whereas a ratio of more than 2 suggests FID/ACD according to Weiss et al. (N Engl J Med 2005; 352:1011-23). According to Thomas et al (Clin Chem 2002; 48: 1066-76) the sTfR/log ferritin index is dependent on the inflammation status and in combination with the reticulocyte hemoglobin content (CHr), an indicator of red cell hemoglobinization, the sTfR/log ferritin index is used to identify FID in iron-replete states.

EP 1 425 589 A2 discloses a method for differential diagnosis and monitoring iron deficiency comprising the determination of ferritin, sTfR and CHr, particularly aiming at a distinction from other disorders of iron metabolism such as ACD.

WO 2008/011158 A2 proposes a patient stratification scheme in the form of a decision tree in order to discriminate anemic patients in those with IDA, ACD and FID/ACD. The discrimination is based on the determination of hepcidin and offsetting the determined values of sTfR and ferritin against one another in terms of the sTfR/log ferritin index. In addition, it is necessary to evaluate the inflammatory status of the patient.

Thus, the hitherto known methods require that at least four parameters have to be assessed in order to distinguish IDA from ACD and FID/ACD or they require the offset of two parameters against one another which renders the methods quite cumbersome and prone for errors.

Current understanding of the regulation of iron metabolism in IDA, ACD and FID/ACD is based on a number of critical soluble proteins, including ferritin, transferrin, sTfR and hepcidin regulating intracellular and extracellular iron metabolism (Deicher R et al. Eur J Clin Invest 2006; 36: 301-9).

Hepcidin, a peptide produced in the liver, distributed in the plasma and excreted in urine is a regulator of intestinal iron absorption and iron recycling by macrophages (Hentze, Cell 2004; 117:285). The peptide regulates intestinal iron absorption, iron mobilization from hepatic stores and iron recycling by macrophages (Nicolas G, et al. J Clin Invest 2002; 110: 1037-44). Hepcidin controls cellular iron efflux on binding to iron export protein ferroportin. Inflammation induces hepcidin production and leads to internalization and degradation of ferroportin, which is present on the cell membrane of enterocytes and macrophages. Thus, an increase in hepcidin leads to a decrease in dietary iron absorption and an increase in macrophage iron. These changes are typically seen in inflammation and lead to decreased circulating iron and disturbances in iron distribution of the body (Fleming ER. J Mol Med 2008; 86:491-4). Hepcidin, a disulfide-rich peptide, is produced by hepatocytes as an 84-amino-acid pre-prohepcidin and subsequent posttranslational processing results in the biologically active 25-amino-acid form (hepcidin-25) that is secreted in the plasma (Park CH et al. J Biol Chem 2001; 276: 7806-10).

It is the object of the present invention to provide a method for diagnosing or distinguishing, in an anemia patient, if the patient suffers from IDA, ACD or FID/ACD, or optionally from latent iron deficiency which method avoids at least some of the shortcomings of the hitherto

known methods. Preferably, it is an object to provide a simple and effective method, more preferably a method which allows for a specific and sensitive differential diagnosis or detection of IDA, ACD or FID/ACD, and optionally latent iron deficiency.

Differentiation between IDA, ACD and FID/ACD is clinically important because erythropoiesis-stimulating agents (ESA) therapy, like EPO administration, may be beneficial for ACD and FID/ACD patients but may be deleterious for IDA patients, especially if these patients suffer from certain malignancies. The (sTfR)/log ferritin index may be useful in distinguishing ACD from FID/ACD, but the ratio is dependent on the inflammatory status of the patient. New markers and parameters, which accurately indicate the need for iron and for erythropoiesis without requiring knowledge of the inflammatory status, are clearly needed.

It is therefore yet another object to provide a method of deciding on the therapy for a patient suffering from IDA, ACD or FID/ACD, or optionally from latent iron deficiency, which preferably does not require knowledge of the inflammatory status, so as to allow the clinical physician to determine a suitable therapy for a patient based on the differential diagnosis reached by way of the method of the present invention.

Furthermore, it is an object to provide a method of monitoring a therapy of an anemia patient suffering from IDA, from ACD or from FID/ACD, or optionally from latent iron deficiency.

It is also an object to provide a device, a kit and a computer program adapted for carrying out the methods of the present invention.

At least some of these objects are accomplished by the provision of the subject matter defined in the claims and herein below.

In a first aspect it is provided a method for diagnosing or distinguishing, in an anemia patient, if the patient suffers from IDA, ACD or FID/ACD, or optionally from latent iron deficiency, the method comprising, the steps of

a) determining the amount of hepcidin or a variant thereof in a sample from the patient;

b) determining a value selected from the mean hemoglobin content per reticulocyte or the the hemoglobin concentration of reticulocytes in a sample from the patient;

c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount;

d) comparing the value determined in step b) to a reference value; and

e) diagnosing or distinguishing if the patient suffers from IDA, from ACD or from FID/ACD or optionally from latent iron deficiency.

Use of a means for detecting hepcidin (preferably an anti-hepcidin antibody) for diagnosing or distinguishing, in an anemia patient, if the patient suffers from IDA, from ACD or from FID/ACD, or optionally from latent iron deficiency, wherein the diagnosis or distinction is based on the detection of hepcidin and the determination of the value of the value of the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes in a sample from the patient.

Surprisingly, it has been found that the above differential diagnosis and distinction of the different forms of anemias can be achieved by using only two independent parameters, i.e. hepcidin and the value of the mean hemoglobin content per reticulocyte. The method of the invention allows for a surprisingly specific and sensitive differential diagnosis of the above diseases and conditions as exemplified in the Examples. Moreover, using the method of the present invention it is not necessary to evaluate the inflammatory status of the patient.

Preferably, the method of the present invention is an ex vivo or in vitro method.

According to a preferred embodiment of the present invention no additional markers or parameters are determined apart from the two mentioned in steps a) and b) such as an inflammation marker like interleukin 6 or C-reactive protein.

Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate to sample pre-treatments or evaluation of the results obtained by the method. The method of the present invention may be also used for monitoring, confirmation, sub classification and therapeutic monitoring the diseases of the present invention (IDA, ACD or FID/ACD, or optionally latent iron deficiency). It is also envisioned that in a preferred embodiment at least one additional marker or parameter may be determined apart from the two mentioned in steps a) and b) but mostly or only for the purpose of obtaining additional diagnostic information beyond the differential diagnosis IDA, ACD or FID/ACD and optionally latent iron deficiency. Such additional parameter may for example be the estimated glomerular filtration rate which allows to additionally diagnose if the patient suffers from an impaired renal clearance or determination of the amount of an inflammation marker like interleukin 6 or C-reactive protein.

The method may be carried out manually and/or assisted by automation. Preferably, step (a), (b), (c), (d) and/or (e) may in total or in part be assisted by automation, e.g., by a suitable

robotic and sensory equipment for the determination in step (a) and/or (b) or a computer-implemented comparison in step (c) and/or (d).

The term "patient" as used herein relates to animals, preferably mammals, preferably dogs, cats, horses, cattle and most preferably humans, preferably men and women.

The term "anemia patient" as used herein relates to a patient suffering from anemia. Anemia is generally known to be associated with a decrease in normal number of red blood cells (RBCs) or less than the normal quantity of hemoglobin (Hb) in the blood. More specifically, it is the concentration of Hb, red blood cell volume, or red blood cell number. While normal Hb distributions vary with age, sex, and physiological status, e.g., during pregnancy the following Hb thresholds are preferably used to classify a given patient living at sea level as anemic (see Table 1):

Table 1: Hemoglobin thresholds used to define anemia

Age or gender group	Hemoglobin threshold (g/l)
Children (0.50–4.99 yrs)	110
Children (5.00–11.99 yrs)	115
Children (12.00–14.99 yrs)	120
Non-pregnant women (> 15.00 yrs)	120
Pregnant women	110
Men (> 15.00 yrs)	130.

Preferably, anemia encompasses anemia as defined by the World Health Organization (2008, see Worldwide prevalence of anemia 1993-2005. Geneva: World Health Organization. ISBN 9789241596657). In a preferred embodiment, Hb values of < 135 g/L for male and < 123 g/L for female persons are indicative of the patient suffering from anemia. The skilled clinical physician is well aware of factors such as smoking, altidude, etc. which may affect the individual patient's Hb level and will take these parameters in to account when diagnosing whether or not a patient is anemic. The clinical physician will also take into account well established signs and symptoms which can be related to the anemia itself, or the underlying cause when deciding whether or not a given patient suffers from anemia. These signs and symptoms inter alia encompass the following: Anemia patients often report non-specific symptoms of a feeling of weakness, or fatigue, general malaise and sometimes poor concentration. They may also report shortness of breath, dyspnea, on exertion. In moderate

and severe anemia, the body may compensate for the lack of oxygen carrying capability of the blood by increasing erythropoietin concentration and cardiac output. The anemia patient may have symptoms related to this, such as palpitations, angina (if preexisting heart disease is present), intermittent claudication of the legs, and symptoms of heart failure. There may be signs of specific causes of anemia, eg koilonychia (in iron deficiency), jaundice (when anemia results from abnormal break down of red blood cells — in haemolytic anemia), bone deformities (found in thalassaemia major). In severe anemia, there may be signs of a hyperdynamic circulation: a fast heart rate (tachycardia), flow murmurs, and cardiac enlargement. As used herein, the anemia patient preferably encompasses patients suffering from IDA, ACD or FID/ACD and optionally also patients displaying latent iron deficiency are also encompassed by the anemia patient of the present invention.

Preferably, the anemia patients of the present invention excludes patients, where the renal clearance of hepcidin or its variant is reduced, e.g. as a result of a chronic kidney disease. This is preferably the case in stage 3, 4 and 5 of chronic kidney disease and in patients which preferably display a glomerular filtration rate below 29 [mL • min -1 • (1.73 m2) -1] (National Kidney Foundation.Am J Kidney Dis 2002;39 (suppl 1): S1-266). The glomerular filtration rate (GfR) is determined as estimated GfR (Levey AS, et al. Clin Chem 2007; 53: 766-72). Preferably excluded from the anemia patient in the sense of the present invention is a patient suffering from hemolytic anemia, thalassemia trait, hereditary spherocytosis or sickle cell anemia.

As used herein "iron-deficiency anemia (IDA)" is characterized by microcytic red blood cells a ferritin serum level below 20 µg/L or below 30 µg/L in the presence of elevated soluble transferrin receptor(sTfR) and/or an increased ferritin index (FI = sTfR/log ferritin). Reference ranges for sTfR are 2.2-5.0 mg/L (male) and 1.9-4.4 mg/L (female) and for FI 3.2 (2.0 if CRP > 5 mg/L). Further preferred signs and symptoms of iron deficiency include the aforementioned clinical symptoms. As used herein, IDA can be either absolute or functional (FID). In absolute iron deficiency, the iron stores are depleted, whereas in FID, iron stores, although replete, cannot be mobilized from the macrophages of the reticulo-endothelial system. FID is associated with an imbalance between the iron needs of the erythroid marrow and iron supply, which is not maintained at a rate sufficient to allow normal hemoglobinization of the red cells, resulting in reduced reticulocytes and erythrocyte cellular hemoglobin (Hb) content. IDA is the most common type of hypochrome anemia, and is also known as sideropenic anemia. It is the most common cause of microcytic anemia (i.e. it is associated with a decreased volume of the erythrocytes) and is estimated to affect some two

billion people, causing almost one million deaths each year. Preferably, an IDA patient is a patient with microcytic red cells.

As used herein "anemia of chronic disease (ACD)", also referred to as anemia of inflammation, is an anemia associated with a chronic inflammatory state and is associated with a number of diseases including infection, a number of cancers and autoimmune diseases (such as rheumatoid arthritis and systemic lupus erythematosus, connective tissue diseases, vasculitis, sarcoidosis and inflammatory bowel disease), chronic rejection after solid organ transplantation and chronic kidney disease and inflammation (Weiss G et al. N Engl J Med 2005; 352: 1011-23). ACD as used herein is preferably associated with a hemoglobin (Hb) level ranging from about 90-120 g/L. Several mechanisms like iron sequestration, inhibition of erythroid progenitor proliferation and decreased red cell survival contribute to ACD (Sears, Med Clin North Am 1992; 76:567-79). In IDA the iron supply depends on the amount of the iron stores, whereas in ACD, the supply depends on its rate of mobilization. In a preferred embodiment of the present invention, the patients with IDA, ACD or FID/ACD are selected from a patient admitted to departments of Oncology, Urology, Internal Medicine, Neurology, Gynecology and Obstetrics. Preferably, an ACD patient is a patient suffering from a disorder selected from a chronic inflammatory disorder, a malignancy, an autoimmune disease, an inflammatory bowel disease and a critical illness.

In the context of the present invention the term "anemia of chronic disease with functional iron deficiency (FID/ACD)" encompasses a combined state of iron-restricted erythropoiesis and ACD. In FID/ACD the serum ferritin level will preferably be in the normal or elevated range between about 30 µg/L to about 1,000 µg/L with mostly an elevated CRP level (preferably > about 5 mg/L) and a log sTfR/ferritin ratio of about < 2.0 that rises quickly to about ≥2.0 once the inflammation has been successfully treated. Total body iron is usually normal or elevated. In FID/ACD a failure exists to provide iron to the erythroblasts despite replete iron stores (Thomas C, et al. Laboratory Hematology 2005; 11:14-23). Without wishing to be bound to any theory, it is generally believed that in FID/ACD is observed in inflammatory diseases when iron is trapped in the reticulo-endothelial system as a result of increased secretion of hepcidin, a hormone that controls iron release from intestinal cells and macrophages (De Domenico I, et al. Nat Rev Mol Cell Biol 2008; 9: 72-81). FID may also occur in response to the therapeutic use of erythropoietin, which places a significant demand on iron stores that may surpass the iron-release capacity of the reticulo-endothelial system. In a preferred embodiment of the present invention, the FID/ACD is selected from ACD and IDA characterized by a CHr ≤ 28 pg, a proportion of hypochromic red cells > 5%, a TSAT

< 16% and an elevated sTfR . Preferably, two of these aforementioned criteria must be fulfilled in FID/ACD patients. Preferably, a patient suffering from FID/ACD is a patient suffering from a disorder selected from a chronic inflammatory disorder, a malignancy, an autoimmune disease, an inflammatory bowel disease and a critical illness.

As used herein, the term "latent iron deficiency" is meant to refer to patients with depleted iron stores but have not yet begun iron-deficient erythropoiesis. In the context of the present invention the term latent iron deficiency encompasses a state of normal erythropoiesis and a Hb level ranging from about 10 to about 20% below the normal range. Patients have ferritin concentrations about < 30 µg/L and no further biochemical or hematologic signs of iron deficiency. Preferably, the patient suffering from latent iron deficiency is a child, an adolescent, a female patient of about ≤50 years of age preferably displaying menstrual blood loss, an athelete, or a body builder.

The term "sample" refers to a sample of a body fluid, to a sample of separated cells or to a sample from a tissue or an organ. Samples of body fluids can be obtained by well known techniques and include, preferably, samples of blood, plasma, serum, or urine, more preferably, samples of blood, plasma or serum. Tissue or organ samples may be obtained from any tissue or organ by, e.g., biopsy. Separated cells may be obtained from the body fluids or the tissues or organs by separating techniques such as centrifugation or cell sorting. Preferably, cell-, tissue- or organ samples are obtained from those cells, tissues or organs which express or produce the peptides referred to herein. Preferably, the sample of step a) and of b) of the method of the invention is the same sample or a different sample. Even more preferably, sample of step a) is a serum or plasma sample and/or the sample of step b) is a blood sample.

The term "diagnosing" as used herein means assessing, identifying, evaluating, or classifying if a given anemia patient from IDA, from ACD, or from FID/ACD or optionally from latent iron deficiency.

As used herein the term "hepcidin", encompasses a peptide produced in the liver, distributed in the plasma and excreted in urine is a regulator of intestinal iron absorption and iron recycling by macrophages (Hentze, Cell 117:285 (2004)). Besides the predominant form of hepcidin which contains 25 amino acids (hepcidin-25), two peptides shorter at the amino terminus were found, hepcidin-22 and hepcidin-20 which are also considered to be hepcidin in the sense of the present invention (Ganz, et al. Blood 2003; 102: 783-8). Preferably, hepcidin is a peptide as defined in WO2008/011158 and WO 2008/097461. The term hepcidin

also preferably encompasses a variant of the aforementioned hepcidin. The variant encompasses a protein or peptide substantially similar to the specific reference hepcidin molecule, preferably to the human hepcidin or hepcidin-25 peptide. The term substantially similar is well understood by the person skilled in the art. In particular, a hepcidin variant may be an isoform or allele which shows at least one amino acid exchange (and preferably up to about 25, more preferably up to about 15, more preferably up to about 10, more preferably up to about 5, most preferably up to about 3 amino acid exchanges) compared to the amino acid sequence of the specific reference hepcidin molecule. Preferably, such a hepcidin variant has a sequence identity to the specific reference hepcidin molecule of at least about 80%, preferably at least about 85%, more preferably at least about 90%, most preferably at least about 95%, most preferably at least about 98%, preferably with respect to human hepcidin or hepcidin-25 peptide, even more preferably over the entire length of the human hepcidin or hepcidin-25 peptide. The degree of identity between two amino acid sequences can be determined by algorithms well known in the art. Preferably, the degree of identity is to be determined by comparing two optimally aligned sequences over a comparison window, where the fragment of amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad Sci. (USA) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment and, thus, the degree of identity. Preferably, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. Variants referred to above may be allelic variants or any other species specific homologs, paralogs, or orthologs. The expression variant also encompasses also degradation products, e.g. proteolytic degradation products, which are still recognized by the

diagnostic means or by ligands directed against the respective full-length protein or peptide. The term "variants" is also meant to cover splice variants. The term "variant" also relates to a post-translationally modified peptide such as glycosylated peptide. A "variant" is also a peptide which has been modified after collection of the sample, for example by covalent or non-covalent attachment of a label, particularly a radioactive or fluorescent label, to the peptide. Preferably, the hepcidin variant possesses essentially the same immunological and/or biological properties of the specific reference peptide, preferably the same immunological and/or biological properties as human hepcidin or hepcidin-25, most preferably the same biological and/or immunological properties as human hepcidin or hepcidin-25 disclosed in WO2008/011158 and WO 2008/097461.Preferably the hepcidin variant displays at least about 70%, preferably at least about 80%, preferably at least about 90%, preferably at least about 95%, preferably at least about 98% of the human hepcidin activity, preferably of the human hepcidin-25 activity. The hepcidin activity, preferably the human hepcidin-25 activity, is the ferroportin binding activity which may for example be determined using the assay described in Nemeth E et al. 2004, Science 306:2090–2093. Preferably the hepcidin variant displays a ferroportin binding activity of at least about 80%, preferably at least about 90%, preferably at least about 95% of hepcidin. Preferably, the hepcidin activity is the stimulation of ferroportin internalization which may be determined using the assay described in Nemeth E et al. 2004, Science 306:2090–2093.Preferably, the hepcidin variant displays an activity of stimulating ferroportin internalization of at least about 80%, preferably at least about 90%, preferably at least about 95% of hepcidin. Alternative assays for the determination of hepcidin activity are described in Ganz T et al. 2006, Am J Physiol Gastrointest Liver Physiol 290:G199–203.

Determining the amount of hepcidin or a variant thereof relates to measuring the amount or concentration, preferably semi-quantitatively or quantitatively. Measuring can be done directly or indirectly. Direct measuring relates to measuring the amount or concentration of the peptide or polypeptide based on a signal which is obtained from the peptide or polypeptide itself and the intensity of which directly correlates with the number of molecules of the peptide present in the sample. Such a signal – sometimes referred to herein as intensity signal -may be obtained, e.g., by measuring an intensity value of a specific physical or chemical property of the peptide or polypeptide. Indirect measuring includes measuring of a signal obtained from a secondary component (i.e. a component not being the peptide or polypeptide itself) or a biological read out system, e.g., measurable cellular responses, ligands, labels, or enzymatic reaction products.

In accordance with the present invention, determining the amount of a hepcidin peptide or polypeptide can be achieved by all known means for determining the amount of a peptide in a sample. Said means comprise immunoassay devices and methods which may utilize labeled molecules in various sandwich, competition, or other assay formats. Said assays will develop a signal which is indicative for the presence or absence of the peptide or polypeptide. Moreover, the signal strength can, preferably, be correlated directly or indirectly (e.g. reverse-proportional) to the amount of polypeptide present in a sample. Further suitable methods comprise measuring a physical or chemical property specific for the peptide or polypeptide such as its precise molecular mass or NMR spectrum. Said methods comprise, preferably, biosensors, optical devices coupled to immunoassays, biochips, analytical devices such as mass- spectrometers, NMR- analyzers, or chromatography devices. Further, methods include micro-plate ELISA-based methods, fully-automated or robotic immunoassays (available for example on ElecsysTM analyzers), CBA (an enzymatic Cobalt Binding Assay, available for example on Roche-HitachiTM analyzers), and latex agglutination assays (available for example on Roche-HitachiTM analyzers), homogenous and heterogeneous immune assays, competitive and non-competitive immune assays.

Preferably, determining the amount of a hepcidin peptide or polypeptide comprises the steps of (a) contacting a cell capable of eliciting a cellular response the intensity of which is indicative of the amount of the peptide or polypeptide with the said peptide or polypeptide for an adequate period of time, (b) measuring the cellular response. For measuring cellular responses, the sample or processed sample is, preferably, added to a cell culture and an internal or external cellular response is measured. The cellular response may include the measurable expression of a reporter gene or the secretion of a substance, e.g. a peptide, polypeptide, or a small molecule. The expression or substance shall generate an intensity signal which correlates to the amount of the peptide or polypeptide.

Also preferably, determining the amount of a hepcidin peptide or polypeptide comprises the step of measuring a specific intensity signal obtainable from the peptide or polypeptide in the sample. As described above, such a signal may be the signal intensity observed at an m/z variable specific for the peptide or polypeptide observed in mass spectra or a NMR spectrum specific for the peptide or polypeptide.

Determining the amount of a hepcidin peptide or polypeptide may, preferably, comprises the steps of (a) contacting the peptide with a specific ligand, (b) (optionally) removing non-bound ligand, (c) measuring the amount of bound ligand. The bound ligand will generate an intensity

signal. Binding according to the present invention includes both covalent and non-covalent binding. A ligand according to the present invention can be any compound, e.g., a peptide, polypeptide, nucleic acid, or small molecule, binding to the peptide or polypeptide described herein. Preferred ligands include antibodies, nucleic acids, peptides or polypeptides such as receptors or binding partners for the peptide or polypeptide and fragments thereof comprising the binding domains for the hepcidin peptides, and aptamers, e.g. nucleic acid or peptide aptamers. Methods to prepare such ligands are well-known in the art. For example, identification and production of suitable antibodies or aptamers is also offered by commercial suppliers. The person skilled in the art is familiar with methods to develop derivatives of such ligands with higher affinity or specificity. For example, random mutations can be introduced into the nucleic acids, peptides or polypeptides. These derivatives can then be tested for binding according to screening procedures known in the art, e.g. phage display. Antibodies as referred to herein include both polyclonal and monoclonal antibodies, as well as fragments thereof, such as Fv, Fab and F(ab)2 fragments that are capable of binding hepcidin antigen or hapten. The present invention also includes single chain antibodies and humanized hybrid antibodies wherein amino acid sequences of a non-human donor antibody exhibiting a desired antigen-specificity are combined with sequences of a human acceptor antibody. Preferably, the anti-hepcidin antibody is an antibody disclosed in WO2008/011158 and WO 2008/097461. The donor sequences will usually include at least the antigen-binding amino acid residues of the donor but may comprise other structurally and/or functionally relevant amino acid residues of the donor antibody as well. Such hybrids can be prepared by several methods well known in the art. Preferably, the ligand or agent binds specifically to the hepcidin peptide or polypeptide. Specific binding according to the present invention means that the ligand or agent should not bind substantially to ("cross-react" with) another peptide, polypeptide or substance present in the sample to be analyzed. Preferably, the specifically bound hepcidin peptide or polypeptide should be bound with at least 3 times higher, more preferably at least 10 times higher and even more preferably at least 50 times higher affinity than any other relevant peptide or polypeptide, e.g. than prohepcidin or pre-prohepcidin. Non-specific binding may be tolerable, if it can still be distinguished and measured unequivocally, e.g. according to its size on a Western Blot, or by its relatively higher abundance in the sample. Binding of the ligand can be measured by any method known in the art. Preferably, said method is semi-quantitative or quantitative. Suitable methods are described in the following.

First, binding of a ligand may be measured directly, e.g. by NMR or surface plasmon resonance.

Second, if the ligand also serves as a substrate of an enzymatic activity of the peptide or polypeptide of interest, an enzymatic reaction product may be measured (e.g. the amount of a protease can be measured by measuring the amount of cleaved substrate, e.g. on a Western Blot). Alternatively, the ligand may exhibit enzymatic properties itself and the "ligand/peptide or polypeptide" complex or the ligand which was bound by the peptide or polypeptide, respectively, may be contacted with a suitable substrate allowing detection by the generation of an intensity signal. For measurement of enzymatic reaction products, preferably the amount of substrate is saturating. The substrate may also be labeled with a detectable label prior to the reaction. Preferably, the sample is contacted with the substrate for an adequate period of time. An adequate period of time refers to the time necessary for a detectable, preferably measurable, amount of product to be produced. Instead of measuring the amount of product, the time necessary for appearance of a given (e.g. detectable) amount of product can be measured.

Third, the ligand may be coupled covalently or non-covalently to a label allowing detection and measurement of the ligand. Labeling may be done by direct or indirect methods. Direct labeling involves coupling of the label directly (covalently or non-covalently) to the ligand. Indirect labeling involves binding (covalently or non-covalently) of a secondary ligand to the first ligand. The secondary ligand should specifically bind to the first ligand. Said secondary ligand may be coupled with a suitable label and/or be the target (receptor) of tertiary ligand binding to the secondary ligand. The use of secondary, tertiary or even higher order ligands is often used to increase the signal. Suitable secondary and higher order ligands may include antibodies, secondary antibodies, and the well-known streptavidin-biotin system (Vector Laboratories, Inc.). The ligand or substrate may also be "tagged" with one or more tags as known in the art. Such tags may then be targets for higher order ligands. Suitable tags include biotin, digoxygenin, His-Tag, Glutathion-S-Transferase, FLAG, GFP, myc-tag, influenza A virus haemagglutinin (HA), maltose binding protein, and the like. In the case of a peptide or polypeptide, the tag is preferably at the N-terminus and/or C-terminus. Suitable labels are any labels detectable by an appropriate detection method. Typical labels include gold particles, latex beads, acridan ester, luminol, ruthenium, enzymatically active labels, radioactive labels, magnetic labels ("e.g. magnetic beads", including paramagnetic and superparamagnetic labels), and fluorescent labels. Enzymatically active labels include e.g. horseradish peroxidase, alkaline phosphatase, beta-Galactosidase, Luciferase, and derivatives thereof.

Suitable substrates for detection include di-amino-benzidine (DAB), 3,3'-5,5'-tetramethylbenzidine, NBT-BCIP (4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, available as ready-made stock solution from Roche Diagnostics), CDP-Star™ (Amersham Biosciences), ECF™ (Amersham Biosciences). A suitable enzyme-substrate combination may result in a colored reaction product, fluorescence or chemoluminescence, which can be measured according to methods known in the art (e.g. using a light-sensitive film or a suitable camera system). As for measuring the enyzmatic reaction, the criteria given above apply analogously. Typical fluorescent labels include fluorescent proteins (such as GFP and its derivatives), Cy3, Cy5, Texas Red, Fluorescein, and the Alexa dyes (e.g. Alexa 568). Further fluorescent labels are available e.g. from Molecular Probes (Oregon). Also the use of quantum dots as fluorescent labels is contemplated. Typical radioactive labels include 35S, 125I, 32P, 33P and the like. A radioactive label can be detected by any method known and appropriate, e.g. a light-sensitive film or a phosphor imager. Suitable measurement methods according the present invention also include precipitation (particularly immunoprecipitation), electrochemiluminescence (electro-generated chemiluminescence), RIA (radioimmunoassay), ELISA (enzyme-linked immunosorbent assay), sandwich enzyme immune tests, electrochemiluminescence sandwich immunoassays (ECLIA), dissociation-enhanced lanthanide fluoro immuno assay (DELFIA), scintillation proximity assay (SPA), turbidimetry, nephelometry, latex-enhanced turbidimetry or nephelometry, or solid phase immune tests. Further methods known in the art (such as gel electrophoresis, 2D gel electrophoresis, SDS polyacrylamid gel electrophoresis (SDS-PAGE), Western Blotting, and mass spectrometry), can be used alone or in combination with labeling or other dectection methods as described above.

More preferably the amount of hepcidin is determined by mass spectrometry method, preferably by isotope-dilution micro-HPLC-tandem mass spectrometry method, preferably by a method as described in the Examples and in Kobold U et al. (Clin Chem 2008; 54: 1584-6).

The amount of a hepcidin peptide or polypeptide may be, also preferably, determined as follows: (a) contacting a solid support comprising a ligand for the peptide or polypeptide as specified above with a sample comprising the peptide or polypeptide and (b) measuring the amount peptide or polypeptide which is bound to the support. The ligand, preferably chosen from the group consisting of nucleic acids, peptides, polypeptides, antibodies and aptamers, is preferably present on a solid support in immobilized form. Materials for manufacturing solid supports are well known in the art and include, inter alia, commercially available column materials, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass and/or

silicon chips and surfaces, nitrocellulose strips, membranes, sheets, duracytes, wells and walls of reaction trays, plastic tubes etc. The ligand or agent may be bound to many different carriers. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for the purposes of the invention. Suitable methods for fixing/immobilizing said ligand are well known and include, but are not limited to ionic, hydrophobic, covalent interactions and the like. It is also contemplated to use "suspension arrays" as arrays according to the present invention (Nolan 2002, Trends Biotechnol. 20(1):9-12). In such suspension arrays, the carrier, e.g. a microbead or microsphere, is present in suspension. The array consists of different microbeads or microspheres, possibly labeled, carrying different ligands. Methods of producing such arrays, for example based on solid-phase chemistry and photo-labile protective groups, are generally known (US 5,744,305).

The mean hemoglobin content (MCH) and the mean corpuscular volume (MCV) of red cells may be determined by methods generally known in the art. The mean hemoglobin content per reticulocyte (= hemoglobin content of the reticulocytes being measured on a per cell basis) may for example be determined as described in Brugnara C, Crit Rev Clin Lab Sci; 2000: 97: 93-130, e.g. using the Advia 120 Hematology System (Siemens Diagnostics, Eschborn, Germany) or as described in Frank S, et al. Clin Chem 2004; 50: 1240-2 using Sysmex Hematology System (Sysmex, Norderstedt, Germany). Preferably, the CHr is used in the methods of the present invention. CHr measurements include mean Hb concentration of reticulocytes (CHCMr) and mean cell volume (MCVr). The CHr is a calculated index (CHr = MCVr x CHCMr). The term "amount" as used herein encompasses the absolute amount of a polypeptide or peptide, the relative amount or concentration of the said polypeptide or peptide as well as any value or parameter which correlates thereto or can be derived therefrom. Such values or parameters comprise intensity signal values from all specific physical or chemical properties obtained from the said peptides by direct measurements, e.g., intensity values in mass spectra or NMR spectra. Moreover, encompassed are all values or parameters which are obtained by indirect measurements specified elsewhere in this description, e.g., response levels determined from biological read out systems in response to the peptides or intensity signals obtained from specifically bound ligands. It is to be understood that values correlating to the aforementioned amounts or parameters can also be obtained by all standard mathematical operations.

The term "about" as used herein encompasses a range of + and – 20% relative to the specific value, amount, concentration, level, etc, e.g. indication of a value of "about 100" is meant to encompass a value of a numerical range of 100 +/- 20%, i.e. a value range from 80 to 120. Preferably the term "about" encompasses a range of + and – 10% relative to the specific value, amount, concentration, level, etc, most preferably a range of + and – 5% relative to the specific value, amount, concentration, level, etc.

The term "comparing" as used herein encompasses comparing the amount of the hepcidin peptide or polypeptide (or another determined parameter like CHr) comprised by the sample to be analyzed with an amount of a suitable reference source specified elsewhere in this description. It is to be understood that comparing as used herein refers to a comparison of corresponding parameters or values, e.g., an absolute amount is compared to an absolute reference amount while a concentration is compared to a reference concentration or an intensity signal obtained from a test sample is compared to the same type of intensity signal of a reference sample. The comparison referred to in step (c) and/or (d) of the method of the present invention may be carried out manually or computer assisted. For a computer assisted comparison, the value of the determined amount may be compared to values corresponding to suitable references which are stored in a database by a computer program. The computer program may further evaluate the result of the comparison, i.e. automatically provide the desired assessment in a suitable output format. Based on the comparison of the amount determined in step a) and the reference amount, the differential diagnosis of the different forms of anemia in a patient is determined. Therefore, the reference amount is to be chosen so that either a difference or a similarity in the compared amounts allows allocation of subjects in different anemia groups, preferably the ones depicted in Figures 3 and 4.

Accordingly, the term "reference amount", "reference level" or reference value" as used herein refers to an amount, level or value which defines a cut-off. An amount, level or value of the parameter above the cut-off results in a different diagnosis when compared to patients displaying a determined amount, level or value of the parameter below the cut-off. Thus, by comparing the actually determined amount, level or value of the parameters hepcidin, on the one hand, and the hemoglobin concentration of reticulocytes, on the other hand and by comparing these values to the respective cut-offs it is possible to diagnose or distinguish patients suffering from anemia of chronic disease, from iron deficiency anemia or from anemia of chronic disease with iron deficiency, or optionally from latent iron deficiency as referred to above.

Of course, the reference amount applicable for an individual subject may vary depending on various physiological parameters such as age, gender, or subpopulation, as well as on the means used for the determination of the polypeptide or peptide referred to herein. A suitable reference amount may be determined by the method of the present invention from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the test sample. The reference amounts of the present invention were confirmed in the Examples.

In a preferred embodiment of the method of the invention,

i) an amount of hepcidin or a variant thereof determined in step a) above to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes above the respective reference value determined in step b) is indicative of the patient suffering from anemia of chronic disease;

ii) an amount of hepcidin or a variant thereof determined in step a) above to the hepcidin reference value the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes below the respective reference value determined in step b) is indicative of the patient suffering from anemia of chronic disease with iron deficiency; and

iii) an amount of hepcidin or a variant thereof determined in step a) below to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes below the respective reference value determined in step b) is indicative of the patient suffering from iron deficiency anemia; optionally

iv) an amount of hepcidin or a variant thereof determined in step a) below to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes above the respective reference value determined in step b) is indicative of the patient suffering from latent iron deficiency.

In a preferred embodiment the reference CHr value is exchanged determined in combination with hepcidin.

In another preferred embodiment of the method of the invention,

i) an amount of at least about 3 nmol/L, preferably of at least about 3,7 nmol/L, preferably at least about 4 nmol/L, preferably at least about 4,3 nmol/L, preferably at

least about 5 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 25 pg, preferably of at least about 27 pg, preferably at least about 28 pg, preferably at least about 29 pg, preferably at least about 30 pg, determined in step b) is indicative of the patient suffering from ACD;

ii) an amount of at least about 3 nmol/L, preferably of at least about 3,7 nmol/L, preferably at least about 4 nmol/L, preferably at least about 4,3 nmol/L, preferably at least about 5 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 30 pg, preferably of less than about 29 pg, preferably of less than about 28 pg, preferably of less than about 27 pg, preferably of less than about 25 pg, determined in step b) is indicative of the patient suffering from FID/ACD; and

iii) an amount of less than about 5 nmol/L, preferably of less than about 4,3 nmol/L, preferably less than about 4 nmol/L, preferably less than about 3,7 nmol/L, preferably less than about 3 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 30 pg, preferably of less than about 29 pg, preferably of less than about 28 pg, preferably of less than about 27 pg, preferably of less than about 25 pg, determined in step b) is indicative of the patient suffering from IDA; optionally

iv) an amount of less than about 5 nmol/L, preferably of less than about 4,3 nmol/L, preferably less than about 4 nmol/L, preferably less than about 3,7 nmol/L, preferably less than about 3 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 25 pg, preferably of at least about 27 pg, preferably at least about 28 pg, preferably at least about 29 pg, preferably at least about 30 pg, determined in step b) is indicative of the patient suffering from latent iron deficiency.

More preferably,

i) an amount of at least about 4 nmol/L of hepcidin or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 28 pg determined in step b) is indicative of the patient suffering from ACD;

i) an amount of at least about 4 nmol/L of hepcidin or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 28 pg determined in step b) is indicative of the patient suffering from FID/ACD; and

iii) an amount of less than about 4 nmol/L of hepcidin or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 28 pg determined in step b) is indicative of the patient suffering IDA; optionally

iv) an amount of less than about 4 nmol/L of hepcidin or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 28 pg determined in step b) is indicative of the patient suffering from latent iron deficiency.

As will be understood by those skilled in the art, such a diagnostic assessment is usually not intended to be correct for all (i.e. 100%) of the patients to be identified. The term, however, requires that a statistically significant portion of patients can be identified (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test etc.. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. More preferably, at least 60%, at least 70%, at least 80% or at least 90% of the patients of a population can be properly identified by the method of the present invention.

In general, for determining the respective amounts/levels allowing to establish the desired diagnosis in accordance with the respective embodiment of the present invention, ("threshold", "reference amount"), the amount(s)/level(s) or amount ratios of the respective peptide or peptides are determined in appropriate patient groups. According to the diagnosis to be established, the patient group comprises only patients suffering from anemia and which also suffer from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency. Said diseases are to be distinguished by the respective parameters (i.e. hepcidin and any one of the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes) using validated analytical methods. The results which are obtained are collected and analyzed by statistical methods known to the person skilled in the art. The obtained threshold values are then established in accordance with the desired probability of suffering from the disease and linked to the particular threshold value. For example, it may be useful to choose the median value, the 60th, 70th, 80th, 90th, 95th or even the 99th percentile of the healthy and/or non-healthy patient collective, in order to establish the threshold value(s) or reference value(s).

A preferred reference amount serving as a threshold may be derived from the upper limit of normal (ULN), i.e. the upper limit of the physiological amount to be found in a population. The ULN for a given population of subjects can be determined by various well known techniques. A suitable technique may be to determine the median of the population for the peptide or polypeptide amounts to be determined in the method of the present invention.

A reference value of a diagnostic marker can be established and confirmed, and the level of the marker in a patient sample can simply be compared to the reference value. The sensitivity and specificity of a diagnostic and/or prognostic test depends on more than just the analytical "quality" of the test-they also depend on the definition of what constitutes an abnormal result. In practice, Receiver Operating Characteristic curves, or "ROC" curves, are typically calculated by plotting the value of a variable versus its relative frequency in "normal" and "disease" populations. For any particular parameter of the invention, a distribution of marker levels for subjects with and without a disease will likely overlap. Under such conditions, a test does not absolutely distinguish normal from disease with 100% accuracy, and the area of overlap indicates where the test cannot distinguish normal from disease. A threshold (cut-off, reference value) is selected, above which (or below which, depending on how a marker changes with the disease) the test is considered to be abnormal and below which the test is considered to be normal. The area under the ROC curve is a measure of the probability that the perceived measurement will allow correct identification of a condition. ROC curves can be used even when test results don't necessarily give an accurate number. As long as one can rank results, one can create an ROC curve. For example, results of a test on "disease" samples might be ranked according to degree (say 1=low, 2=normal, and 3=high). This ranking can be correlated to results in the "normal" population, and a ROC curve created. These methods are well known in the art. See, e.g., Hanley et al, Radiology 1982;143: 29-36 .

In certain embodiments, markers and/or marker panels are selected to exhibit at least about 70% sensitivity, more preferably at least about 80% sensitivity, even more preferably at least about 85% sensitivity, still more preferably at least about 90% sensitivity, and most preferably at least about 95% sensitivity, combined with at least about 70% specificity, more preferably at least about 80% specificity, even more preferably at least about 85% specificity, still more preferably at least about 90% specificity, and most preferably at least about 95% specificity. In particularly preferred embodiments, both the sensitivity and specificity are at least about 75%, more preferably at least about 80%, even more preferably at least about 85%, still more preferably at least about 90%, and most preferably at least about 95%.

Surprisingly, based on ROC analysis it was revealed that hepcidin enabled differentiation of IDA from ACD and from FID/ACD at 3.2 nmol/L (sensitivity 90.4%, specificity 85.1%), 3.7 nmol/L (sensitivity 96.2, specificity 76.1), 4.0 nmol/L (sensitivity 98.1%, specificity 74.6%), and 4.3 nmol/L (sensitivity 98.1%, specificity 73.1%). The CHr enabled differentiation of IDA from ACD and FID/ACD at 25 pg (sensitivity 100%, specificity 33.3%), 27 pg (sensitivity 95.7%, spec. 75%), 28 pg (sensitivity 85.7%, specificity 100%), 29 pg (sensitivity 64.3%, specificity 100%), and 30 pg (sensitivity 45.7%, specificity 100%).

In other embodiments, a positive likelihood ratio, negative likelihood ratio, or odds ratio is used as a measure of a test's ability to predict risk or diagnose a disease. In the case of a positive likelihood ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a positive result is more likely in the diseased group; and a value less than 1 indicates that a positive result is more likely in the control group. In the case of a negative likelihood ratio, a value of 1 indicates that a negative result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a negative result is more likely in the test group; and a value less than 1 indicates that a negative result is more likely in the control group. In certain preferred embodiments, markers and/or marker panels are preferably selected to exhibit a positive or negative likelihood ratio of at least about 1.5 or more or about 0.67 or less, more preferably at least about 2 or more or about 0.5 or less, still more preferably at least about 5 or more or about 0.2 or less, even more preferably at least about 10 or more or about 0.1 or less, and most preferably at least about 20 or more or about 0.05 or less. The term "about" in this context refers to +/- 5% of a given measurement.

In the case of an odds ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a positive result is more likely in the diseased group; and a value less than 1 indicates that a positive result is more likely in the control group. In certain preferred embodiments, markers and/or marker panels are preferably selected to exhibit an odds ratio of at least about 2 or more or about 0.5 or less, more preferably at least about 3 or more or about 0.33 or less, still more preferably at least about 4 or more or about 0.25 or less, even more preferably at least about 5 or more or about 0.2 or less, and most preferably at least about 10 or more or about 0.1 or less. The term "about" in this context refers to +/- 5% of a given measurement.

Panels may comprise at least one additional marker; both specific markers of a disease (e.g., markers that are increased or decreased in bacterial infection, but not in other disease states)

and/or non-specific markers (e.g., markers that are increased or decreased due to inflammation, regardless of the cause; markers that are increased or decreased due to changes in hemostasis, regardless of the cause, etc.). While certain markers may not be individually definitive in the methods described herein, a particular "fingerprint" pattern of changes may, in effect, act as a specific indicator of disease state. As discussed above, that pattern of changes may be obtained from a single sample, or may optionally consider temporal changes in one or more members of the panel (or temporal changes in a panel response value).

According to another aspect the present invention it is provided a method of recommending, deciding on and/or initiating or discontinuing the therapy for a patient suffering from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency, comprising the steps of:

a) determining the amount of hepcidin or a variant thereof in a sample from the patient;

b) determining the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes in a sample from the patient;

c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount;

d) comparing the value determined in step b) to a reference value;

e) diagnosing or distinguishing if the patient suffers ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency; and

f) recommending, deciding on and/or initiating or discontinuing the therapy for the patient based on the diagnosis or distinction of step e).

Unless specified differently, the definitions and preferred embodiments described with respect to the method of diagnosing or distinguishing above also apply to the present aspect of the invention.

According to a preferred embodiment of the method,

i) an amount of at least about 3 nmol/L, preferably of at least about 3,7 nmol/L, preferably at least about 4 nmol/L, preferably at least about 4,3 nmol/L, preferably at least about 5 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 25 pg, preferably of at least about 27 pg, preferably at least about 28 pg, preferably at least about 29 pg, preferably at

least about 30 pg, determined in step b) is indicative of the patient suffering from anemia of chronic disease;

ii) an amount of at least about 3 nmol/L, preferably of at least about 3,7 nmol/L, preferably at least about 4 nmol/L, preferably at least about 4,3 nmol/L, preferably at least about 5 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 30 pg, preferably of less than about 29 pg, preferably of less than about 28 pg, preferably of less than about 27 pg, preferably of less than about 25 pg, determined in step b) is indicative of the patient suffering from anemia of chronic disease with iron deficiency; and

iii) an amount of less than about 5 nmol/L, preferably of less than about 4,3 nmol/L, preferably less than about 4 nmol/L, preferably less than about 3,7 nmol/L, preferably less than about 3 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of less than about 30 pg, preferably of less than about 29 pg, preferably of less than about 28 pg, preferably of less than about 27 pg, preferably of less than about 25 pg, determined in step b) is indicative of the patient suffering from iron deficiency anemia; optionally

iv) an amount of less than about 5 nmol/L, preferably of less than about 4,3 nmol/L, preferably less than about 4 nmol/L, preferably less than about 3,7 nmol/L, preferably less than about 3 nmol/L of hepcidin, or a variant thereof determined in step a) and a mean hemoglobin content per reticulocyte of at least about 25 pg, preferably of at least about 27 pg, preferably at least about 28 pg, preferably at least about 29 pg, preferably at least about 30 pg, determined in step b) is indicative of the patient suffering from latent iron deficiency.

According to a preferred embodiment of the method,

i) the patient suffers from anemia of chronic disease and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an erythropoiesis-stimulating agents (ESA) therapy, and/or said therapy is initiated;

ii) the patient suffers from iron deficiency anemia and it is recommended or decided to subject the patient to an iron supplementation therapy and to refrain from ESA therapy; in the event the patient underwent ESA therapy at, around or shortly before (i.e. the last ESA administration was carried out within 3 weeks, preferably within 2 weeks, preferably within 1 week, preferably within 3 days, preferably within 2 days, preferably within 12 hours, prior to the sampling) the time the sample was collected it

is decided to discontinue the ESA therapy and to treat the patient by an iron supplementation therapy; and/or said therapy is initiated or discontinued; or

iii) the patient suffers from anemia of chronic disease with iron deficiency and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an ESA therapy, and/or said therapy is initiated; optionally

iv) the patient suffers from latent iron deficiency it is recommended or decided to subject the patient to an iron supplementation therapy and/or said therapy is initiated.

According to another aspect the present invention it is provided a method of recommending, deciding on and/or initiating or discontinuing the therapy for a patient suffering from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency, wherein

i) the patient suffers from anemia of chronic disease and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an ESA therapy, and/or said therapy is initiated;

ii) the patient suffers from iron deficiency anemia and it is recommended or decided to subject the patient to an iron supplementation therapy and to refrain from ESA therapy; in the event the patient underwent ESA therapy at, around or shortly before (i.e. the last ESA administration was carried out within 3 weeks, preferably within 2 weeks, preferably within 1 week, preferably within 3 days, preferably within 2 days, preferably within 12 hours, prior to the sampling) the time the sample was collected it is decided to discontinue the ESA therapy and to treat the patient by an iron supplementation therapy; and/or said therapy is initiated or discontinued; or

iii) the patient suffers from anemia of chronic disease with iron deficiency and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an ESA therapy, and/or said therapy is initiated; optionally

iv) the patient suffers from latent iron deficiency it is recommended or decided to subject the patient to an iron supplementation therapy and/or said therapy is initiated.

The therapeutic implication of the invention is to differentiate patients into those who should be administered iron, erythropoietin or a combination of erythropoietin and iron.

Differentiation between IDA, ACD and FID/ACD is clinically important because ESA therapy, like EPO administration, may be beneficial for ACD and FID/ACD patients but may be deleterious for IDA patients, especially if these patients suffer from certain malignancies. The present invention provides an effective means to accomplish this task and may help to

decide on an appropriate and effective therapy for anemia patients once the differential diagnosis of the present invention has been carried out. Thus, expensive and ineffective therapies are readily recognized and can be avoided, which in turn contributes to effective spending of resources to the benefit of the patient and the society as a whole.

As used herein the term „iron supplementation therapy" is meant to refer to any therapy which is suitable to increase the iron content in the body, preferably the iron content in the erythron. Preferably, iron supplementation therapy encompasses oral or parenteral administration of iron containing compositions. The latter for example comprise iron (III)- gluconate saccharose complex or iron (III) hydroxide saccharose or iron ascorbate. Administration is preferably accomplished orally or parenterally, preferably by infusion. Responders show an increase in ferritin concentration in the normal range.

As used herein the term „ erythropoiesis-stimulating agents (ESA) therapy" is meant to refer to any therapy which results in the increase of erythropoietic activity; preferred examples include the administration of drugs or agents having erythropoietic activity like erythropoietin (EPO) and variants and mimetics thereof which possess erythropoietic activity, preferably agents like Aranesp, Darbepoetin, Epo TheraPEG, EPEG, ErepoXen, Albupoetin, EPO Dimer PT-401, Epo-Fc, synthetic erythropoiesic protein (SEP), Hematide, PBI-1402, HemoMer, ABT007, Continuous Erythropoiesis Receptor Activator (CERA, Mircera); growth hormones, cytokines including IL-3 and IL-9. The erythropoietic activity can be easily determined, for example by measuring the increase in Hb value, the increase of reticulocyte count or the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes. Administration is preferably accomplished parenterally, preferably by infusion, subcutaneously or by i.v.. Responders to ESA therapy show an increase of hemoglobin of more than about 10 g/L within 4 weeks.

According to yet another aspect of the present invention it is provided a method of monitoring a therapy of a patient suffering from ACD, from IDA and from FID/ACD, or optionally from latent iron deficiency, comprising the steps of

a) determining the amount of hepcidin, or a variant thereof in a sample from the patient;

b) determining the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes in a sample from the patient;

c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount;

d) comparing the value determined in step b) to a reference value;

e) determine the status of the monitored disease; and optionally

f) recommending, deciding on and/or continuing, modulating or discontinuing the therapy for the patient based on status of the monitored disease determined in step e).

Preferably, anemic patients with chronic infections, malignancies, autoimmune diseases and the critically ill should be monitored. Preferably, indicators of successful therapy are the increase in Hb value of more than about 10 g /L, the increase of reticulocyte hemoglobin content to about > 28 pg, a decrease of hepcidin in ACD or FID/ACD and an increase of hepcidin in IDA. Preferably at least one, preferably at least 2, preferably at least 3 of the above criteria are met to consider the patient a responder. Preferably, the Hb value and reticulocyte hemoglobin content are direct markers of the responding bone marrow and are indicators of successful therapeutic intervention .

Preferably, the patient to be monitored or monitored suffers from ACD or FID/ACD (e.g. chronic inflammatory disorder, malignancy, autoimmune disease, inflammatory bowel disease and critical illness). In these patients the following parameters are an indicator of a response: Increase in Hb value > about 10g/L, of reticulocyte hemoglobin content > about 28 pg and decrease in of serum hepcidin from high value to > about 4 nmol/L or movement of the patient data point from quadrant 3 to quadrant 2 in the hepcidin plot (Fig. 3). Preferably at least one, preferably at least 2, preferably at least 3 of the above criteria are met to consider the patient a responder. Non responders to treatment preferably show no adequate increase in Hb and mean hemoglobin content per reticulocyte and no movement of patient data in the hepcidin plot.

Preferably, the patient to be monitored or monitored suffers from IDA and microcytic red cells (e.g. chronic blood loss). In these patients the following parameters are an indicator of a response: Increase in Hb value > about 10g/L, of serum ferritin > about 30 µg/L, of reticulocyte hemoglobin content > about 28 pg and increase of serum hepcidin from low value to > about 4 nmol/L or movement of the patient data point from quadrant 4 to quadrant 1 in the hepcidin plot (Fig. 3). Preferably at least one, preferably at least 2, preferably at least 3 of the above criteria are met to consider the patient a responder. Preferably, non responders to treatment show no adequate increase in serum ferritin, Hb, and mean hemoglobin content per reticulocyte and no movement of patient data in the hepcidin plot.

Preferably, the patient to be monitored or monitored suffers from latent iron deficiency (e.g. children, adolescents, female persons ≤about 50 years of age with menstrual blood loss,

athletes, body builder). In these patients the following parameters are an indicator of a response: Increase in Hb value > about 10g/L, of serum ferritin > about 30 µg/L. Preferably at least one, preferably at least 2 of the above criteria are met to consider the patient a responder.

Unless specified differently, the definitions and preferred embodiments described with respect to the method of diagnosing or distinguishing above also apply to the present aspect of the invention.

According to yet another aspect of the invention it is provided a device adapted for diagnosing or distinguishing in an anemia patient if the patient suffers from anemia of chronic disease, from ACD, from IDA and from FID/ACD, or optionally from latent iron deficiency, preferably according to a method described above, comprising:

a) a first analyzing unit comprising a detection agent for hepcidin or a variant thereof, wherein the analyzing unit is adapted for determining the amount of the hepcidin detected by the detection agent;

b) a second analyzing unit for detecting the the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, wherein the second analyzing unit is adapted for determining the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes;

c) an evaluation unit comprising a computer comprising tangibly embedded a computer program code for carrying out the comparison of the determined amount obtained from the first and second analyzing units, respectively, with a suitable data base comprising a corresponding reference amount as specified above; preferably the first and second analyzing units and preferably also the evaluation units are operatively linked to each other.

Preferably, the device further comprises means for outputting the required treatment and/or therapy on the basis of the classification of the disorder of iron metabolism. More preferably the device further comprises means for outputting the progress and/or response to a treatment and/or therapy of a disorder of iron metabolism.

According to yet another aspect of the invention it is provided a kit adapted for carrying out the method describe above, comprising:

means for determining the amount of hepcidin or a variant thereof;

means for comparing the determined amount of hepcidin or a variant thereof with reference amounts;

means for determining the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes; and

means for comparing the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes with reference amounts; and

means for comparing the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes with reference amounts; and

instructions for carrying out the method.

According to yet another aspect of the invention it is provided a computer program comprising computer program code which is suitable for carrying out a method of the invention when the computer program is run on a computer.

In another aspect of the invention it is provided a computer readable medium with a computer program of the invention stored thereon.

In another aspect of the invention it is provided a computer program product with a computer program of the invention stored thereon. Preferably, the computer program further comprises means for outputting the required treatment and/or therapy on the basis of the diagnosed disease, the means being stored on a computer readable medium.

Preferably, the determined measurements are represented graphically in the form of diagrams in order to easily assign the measuring ranges to the various iron states. For example the measured level of hepcidin can be plotted on the abscissa and the measured level of CHr or alternatively the Ret-He can be plotted on the ordinate. This results in various measuring ranges (fields in the diagram) for the various iron states and in particular ACD, IDA and FID/ACD, and optionally mild IDA can be distinguished from each other.

Brief description of the drawings

Figure 1 Diagnostic plot using the ferritin index (sTfR/log ferritin index plot) as indicator of iron supply and CHr as indicator of iron demand for erythropoiesis. In patients with CRP > 5 mg/L the cutoff for the sTfR/log ferritin index was 2.0

instead of 3.2 measured with the Roche sTfR assay and 0.8 and 1.5 in the Siemens assay. The plot enables differentiation of four different categories of iron status (Thomas C, et al. Clin Chem 2002; 48: 1066-76).

Figure 2 ROC curve analysis showing the ability of hepcidin-25 (cutoff ≤4 nmol/L) to discriminate storage ID from iron-repletion (line 1) and from the combined state of iron repletion and FID (line 2). Line 3 represents the inability of the parameter hepcidin-25 (take alone) to differentiate patients with iron repletion from the combined state.

Figure 3 Hepcidin-25 plot using serum hepcidin-25 concentration as indicator of iron supply and CHr ≤25-30 pg as indicator of iron demand for erythropoiesis. A cutoff value < 3 to < 5 nmol/L differentiates iron replete from iron-depleted states. The hepcidin-25 plot enables differentiation of IDA from ACD and from FID/ACD, or optionally from latent iron deficiency.

Figure 4 Hepcidin-25 plot using serum hepcidin-25 concentration as indicator of iron supply and CHr ≤28 pg as indicator of iron demand for erythropoiesis. A cutoff value < 4 nmol/L differentiates iron replete from iron-depleted states. The hepcidin-25 plot enables differentiation of IDA from ACD and from FID/ACD, or optionally from latent iron deficiency

The present invention is now described in more detail with the following examples and figures which are not intended to be interpreted as limiting the invention.

Examples

Materials and Methods

STUDY DESIGN

The clinical laboratory department of the University Hospital Northwest in Frankfurt, Germany, implemented a medical decision-oriented testing program for anemia differentiation in 2002. The program is requested mainly from the physicians in place of a menu of individual tests in patients with chronic disease (e.g. cancer, chemotherapy-associated anemia, infection, autoimmune disease, inflammatory bowel disease and critical illness. For this study the anemia program was extended by the measurement of serum hepcidin-25. The criteria used for anemia were Hb < 135 g/L for male and < 123 g/L for female persons. The anemia program included the CBC, reticulocyte count, biochemical

markers and hematologic indices of iron metabolism, haptoglobin, a screening program that facilitates exclusion of thalassemia trait, and the RBC scatter diagram of the hematology analyzer. In this randomized study, patients were allocated to one of the following groups representing different iron states: (i) iron-replete state in chronic disease (ii) iron-depleted state, patients may have storage iron deficiency and FID or latent ID (recently depleted iron stores but no FID); or (iii) functional ID in iron-replete state.

According to clinical findings and laboratory investigations patients with iron state (i) were consistent with ACD, patients with iron state (ii) were consistent with IDA or latent ID, and patients with iron state (iii) were consistent with FID/ACD.

The diagnostic criteria for selection of the different patient groups were besides clinical findings (Tab.2) the following markers of iron metabolism:

Group I (ACD):	The following arrangement of parameters; Normocytic red blood cells, ferritin > 100 µg/L, FI ≤2.0 if CRP was > 5 mg/L or otherwise ≤3.2, TSAT < 16%.
Group II (IDA or latent iron deficiency):	The following arrangement of parameters; Microcytic red blood cells, Ferritin < 20 µg/L or < 30 µg/L in the case of elevated sTfR*, FI > 2.0 if CRP was > 5 mg/L or otherwise > 3.2.
Group III (FID/ACD):	The following arrangement of parameters; Microcytic or normocytic red blood cells, CHr ≤ 28 pg, HYPO > 5%, TSAT < 16%, elevated sTfR*.

Patients with hemolytic anemia, thalassemia trait, hereditary spherocytosis and sickle cell anemia were excluded from the study. The diagnosis of functional ID for all patients was based on the examination of TSAT and red cell hemoglobin content, using the CHr test and the proportion of hypochromic red cells (HYPO) test as the gold standards (Thomas C, et al., Clin Chem 2002; 48:1066-76). Normally, in non-anemic individuals CHr is greater than the mean mature CH and CHr:CH ratios below 1 are consistent with recently developed FID (Brugnara C. Crit Rev Clin Lab Sci 2000;97: 93-130).

PATIENTS

Adult patients who were admitted to the hospital setting underwent an initial clinical and laboratory screen within 2 hours of admission. From October 2008 to March 2009 all patients

were included in the study physicians requested the anemia program. The biochemical markers and hematologic indices were determined on the day of admission and hepcidin-25 in two batches in March and June 2009. The patients were representative samples of University Hospital Northwest chronic disease patients with a mean age of 68 ± 16 years and a male/female ratio of 64/91. We studied 155 patients who were admitted to the departments of Oncology, Urology, Internal Medicine, Neurology and Gynecology and Obstetrics. Of the patients included 86 had solid tumors (prostate, lung, breast, colon, uterus, ovary, melanoma) mainly with metastasis, 45 had acute or chronic active inflammatory diseases (urinary tract, respiratory disease, inflammatory bowel disease, rheumatic disease) and 24 had heterogeneous diseases of only one or two causes (chronic gynecologic affliction, chronic heart failure, neurologic disease). No patient had an estimated glomerular filtration rate below 42 [mL • min -1 • (1.73 m2) -1].The study was conducted in compliance with the Helsinki Declaration (World Medical Association. World Medical Association of Helsinki: ethical principles for medical research involving human subjects, http://www.wma.net/e/policy/b3.htm).

DIAGNOSTIC TESTING

Blood samples were collected in EDTA tubes for analysis of complete blood count (CBC) including HYPO, reticulocyte count and the CHr, which were analyzed with a standard hematology analyzer, i.e. with Advia 120 Hematology System (Siemens Diagnostics, Eschborn, Germany) within 8 h of venipuncture. Serum specimens were used to determine the standard biochemical markers of iron metabolism (ferritin, TSAT, sTfR), CRP and hepcidin-25. Haptoglobin was determined with immunoturbidimetry (BN-Prospect, Siemens Diagnostics, Eschborn), ferritin, transferrin, iron, sTfR and CRP were measured using a standard clinical chemistry analyzer, i.e. the Cobas 6000 clinical chemistry analyzer (Roche, Mannheim, Germany). The ferritin index (FI) was calculated as described (Thomas C, et al., Clin Chem 2002; 48:1066-76). TSAT was calculated using the formula [serum iron (µmol/L) x 0.038]/serum transferrin (g/L) x 100. For the determination of hepcidin-25 1 mL serum was separated from the samples for the clinical chemistry routine and kept frozen at minus 28 ^{0}C. All parameters used in the study were determined from specimens of one blood collection in the morning. Reference range data (normal range) for CHr were 28-35 pg (Thomas C, et al. Clin Chem. 2002; 48:1066-76) and 1-5% for HYPO (Thomas C, et al. Clin Chem. 2002; 48:1066-76), for ferritin 27-365 µg/L (male and postmenopausal female) and 13-148 µg/L (premenopausal female) (Lotz J et al. Clin Chem Lab Med 1999; 37: 821-5), sTfR 2.2-5.0 mg/L (male) and 1.9-4.4 mg/L (female) (Kolbe-Busch S, et al., Clin Chem Lab Med 2002; 40:

529-36), TSAT 16-50%, and FI ≤2.0 in patients with CRP > 5 mg/L instead of ≤3.2 (Thomas C, et al. Clin Chem. 2002; 48:1066-76. Quantification of serum hepcidin-25 was assessed using isotope-dilution micro-HPLC-tandem mass spectrometry method (Kobold U, et al., Clin Chem 2008; 54: 1584-6). The limit of quantification was < 0.2 nmol/L, the imprecision, linearity, recovery of the method were described recently (Kobold U et al., Clin Chem 2008; 54: 1584-6). The hepcidin-25 conversion factor was nmol/L x 2.789 = µg/L.

STATISTICAL ANALYSIS

Data were evaluated using standard parametric tests, and calculations were performed with the MedCalc a standard statistical software package of biomedical research, i.e. Schoonjans F. MedCalc statistics for biomedical research, http://www.medcalc.be.

Results

CORRELATION OF TESTS

In the 155 patients studied, correlation between hepcidin-25 and ferritin showed a smaller increase of hepcidin-25 in the ferritin range ≤200 µg/L as in the selected ranges above (Tab. 3). The relationship between hepcidin-25 and ferritin in the range of ≤200 µg/L was y = -0.2230 + 0.0478x (N=81, r^2 = 0.323, p < 0.001) and y = 16.1035 + 0.0139x (N= 70, r^2 = 0.157, p 0.001) in the ferritin range of 201 to 2,000 µg/L. The regression line in all patients between hepcidin-25 and TSAT was y= -12.1 + 0.86x (N=155, r^2=0.487, p < 0.001) and between hepcidin-25 and CRP y= 1.13 + 0.092x (N=155, r^2 = 0.281, p= 0.02). There were negative relationships between hepcidin-25 and both the sTfR (y = 5.224 - 0.3371x, N= 80, r^2 = 0.067, p=0.020) and the FI (y = 4.844 - 0.3815x, N= 80, r^2 = 0.102, p=0.006) in the ferritin range ≤200 µg/L. The data indicate that low hepcidin-25 concentration correlate with iron-deficient states and high hepcidin-25 concentrations with iron-replete states.

Spearman's rank correlation showed no significant relationship between hepcidin-25 and Hb (R=-0.081, p= 0.316), HYPO (R= -0.260, p= 0.120), CH (R= 0.191, p= 0.118) and CHr (R= 0.128, p=0.112), respectively. The data indicate no correlation of hepcidin-25 with the hematologic markers of iron deficiency.

HEMATOLOGIC AND BIOCHEMICAL MARKERS IN IRON DEFICIENCY

There were significant differences among the three groups of patients with different states of erythropoiesis and iron states (see Tab. 4). Group-I patients, essentially corresponding to ACD patients, had normocytic anemia, normal CH, CHr, TSAT, sTfR, FI and normal to

elevated ferritin. Microcytic erythropoiesis with decrease in ferritin, TSAT and CHr, but elevated sTfR, FI and HYPO was measured in the group-II patients, which essentially correspond to IDA or latent IDA patients. Group-III patients which essentially correspond to FID/ACD presented an intermediate state between group I and group II. These patients differentiated from the group I with a CHr ≤30, preferably ≤29, preferably ≤28, preferably ≤27, preferably ≤26, preferably ≤25 pg (for determination of the optimal value from ROC-analysis, see Thomas C, et al., Clin Chem 2002; 48:1066-76) a higher proportion of patients with HYPO > 5%, a significant increase of sTfR (p=0.004) and a significant decline of TSAT (p=0.003) (Tab. 4).

HEPCIDIN-25

The concentration of hepcidin-25 was lower in group-II patients (ID group) than in group I (iron-replete group) and group-III patients (combined state) (Tab.4). We drew ROC curves to further confirm the concordance of low hepcidin-25 with the IDA group. As can be seen from Fig. 2, hepcidin-25 displayed a high area under the ROC curve (AUCROC) and high sensitivity and specificity both between IDA and iron-replete group (i.e. ACD) (AUCROC 0.968; 95% CI 0.915- 0.992; sensitivity 98.1%, specificity 84.5%; p=0.0001) as well as between IDA group and the patients with functional ID in iron-replete state (i.e. FID/ACD) (AUCROC 0.995; 95% CI 0.948-1.000; sensitivity 98.1%, specificity 97.1%; p= 0.0001). However, this was not the case between the iron-replete groups with (i.e.FID/ACD) and without FID (i.e. ACD) (AUCROC 0.569; 95% CI 0.461-0.671; sensitivity 42.3%, specificity 75.0%; p= 0.2590).The optimal cutoff value (highest sum of sensitivity and specificity from ROC curves) for ID was a hepcidin-25 concentration of 4 nmol/L. Suboptimal cutoffs were in the range of 3-5 nmol/L with sensitivity of 90.4% and specificity of 85.1% at the criterion ≤3.2 nmol/L and sensitivity of 98.1% and specificity of 68.1% at the citerion ≤5 nmol/L.

INTEGRATION OF THE PATIENT GROUPS INTO DIAGNOSTIC PLOTS

Using the well known assay of sTfR/log ferritin index as an indicator of iron supply in combination with CHr as indicator of FID the erythropoiesis and iron status of anemia patients was differentiated into four states recently (Fig. 1). Data of CHr and sTfR/log ferritin index of the 3 patient groups were placed in the sTfR/log ferritin index plot and showed mismatching of thirteen from 155 (8.4%) patients. Eleven of the 13 mismatches resulted from too high values of the FI, resulting in more patients with iron depletion.

Serum hepcidin-25 if used alone was unable to differentiate ACD from FID/ACD. For the identification of both states we combined the CHr and hepcidin-25 in a new plot (Fig. 4). Data

points in Q1 and Q2 included patients with either depleted (Q2) or recently depleted (Q1) iron stores and patients with data points in Q3 and Q4 were consistent with iron-replete stores. The optimal cutoff value for FID was CHr ≤28 pg and the optimal hepcidin cutoff value for iron depletion was ≤4 nmol/L. If hepcidin-25 and CHr data of the three patient groups were placed into the plot only 1 of 52 (1.9%) patients with IDA or latent ID was mismatched with FID/ACD or ACD. However, 20 of 67 (29.9%) ACD patients had data points in the area of latent ID, indicating latent iron deficiency.

Summarizing the above, the hepcidin and CHr-based diagnosis allows for a considerable sensitive and specific differential diagnosis of ACD, IDA or FID/ACD, or optionally latent iron deficiency.

Discussion

The comparison of hepcidin-25 with the biochemical markers and hematologic indices commonly used for the differentiation of iron states showed a significant relation with the biochemical markers but not with the hematologic indices in our 155 anemia patients. In our studies the most important increase in hepcidin-25 was measured at ferritin concentrations > 200 µg/L, a range where iron overload starts, and > 1,000 µg/L were iron overload is evident. The correlation analysis between ferritin values ≤200 µg/L and hepcidin-25 as well as TSAT and hepcidin-25 indicated that hepcidin-25 concentrations were below the detection level of 0.2 nmol/L at ferritin < 9 µg/L and TSAT < 14.3%. Hepcidin-25 production is induced by inflammation and levels correlated with IL-6 (Tomosugi N, et al. Blood 2006; 108:1381-7). In our patients the regression analysis showed only a low slope of the regression line between hepcidin-25 and CRP, indicating that CRP is of little value for the assessment whether the hepcidin-25 concentration was influenced by inflammation.

Serum hepcidin-25 concentrations ≤4 nmol/L surprisingly differentiated states of storage ID (latent ID and IDA) from ACD and FID/ACD (Tab.4). Patients with storage ID who have not yet begun iron-deficient erythropoiesis had comparable hepcidin-25 values than patients with IDA, again indicating that hepcidin-25 is no marker of FID.

If hepcidin-25 was used together with the CHr, a marker of FID, in the hepcidin-25 plot ACD was distinguished from FID/ACD and IDA with the same accuracy as with the diagnostic assessment shown in Tab. 2 and better accuracy in comparison to the sTfR/log ferritin index plot. Thus, the hepcidin-25 plot provides a versatile, simple-to-use and attractive tool for the diagnosis and monitoring of iron states in anemia patients.

Table 2 Diagnostic criteria for the classification of anemic patients

Iron state	Diagnostic criteria
Group I (ACD)	In addition to clinical findings (chronic infection, autoimmune disease, malignancy) at least two of the following criteria in addition: Normocytic RBC, ferritin > 100 µg/L, FI ≤2.0 if CRP was > 5 mg/L or otherwise ≤3.2, TSAT < 16%.
Group II (storage ID)	Ferritin < 20 µg/L or < 30 µg/L in the case of elevated sTfR*, FI > 2.0 if CRP was > 5 mg/L or otherwise > 3.2. At least 2 criteria were required, and in the case of IDA microcytic anemia in addition.
Group III (FID/ACD)	Criteria of ACD and two criteria of FID. Criteria of FID were: CHr ≤28 pg, HYPO > 5%, CHr:CH ratio < 1.0, TSAT < 16%, elevated sTfR*.

* In the absence of reticulocytosis

Table 3 Association between hepcidin-25 and ferritin in serum

Ferritin (µg/L)	N	Hepcidin-25 (nmol/L)		
		Mean ± SD	Median	IQR
≤20	16	1.01 ± 0.93	0.65	1.25
≤30	25	1.09 ± 0.88	0.80	1.20
31-100	32	2.35 ± 2.57	1.35	2.80
101-200	21	6.96 ± 7.15	4.00	4.39
201-500	29	17.94 ± 11.84	17.00	11.63
501-1000	22	26.50 ± 20.45	20.15	27.47
> 1000	20	48.55 ± 29.39	43.90	35.64

IQR= Interquartile range

Table 4 Baseline characteristics and iron status measurements in anemic patients (N= 155)

Variable	Group I (iron-replete state)	Group II (depleted iron stores)	Group III (FID in iron-replete state)
N	67	52	36
Blood count			
Hb (g/L)	97 (72-133)	101(75-128)	98 (51-127)
RBC (10^{12}/L)	3.6 (2.5-4.9)	4.1 (2.6-5.4)	3.9 (2.9-5.0)
MCV (fl)	88.8 (76.1-104)	77.6 (64.5-90.1)	83.3 (74.6-94.4)
CH (pg)	28.7 (25.7-36.4)	25.9 (18.7-33.2)	26.8 (18.3-30.2)
Reticulocytes (10^9/L)	70.1 (2.4-246)	87.2 (5.0-215)	60.5 (2.0-131)
CHr (pg)	30.5 (26.8-34.6)	25.9 (19.8-30.4)	26.1 (20.6-27.8)
HYPO (%) (% prevalence > 5%)	2.7 (0.1-41.3) 41.8	15.5 (0.7-63.9) 82.7	6.3 (0.4-69.7) 55.6
CHr:CH ratio < 1 (N)* (% prevalence of < 1)	8 11.9	22 42.3	20 55.5
Serum measurements			
Ferritin (µg/L) (% prevalence > 100 µg/L)	384 (26.2-10467) 89.6	35.5 (5.0-137.6) 9.6	406 (42.4-2134) 94.5
sTfR (mg/L)	2.9 (1.6-8.3)	6.7 (3.5-23.4)	3.9 (1.3-11.2)
sTfR/log ferritin	1.2 (0.6-7.1)	4.6 (1.8-18.6)	1.6 (0.8-5.1)
TSAT (%) (% prevalence ≤16%)	27.4 (14.3-92.2) 8.4	14.4 (6.2-22.7) 77.3	15.2 (8.7-39.9) 51.6
CRP (mg/L) (% prevalence > 5 mg/L)	21 (1-297) 76.1	13 (1-210) 69.2	99 (8-289) 100
Hepcidin (nmol/L) (% prevalence > 2 nmol/L) (% prevalence > 4 nmol/L)	11.7 (0.4-87.1) 92.5 61.2	1.0 (< 0.2-5.4) 15.3 1.9	21 (3.1-88.5) 100 97.2

Values are given as median and range. *Number of patients

Claims

1. A method for diagnosing or distinguishing, in an anemia patient, if the patient suffers from anemia of chronic disease (ACD), from iron deficiency anemia (IDA) or from anemia of chronic disease with functional iron deficiency (FID/ACD), or optionally from latent iron deficiency, the method comprising the steps of

   a) determining the amount of hepcidin or a variant thereof in a sample from the patient;

   b) determining a value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, in a sample from the patient;

   c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount;

   d) comparing the value determined in step b) to a reference value; and

   e) diagnosing or distinguishing if the patient suffers from ACD, from IDA or from FID/ACD or optionally from latent iron deficiency.

2. The method according to claim 1, wherein

   i) an amount of hepcidin or a variant thereof determined in step a) above to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, above the respective reference value determined in step b) is indicative of the patient suffering from anemia of chronic disease;

   ii) an amount of hepcidin or a variant thereof determined in step a) above to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, below the respective reference value determined in step b) is indicative of the patient suffering from anemia of chronic disease with iron deficiency; and

   iii) an amount of hepcidin or a variant thereof determined in step a) below to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes

below the respective reference value determined in step b) is indicative of the patient suffering from iron deficiency anemia; optionally

iv) an amount of hepcidin or a variant thereof determined in step a) below to the hepcidin reference value and the value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, above the respective reference value determined in step b) is indicative of the patient suffering from latent iron deficiency.

3. The method of any one of claims 1 to 2, wherein the patient suffering from ACD is a patient suffering from a disorder selected from a chronic inflammatory disorder, a malignancy, an autoimmune disease, an inflammatory bowel disease and a critical illness.

4. The method of any one of claims 1 to 3, wherein the patient suffering from IDA is a patient with microcytic red cells.

5. The method of any one of claims 1 to 4, wherein the patient suffering from FID/ACD is a patient suffering from a disorder selected from a chronic inflammatory disorder, a malignancy, an autoimmune disease, an inflammatory bowel disease and a critical illness.

6. The method of any one of claims 1 to 5, wherein in step a) the sTfR/log ferritin index is determined instead of hepcidin; and in step c) the sTfR/log ferritin index is compared to a reference value.

7. The method of any one of claims 1 to 6, wherein the patient suffering from latent iron deficiency is a patient suffering selected from a patients with increased iron demand, preferably a child, an adolescent, a female patient of up to about 50 years of age having menstrual blood loss, and an athelete.

8. A method of recommending, deciding on and/or initiating or discontinuing the therapy for an anemia patient suffering from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency; preferably a patient diagnosed according to a method of any one of claims 1 to 7; wherein

i) the patient suffers from ACD and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an erythropoiesis-stimulating agents (ESA) therapy, and/or said therapy is initiated;

ii) the patient suffers from IDA and it is recommended or decided to subject the patient to an iron supplementation therapy and to refrain from ESA therapy; in the event the patient underwent ESA therapy at, around or shortly before the time the sample was collected it is decided to discontinue the ESA therapy and to treat the patient by an iron supplementation therapy; and/or said therapy is initiated or discontinued; or

iii) the patient suffers from FID/ACD and it is recommended or decided to subject the patient to an iron supplementation therapy and/or an ESA therapy, and/or said therapy is initiated; optionally

iv) the patient suffers from latent iron deficiency it is recommended or decided to subject the patient to an iron supplementation therapy and/or said therapy is initiated.

9. A method of monitoring a therapy of a patient suffering from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency, comprising the steps of

a) determining the amount of hepcidin or a variant thereof in a sample from the patient;

b) determining a value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes in a sample from the patient;

c) comparing the amount of hepcidin or a variant thereof determined in step a) to a reference amount;

d) comparing the value determined in step b) to a reference value;

e) determining the status of the monitored disease; and optionally

f) recommending, deciding on and/or continuing, modulating or discontinuing the therapy for the patient based on status of the monitored disease determined in step e).

10. A device adapted for carrying out the method of any of claims 1 to 9, or a device adapted for diagnosing or distinguishing in an anemia patient if the patient suffers from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency; preferably according to a method of any one of claims 1 to 7; comprising:

a) a first analyzing unit comprising a detection agent for hepcidin or a variant thereof, wherein the analyzing unit is preferably adapted for determining the amount of the hepcidin detected by the detection agent;

b) a second analyzing unit for detecting a value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes, wherein the second analyzing unit is preferably adapted for determining the value of the mean hemoglobin content per reticulocyte;

c) an evaluation unit comprising a computer comprising tangibly embedded a computer program code for carrying out the comparison of the determined amount obtained from the first and second analyzing units, respectively, with a suitable data base comprising a corresponding reference amount; preferably the first and second analyzing units and preferably also the evaluation units are operatively linked to each other.

11. A kit adapted for carrying out the method of any of claims 1 to 9, comprising:

means for determining the amount of hepcidin or a variant thereof;

means for comparing the determined amount of hepcidin or a variant thereof with reference amounts;

means for determining a value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes; and

means for comparing the determined value selected from the mean hemoglobin content per reticulocyte and the hemoglobin concentration of reticulocytes with reference amounts; and

instructions for carrying out the method.

12. A computer program comprising computer program code which is suitable for carrying out a method according to any one of claims 1 to 9 when the computer program is run on a computer.

13. A computer readable medium with a computer program according to claim 12 stored thereon.

14. Use of a means for detecting hepcidin or a variant thereof, preferably an antibody specifically binding hepcidin or a variant thereof, for diagnosing or distinguishing, in an anemia patient, preferably in a sample obtained from the patient, if the patient suffers from ACD, from IDA or from FID/ACD, or optionally from latent iron deficiency, wherein the diagnosis or distinction is based on the detection of hepcidin and the determination of the value selected from the mean hemoglobin content per reticulocyte or the mean hemoglobin concentration in a sample from the patient.

Figure 1/4

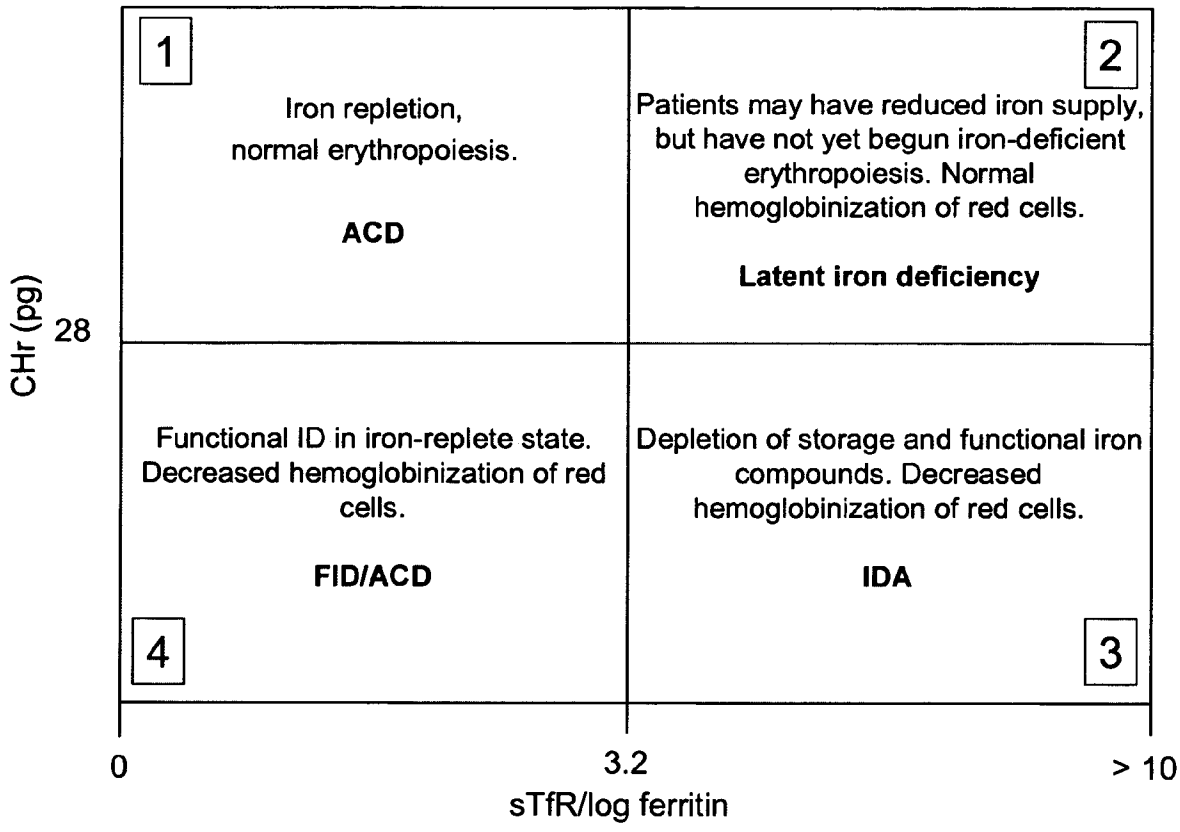

1
Iron repletion,
normal erythropoiesis.
ACD
2
Patients may have reduced iron supply, but have not yet begun iron-deficient erythropoiesis. Normal hemoglobinization of red cells.
Latent iron deficiency
CHr (pg)
28
Functional ID in iron-replete state. Decreased hemoglobinization of red cells.
FID/ACD
Depletion of storage and functional iron compounds. Decreased hemoglobinization of red cells.
IDA
4
3
0
3.2
> 10
sTfR/log ferritin

Figure 2/4

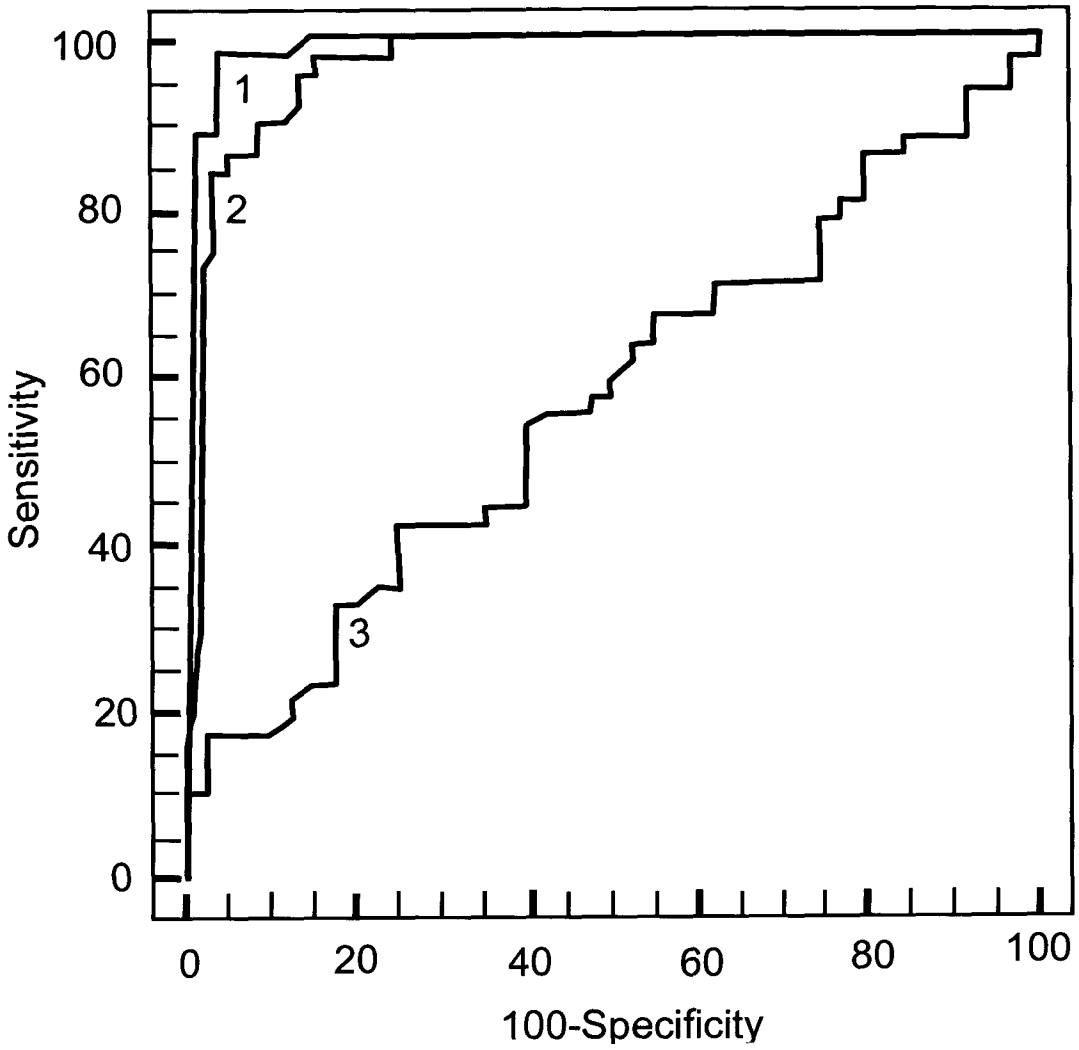

100
80
60
40
20
0
Sensitivity
1
2
3
0
20
40
60
80
100
100-Specificity

Figure 3/4

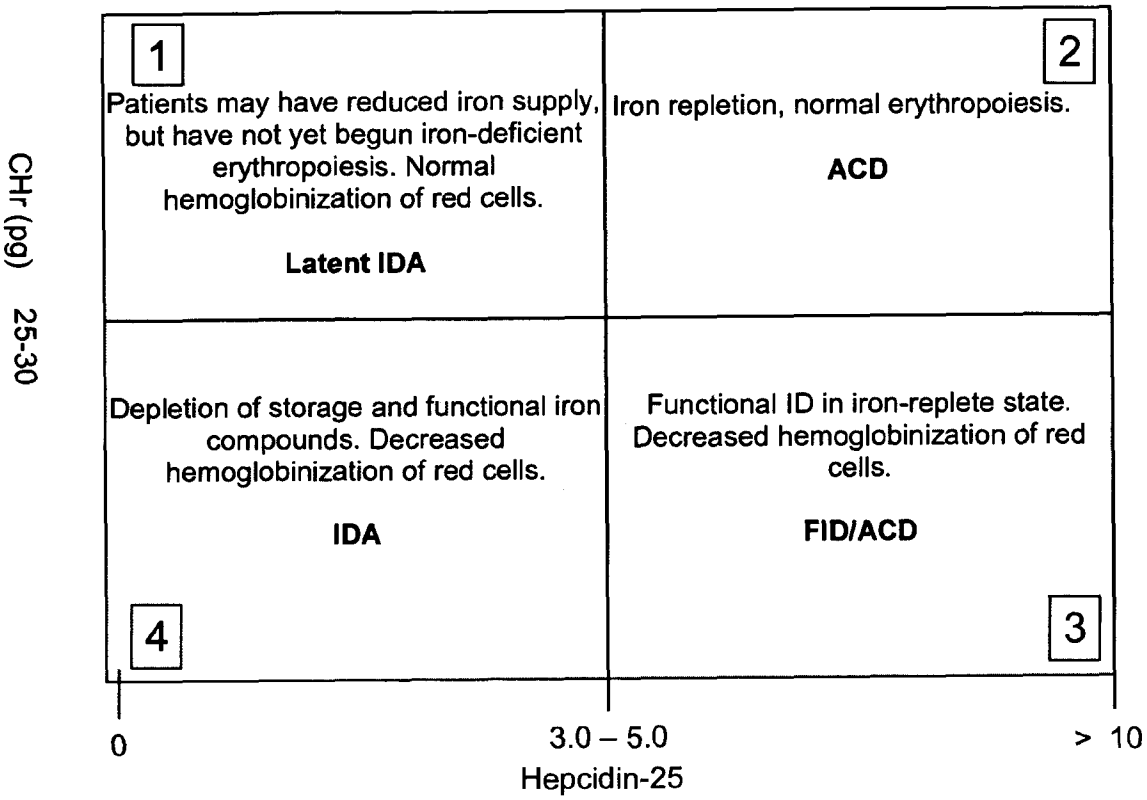
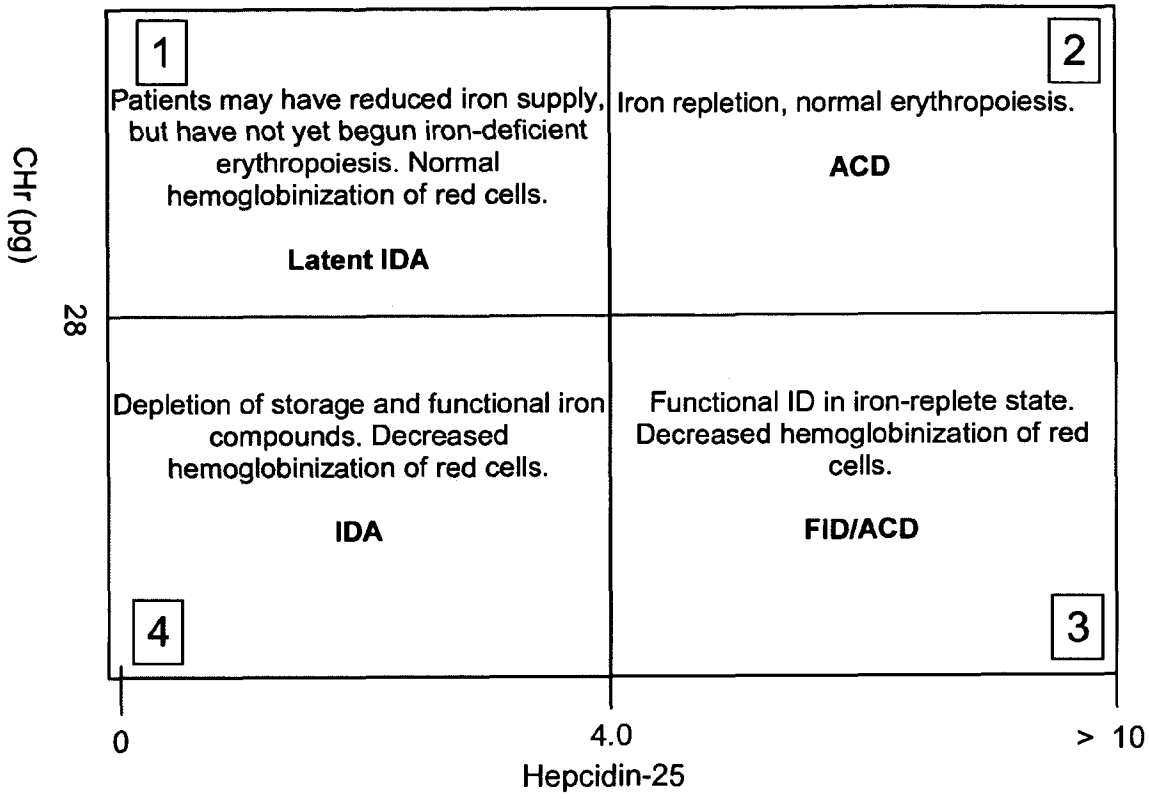

1
Patients may have reduced iron supply, but have not yet begun iron-deficient erythropoiesis. Normal hemoglobinization of red cells.
Latent IDA
2
Iron repletion, normal erythropoiesis.
ACD
Depletion of storage and functional iron compounds. Decreased hemoglobinization of red cells.
IDA
Functional ID in iron-replete state. Decreased hemoglobinization of red cells.
FID/ACD
4
3
CHr (pg) 25-30
0
3.0 – 5.0
> 10
Hepcidin-25

Figure 4/4


1
Patients may have reduced iron supply, but have not yet begun iron-deficient erythropoiesis. Normal hemoglobinization of red cells.
Latent IDA
2
Iron repletion, normal erythropoiesis.
ACD
Depletion of storage and functional iron compounds. Decreased hemoglobinization of red cells.
IDA
Functional ID in iron-replete state. Decreased hemoglobinization of red cells.
FID/ACD
4
3
CHr (pg)
28
0
4.0
> 10
Hepcidin-25

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/006762

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE MAST QUIRIJN ET AL: "Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection", BRITISH JOURNAL OF HAEMATOLOGY, vol. 145, no. 5, June 2009 (2009-06), pages 657-664, XP002570909, ISSN: 0007-1048 * abstract page 658, left-hand column, paragraph 2 page 659, left-hand column, paragraph 2 - right-hand column, paragraph 1 page 663, left-hand column, last paragraph table II ----- -/--	1-5,7, 9-14

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2011	24/02/2011

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/006762

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN DONGEN-LASES E C ET AL: "DIAGNOSING IRON DEFICIENCY ANEMIA IN PATIENTS WITH RHEUMATOID ARTHRITIS", HAEMATOLOGICA-THE HEMATOLOGY JOURNAL, vol. 94, no. Suppl. 2, June 2009 (2009-06) , pages 240 URL-HTTP://WW, XP002570910, & 14TH ANNUAL MEETING OF THE EUROPEAN-HEMATOLOGY-ASSOCIATION; BERLIN, GERMANY; JUNE 04 -07, 2009 * abstract -----	10,11
X	THOMAS C ET AL: "Anemia of chronic disease: Pathophysiology and laboratory diagnosis", LABORATORY HEMATOLOGY 2005 US, vol. 11, no. 1, 2005, pages 14-23, XP008119689, ISSN: 1080-2924 cited in the application * abstract page 20, left-hand column page 21, left-hand column, paragraph 2 page 21, right-hand column, paragraph 2 - page 22, left-hand column, paragraph 1 figures 7,8 -----	6,8
X	WO 2008/011158 A2 (AMGEN INC [US]; LI HONGYAN [US]; BREAU ALAN [US]; SASU BARBRA [US]) 24 January 2008 (2008-01-24) cited in the application paragraphs [0069], [0084] - [0088] -----	11
A	CLARK S F: "Iron deficiency anemia: Diagnosis and management", CURRENT OPINION IN GASTROENTEROLOGY 200903 US, vol. 25, no. 2, March 2009 (2009-03), pages 122-128, XP002570912, ISSN: 0267-1379 the whole document -----	1-14
A	GANZ TOMAS ET AL: "Immunoassay for human serum hepcidin.", BLOOD 15 NOV 2008, vol. 112, no. 10, 15 November 2008 (2008-11-15), pages 4292-4297, XP002570913, ISSN: 1528-0020 the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/006762

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		EP 2057472 A2	13-05-2009
		JP 2009544941 T	17-12-2009
		US 2009173876 A1	09-07-2009