CAT
Critically Appraised Topic

Potential role of hepcidin in the investigation of anemia: diagnostic value and (pre)analytical implications

Author:	Apr. Vermeulen Edith
Supervisor:	Prof. dr. Vermeersch Pieter
Search/methodology verified by:	Prof. dr. Vermeersch Pieter
Date:	21/04/2010

CLINICAL BOTTOM LINE

Hepcidin plays a key role in regulating plasma iron levels through inhibition of iron export by ferroportin from enterocytes and macrophages. Hepcidin levels respond to inflammation (as an acute-phase reactant), body iron, hypoxia and erythropoiesis. Hepcidin is a promising marker in the investigation of anemia, especially to fill out the 'diagnostic blanks' in iron deficiency that still exist, as in anemia of chronic disease, in infants or in chronic kidney disease. The aim of this *critically appraised topic* was to review the evidence for the diagnostic utility of hepcidin determination and the (pre)analytical problems involved.

Quantification of hepcidin-25 was long problematic. Many studies therefore measured the precursor peptide 'prohepcidin' as a surrogate marker. However, prohepcidin levels do not correlate with iron status and are not physiologically relevant. The first quantitative hepcidin ELISA was described in 2008 and a commercial ELISA became available in 2009. Both serum and urine analysis are possible. Serum seems the preferable marker, but this topic still needs further elucidation. Due to diurnal variation, standardized sampling time is needed.

The available evidence regarding the diagnostic value of hepcidin in the investigation of anemia is promising, but further confirmation is needed. Studies prospectively validating a cut-off for hepcidin for the diagnosis of iron deficiency anemia are notably lacking. Significantly differing hepcidin levels were described in clear cases of anemia of chronic disease with or without concomitant iron deficiency. In chronic kidney disease hepcidin levels do not seem to predict hyporesponsiveness to erythropoiesis-stimulating agents, as they possibly reflect ESA dose.

Clearance of the analytical barriers in hepcidin determination will allow investigators to further explore the diagnostic applications of hepcidin.

ABBREVIATIONS

Ab	antibodies
ACD	anemia of chronic disease
BMP	bone morphogenetic proteins
CHr	reticulocyt hemoglobin content
CKD	chronic kidney disease
CV	coefficient of variation
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
ESA	erythropoiesis stimulating agents
HAMP	human antimicrobial peptide (gene encoding for hepcidin)
Hb	hemoglobin
HH	hereditary hemochromatosis
ID	iron-deficiency
IDA	iron-deficiency anemia
ICU	intensive care unit
IRIDA	iron refractory iron-deficiency anemia
IV	intravenous
LC	liquid chromatography
LPS	lipopolysaccharids
MS	mass spectrometry
OMIM	Online Mendelian Inheritance in Men
RES	reticulo-endothelial system
RIA	radioimmuno-assay
rHuEPO	recombinant human erytropoietin
SELDI-TOF	surface-Enhanced Laser Desorption/Ionisation Time Of Flight
sTfR	serum transferrin receptor
TSAT	transferrin saturation

CLINICAL/DIAGNOSTIC SCENARIO

Introduction: Role of hepcidin in iron metabolism

Hepcidin is a circulating peptide hormone encoded by the *HAMP* (human antimicrobial peptide) gene and mainly produced by the liver. Hepcidin is a negative regulator of iron transport in plasma (1,2,3). The mechanism involved in this regulation is the binding of hepcidin to the iron exporter ferroportin (4,5,6). This binding leads to internalization and degradation of ferroportin, resulting in macrophage iron retention and inhibition of gut iron absorption (see attachment 1).
Besides "systemic" liver production of hepcidin in control of the body iron stores, there is evidence for a local production of hepcidin in the kidney, heart, pancreas, macrophages and adipose tissue (7), but the biological relevance of this extrahepatic production is still unclear.
Four pathways control hepcidin production to match iron supply to erytropoietic demand and to maintain adequate plasma iron levels: body iron, inflammation, hypoxia, erythropoiesis. Attachment 2 gives an overview of regulating mechanisms.

The possible additional diagnostic value of hepcidin determination depends on the restrictions of current iron markers (summarized in table 1). Especially in patients with inflammation, assessment of iron status can be troublesome.

Table 1: Iron markers and their limitations in diagnosis of iron disorders

Marker	Use	Restrictions
Serum Iron	– Low (or normal) in ID – High in iron overload	– Diurnal variation – Elevated premenstrually, low at menstruation – Variability according to methodology and hemolysis – High levels in oral contraceptives – Increased in oral iron intake or IV iron – High levels in hepatitis (see ferritin) – Low to normal in inflammation
Ferritin	– Reflects the body iron content – Declines early in development of ID (sensitive)	– High values in inflammation; often normal values in patients with chronic infections or malignancies and ID – Increase in hepatitis or toxic liver injury (alcoholism) – Not indicative of iron stores in hyperthyroidism – Less sensitive in detecting early iron overload than serum iron, TIBC, TSAT – Not predictive of ID(A) in toddlers 1-2y
Transferrin	– Increases in ID, with low saturation – Useful in DD of microcytic anemia (no change in partial α-thalassemia, low in inflammation) – Very low levels in hereditary atransferrinemia	– High levels in pregnancy and estrogen therapy – Low levels in inflammation or malignancy – Decreased synthesis in chronic liver disease or malnutrition – Low levels due to loss in nephrotic syndrome or protein-losing enteropathy
Transferrin saturation (TSAT)	– Low saturation in ID – Normal concentration with increased saturation in hemochromatosis	– TSAT up to 100% in oral iron intake – Rises to 75% in oral contraceptives – 100% during weeks after IV iron (due to circulating iron dextran) – TSAT normal or low in inflammation
Serum transferrin receptor (sTfR)	– Increases early in functional iron deficit, before microcytosis develops – Inappropriate low sTfR in anemia is indicative of decreased bone marrow erytropoietic activity – sTfR/log ferritin ratio (R/F) reflects body iron stores – Changes in ratio reflect absorption and true increment of body iron after oral supplementation	– Lack of assay standardization (no common reference material) – Increased in high turnover states – R/F ratio of limited use in inflammation – Little or no diagnostic value in young children
Reticulocyte hemoglobin content (CHr)	– Reliable measure of early iron deficient erythropoiesis, more sensitive than hemoglobin – Early and significant change in functional iron deficiency in rHuEPO therapy	– Lower specificity than hemoglobin reported – Availability still limited (though increasing) – Problematic in assessing iron status in thalassemia or patients under chemotherapy
Marrow iron	– Most reliable in complex diagnostic situations	– Misleading in insufficient sample size, insufficient observer skills, patients treated with parenteral iron or myeloproliferative disease

Abbreviations: ID iron deficiency; IDA iron deficiency anemia; TIBC total iron binding capacity; DD differential diagnosis; sTfr serum transferrin receptor; rHuEPO recombinant human erytropoietin. This table was composed with data from (10)(11)(12)

Three pathological situations can result from disturbances in iron metabolism: absolute iron deficiency, iron overload and functional iron deficiency characterized by retention of iron in the reticulo-endothelial system leading to a limited bio-availability. The conditions in which a diagnostic challenge still exists are these where hepcidin determination is potentially useful. These conditions are described below.

1. Iron deficiency anemia

Iron deficiency anemia (IDA) is the most common anemia worldwide, characterized by microcytosis with low ferritin, low serum iron and low transferrin saturation. An overview of causes of IDA is given in attachment 3. IDA can result from increased iron loss, decreased intake or decreased absorption. Once the underlying blood loss or nutritional deficit is corrected, iron supplementation leads to improvement of anemia in the vast majority of cases.
However, the diagnosis of IDA can be less clear-cut in some specific situations, as in early childhood and in recombinant human EPO therapy (10). The diagnosis of IDA in the setting of chronic disease is discussed separately.

- Early childhood

The inability of infants to regulate gut iron absorption predisposes them to ID when dietary iron is low and to iron overload when dietary levels are high. Prompt identification of ID in young children is important to prevent irreversible alteration in neurodevelopment. Early iron supplementation in premature infants is shown to have some beneficial effects on cognitive and psychomotor function. Preventive measurement of iron status is recommended at 9 months of age and at 3 months for premature babies. However, conventional laboratory parameters of iron status fail to distinguish neonates with iron deficient erythropoiesis: ferritin is not a good predictor of ID or IDA in these children, levels of transferrin are always low in newborns and sTfR has no diagnostic value (10). Furthermore, hemoglobin (Hb) can be low in asymptomatic infants while symptomatic infants have a normal hemoglobin concentration. Reticulocyt hemoglobin content (CHr) expresses the amount of Hb in the reticulocytes, reflecting the availability of iron to the bone marrow for incorporation of only a few days before. CHr seems the best predictor of ID in infants. However, the availability of this assay is still limited. Inherited forms of iron-deficiency anemia are rare. Iron-refractory iron deficiency anemia (IRIDA) is an autosomal recessive disease characterised by congenital hypochromic microcytic anaemia, with no response to oral iron treatment. Early identification is needed, but difficult, to implement the appropriate (parenteral) iron supplementation.

- Recombinant human erytropoietin therapy (rHuEPO)

The substantial increase in erytropoietic activity induced by rHuEPO is accompanied by a sharply increased requirement for bio-available iron. Even with sufficient body iron stores and oral iron supplementation, a course of rHuEPO can induce a decrease in serum iron and desaturation of transferrin, resulting in iron-deficient erythropoiesis. Identification of this condition is needed to adapt dosage or administer IV iron to obtain optimal respons (16). A low baseline ferritin predicts this functional iron deficiency, but is difficult to interpret as inflammation-induced effects are common. The percentage of hypochromic red cells can identify developing ID, but a prolonged state of iron-decicient erythropoiesis is needed to see changes in this marker. Reticulocyt counts and hemoglobin change can identify patients as “responders” or “non-responders”. CHr seems to be a promising marker in the setting of rHuEPO therapy (12,16).

2. Iron overload syndromes

Iron overload can be primary (hereditary hemochromatosis, HH) or secondary (as in thalassemia, myelodysplastic syndromes, chronic liver disease, metabolic syndrome, transfusions). *HFE*-hemochromatosis is the most common form of genetic iron overload. Here, the C282Y homozygous *HFE* genotype is responsible for reduced expression of hepcidin, resulting in parenchymal iron accumulation (14,16). Other mutations affecting the synthesis, activity or regulation of hepcidin cause less frequent forms of HH. Diagnosis is often made based on familial screening, when there is an index case in the family.
Elevation of transferrin saturation is the earliest phenotypic abnormality and is a reliable indicator of risk of iron overload (16). The level is not age-related and does not correlate with symptoms. Serum ferritin correlates with total body iron stores and hepatic iron concentration in HH and is a sensitive predictor of cirrhosis. However, elevated ferritin levels lack specificity as hyperferritinemia is also associated with secondary iron overload syndromes and inflammation. It is essential to diagnose HH presymptomatic, before irreversible tissue damage arises and to differentiate between ongoing iron accumulation and increasingly prevalent disorders with elevated serum ferritin such as the metabolic syndrome (16). Therapy for HH is phlebotomy, which can only be monitored by ferritin, with the same problems of aspecificity of this marker. A rational ‘consensus’ target of therapy is defined as ferritin <50 µg/L, without solid evidence of true ‘physiological’ relevance of this target.

3. Anemia of chronic disease

Anemia of chronic disease (ACD) is a normochromic normocytic anemia with a low reticulocyte count, indicating inhibition of erythropoiesis (8). ACD is characterized by macrophage iron retention induced by cytokines (8,9). Hepcidin plays a central role in the pathogenesis of ACD, as demonstrated in attachment 4.
The diagnosis of ACD is difficult, as coexisting blood loss, the effects of medications or inborn errors of hemoglobin synthesis may interfere. It is clinically important to distinguish between ACD and ACD combined with a true iron deficiency (ACD+ID) because this difference implicates treatment, making iron supplements necessary in the second condition and contraindicated in the first. Most of the biochemical iron markers, e.g. ferritin, are affected by acute-phase reaction. This makes ferritin levels difficult to interpret in the differentiation between ACD with or without iron deficiency. Both diseases present with low serum iron and low transferrin saturation. As compared with patients who have ACD alone, patients with concomitant iron-deficiency anemia more frequently have microcytes, and their anemia tends to be more severe. A newer marker is the soluble transferrin receptor (sTfR): a truncated fragment of the membrane receptor that is increased in ID, when the availability of iron for erythropoiesis is low. The ratio of soluble transferrin receptor (sTfR)/log ferritin may be useful in distinguishing ACD from ACD+IDA, but due to the lack of standardization of sTfR assays, this ratio cannot be widely used in clinical practice. (16)

QUESTIONS

1) Which (pre-)analytical aspects influence hepcidin determination?

2) Would hepcidin determination improve the diagnosis of iron deficiency anemia in children and adults? Is hepcidin a useful marker to differentiate anemia of chronic disease and iron deficiency anemia?

3) Is hepcidin of value in the diagnosis or monitoring of hemochromatosis?

SEARCH TERMS

1) MeSH Database (PubMed): MeSH term:
"hepcidin"[Substance Name]; "hepcidin (6-25), human"[Substance Name]; "Hamp2 protein, mouse "[Substance Name]; "hepcidin 25, human"[Substance Name]; "Anemia, Iron-Deficiency"[Mesh]; "Hemochromatosis"[Mesh]; "Kidney Failure, Chronic"[Mesh]; Peritoneal Dialysis"[Mesh]; "Erythropoietin, Recombinant"[Mesh]; "Phlebotomy"[Mesh]

2) PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters

3) UpToDate Online version 18.1

1) Systematic Reviews, Meta-analyses, Reviews

(3) Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: from research to clinic. World J Gastroenterol 2009; 15(5): 538-51
(7) Kemna EHJM, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. Haematologica 2008; 93(1): 90-7
(8) Weiss G, Goodnough L. Anemia of chronic disease. N Engl J Med 2005; 352: 1011
(10) Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem 2003; 49(10):1573-78
(12) Wish B. Assessing iron status: beyond serum ferritin and transferrin saturation. Clin J Am Soc Nephrol 2006; 1: S4-S8
(14) Andrews N. Disorders of iron metabolism. N Engl J Med 1999; 341(26): 1986-95
(15) Ferrara M, Coppola L, Coppola A, Capozzi L. Iron deficiency in childhood and adolescence: retrospective review. Hematology 2006; 11(3): 183-6.
(16) Alexander J, Kowdley K. HFE-associated hereditary hemochromatosis. Genet Med. 2009; 11(5): 307-13
(18) Hugman A. Hepcidin: an important new regulator of iron homeostasis. Clin Lab Haematol 2006; 28: 75-83
(54) Castagna A, Campostrini N, Zaninotto F, Girelli D. Hepcidin assay in serum by SELDI-TOF-MS and other approaches. J Proteomics 2010; 73(3): 527-36
(66) Babitt JL, Lin HY. Molecular Mechanisms of Hepcidin Regulation: Implications for the Anemia of CKD. Am J Kidney Dis 2010 Feb 25. [Epub ahead of print]
(77) Finberg KE. Iron-refractory iron deficiency anemia. Semin Hematol 2009; 46(4): 378-86.

2) Original Articles

(1) Nicolas G, Bennoun M, Devaux L, Beaumont C, Grandchamp B, *et al.* Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knock-out mice. Proc Natl Acad Sci USA 2001; 98: 8780-5
(2) Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, *et al.* Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003; 33: 21-2
(4) Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004; 306: 2090-3
(5) De Domenico I, Ward DM, Nemeth E, Vaugh MB, Musci G, *et al.* The molecular basis of ferroportin-linked hemochromatosis. Proc Natl Acad Sci USA 2005; 102: 8955-60
(6) De Domenico I, Ward DM, Langelier C, Vaughn MB, Nemeth E, *et al.* The molecular mechanism of hepcidin-mediated ferroportin downregulation. Mol Biol Cell 2007; 18(7): 2569-78
(9) Theurl I, Aigner E, Theurl M, Nairz M, Seifert M, *et al.* Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. Blood 2009; 113: 5277-86
(17) Gagliardo B, Kubat N, Faye A, Jaouen M, Durel B, *et al.* Pro-hepcidin is unable to degrade the iron exporter ferroportin unless maturated by a furin-dependent process. J Hepatol 2009; 50(2): 394-401
(19) Tiker F, Celik B, Tarcan A, Kilicdag H, Ozbek N, *et al.* Serum pro-hepcidin levels and relationships with iron parameters in healthy preterm and term newborns. Pediatr Hematol Oncol 2006; 23(4): 293-7
(20) Roe MA, Spinks C, Heath AL, Harvey LJ, Foxall R, *et al.* Serum prohepcidin concentration: no association with iron absorption in healthy men; and no relationship with iron status in men carrying HFE mutations, hereditary haemochromatosis patients undergoing phlebotomy treatment, or pregnant women. Br J Nutr 2007; 97(3): 544-9
(21) Balogh A, Szabó M, Kelen D, Bokodi G, Prechl J, *et al.* Prohepcidin levels during human perinatal adaptation. Pediatr Hematol Oncol 2007; 24(5): 361-8
(22) Ulukol B, Orhon FS, Hanoluk A, Akar N. Serum pro-hepcidin levels and relationship with ferritin in healthy non-anaemic infants. Acta Haematol 2007; 118(2): 70-2
(23) Kemna EH, Kartikasari AE, van Tits LJ, Pickkers P, Tjalsma H, *et al.* Regulation of hepcidin: insights from biochemical analyses on human serum samples. Blood Cells Mol Dis 2008; 40(3): 339-46
(24) Huang X, Fung ET, Yip C, Zeleniuch-Jacquotte A. Serum prohepcidin is associated with soluble transferrin receptor-1 but not ferritin in healthy post-menopausal women. Blood Cells Mol Dis 2008; 41(3): 265-9
(25) Hadley KB, Johnson LK, Hunt JR. Iron absorption by healthy women is not associated with either serum or urinary prohepcidin. Am J Clin Nutr 2006; 84(1): 150-5
(26) Luukkonen S, Punnonen K. Serum pro-hepcidin concentrations and their responses to oral iron supplementation in healthy subjects manifest considerable inter-individual variation. Clin Chem Lab Med 2006; 44(11): 1361-2
(27) Young MF, Glahn RP, Ariza-Nieto M, Inglis J, Olbina G, *et al.* Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. Am J Clin Nutr 2009; 89(2): 533-8
(28) Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood 2005; 106(5): 1864-6

(29) Hoppe M, Lönnerdal B, Hossain B, Olsson S, Nilsson F, *et al.* Hepcidin, interleukin-6 and hematological iron markers in males before and after heart surgery. J Nutr Biochem 2009 Jan; 20(1): 11-6
(30) Kulaksiz H, Gehrke S, Janetzko A, Rost D, Bruckner T, *et al*. Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004; 53: 735–743
(31) Orhon FS, Ulukol B, Hanoluk A, Akar N. Serum pro-hepcidin levels in infants with iron deficiency anaemia. Int J Lab Hematol 2008; 30(6): 546-7
(32) Kato A, Tsuji T, Luo J, Sakao Y, Yasuda H, *et al*. Association of prohepcidin and hepcidin-25 with erythropoietin response and ferritin in hemodialysis patients. Am J Nephrol 2008; 28(1): 115-21
(33) Beirão I, Almeida S, Swinkels D, Costa PM, Moreira L, *et al.* Low serum levels of prohepcidin, but not hepcidin-25, are related to anemia in familial amyloidosis TTR V30M. Blood Cells Mol Dis 2008; 41(2): 175-8
(34) Boinska J, Zekanowska E, Kwapisz J. Pro-hepcidin and iron metabolism parameters in multi-time blood donors. Int J Lab Hematol 2009 Nov 30. [*Epub ahead of print*]
(35) Malyszko J, Malyszko JS, Mysliwiec M. A possible role of hepcidin in the pathogenesis of anemia among kidney allograft recipients. Transplant Proc 2009; 41(8): 3056-9
(36) Olmez OF, Gurel S, Yilmaz Y. Plasma prohepcidin levels in patients with chronic viral hepatitis: relationship with liver fibrosis. Eur J Gastroenterol Hepatol 2009 Nov 24. [*Epub ahead of print*]
(37) Anderson DS, Heeney MM, Roth U, Menzel C, Fleming MD, *et al.* High-throughput matrix-assisted laser desorption ionization-time-of-flight mass spectrometry method for quantification of hepcidin in human urine. Anal Chem 2010; 82(4): 1551-5
(38) Arnold J, Sangwaiya A, Bhatkal B, Geoghegan F, Busbridge M. Hepcidin and inflammatory bowel disease: dual role in host defence and iron homoeostasis. Eur J Gastroenterol Hepatol 2009; 21(4): 425-9
(39) Ashby DR, Gale DP, Busbridge M, Murphy KG, Duncan ND, *et al.* Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009; 75(9): 976-81
(40) Bansal SS, Halket JM, Bomford A, Simpson RJ, Vasavda N, *et al.* Quantitation of hepcidin in human urine by liquid chromatography-mass spectrometry. Anal Biochem 2009; 384(2): 245-53
(41) Bansal SS, Halket JM, Fusova J, Bomford A, Simpson RJ, *et al.* Quantification of hepcidin using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2009; 23(11): 1531-42
(42) Busbridge M, Griffiths C, Ashby D, Gale D, Jayantha A, *et al.* Development of a novel immunoassay for the iron regulatory peptide hepcidin. Br J Biomed Sci 2009;66(3):150-7
(43) De Domenico I, Nemeth E, Nelson JM, Phillips JD, Ajioka RS, *et al.* The hepcidin-binding site on ferroportin is evolutionarily conserved. Cell Metab 2008; 8(2): 146-56
(44) Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. Immunoassay for human serum hepcidin. Blood 2008; 112(10): 4292-7
(45) Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, den Heijer M, Tjalsma H, *et al.* High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol 2009; 146(3): 317-25
(46) Kobold U, Dülffer T, Dangl M, Escherich A, Kubbies M, *et al.* Quantification of hepcidin-25 in human serum by isotope dilution micro-HPLC-tandem mass spectrometry. Clin Chem 2008; 54(9): 1584-6
(47) Koliaraki V, Marinou M, Vassilakopoulos TP, Vavourakis E, Tsochatzis E, *et al.* A novel immunological assay for hepcidin quantification in human serum. PLoS One 2009; 4(2): e4581.
(48) Li H, Rose MJ, Tran L, Zhang J, Miranda LP. Development of a method for the sensitive and quantitative determination of hepcidin in human serum using LC-MS/MS. J Pharmacol Toxicol Methods 2009; 59(3): 171-80
(49) Murao N, Ishigai M, Yasuno H, Shimonaka Y, Aso Y. Simple and sensitive quantification of bioactive peptides in biological matrices using liquid chromatography/selected reaction monitoring mass spectrometry coupled with trichloroacetic acid clean-up. Rapid Commun Mass Spectrom 2007; 21(24): 4033-8
(50) Murphy AT, Witcher DR, Luan P, Wroblewski VJ. Quantitation of hepcidin from human and mouse serum using liquid chromatography tandem mass spectrometry. Blood 2007; 110(3): 1048-54
(51) Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, *et al*. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008; 3(7): e2706
(52) Ward DG, Roberts K, Stonelake P, Goon P, Zampronio CG, *et al.* SELDI-TOF-MS determination of hepcidin in clinical samples using stable isotope labelled hepcidin as an internal standard. Proteome Sci 2008; 14(6): 28
(53) Kroot JJ, Kemna EH, Bansal SS, Busbridge M, Campostrini N, *et al.* Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. Haematologica 2009; 94(12): 1748-52
(55) Kemna EH, Tjalsma H, Podust VN, Swinkels DW. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin Chem 2007; 53(4): 620-8
(56) Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, *et al.* Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003; 101(7): 2461-3
(57) Détivaud L, Nemeth E, Boudjema K, Turlin B, Troadec MB, *et al.* Hepcidin levels in humans are correlated with hepatic iron stores, hemoglobin levels, and hepatic function. Blood 2005; 106(2): 746-8
(58) Kroot JJ, Hendriks JC, Laarakkers CM, Klaver SM, Kemna EH, *et al.* (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem 2009; 389(2): 124-9
(59) Bergamaschi G, Villani L. Serum hepcidin: a novel diagnostic tool in disorders of iron metabolism. Haematologica 2009; 94(12): 1631-3

(60) Hunter HN, Fulton DB, Ganz T, Vogel HJ. The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. J Biol Chem 2002; 277(40): 37597-603
(61) Aeberli I, Hurrell RF, Zimmermann MB. Overweight children have higher circulating hepcidin concentrations and lower iron status but have dietary iron intakes and bioavailability comparable with normal weight children. Int J Obes (Lond) 2009; 33(10): 1111-7
(62) Peeling P, Dawson B, Goodman C, Landers G, Wiegerinck ET, *et al.* Effects of exercise on hepcidin response and iron metabolism during recovery. Int J Sport Nutr Exerc Metab 2009; 19(6): 583-97
(63) Brugnara C. An immunoassay for fuman serum hepcidin at last: Ganz klar? Blood 2008; 112(10): 3922-23
(64) Lasocki S, Baron G, Driss F, Westerman M, Puy H, *et al.* Diagnostic accuracy of serum hepcidin for iron deficiency in critically ill patients with anemia. Intensive Care Med 2010 Mar 6. [*Epub ahead of print*]
(65) Cherian S, Forbes DA, Cook AG, Sanfilippo FM, Kemna EH, *et al.* An insight into the relationships between hepcidin, anemia, infections and inflammatory cytokines in pediatric refugees: a cross-sectional study. PLoS One 2008; 3(12): e4030
(67) Peters HP, Laarakkers CM, Swinkels DW, Wetzels JF. Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010; 25(3): 848-53
(68) Weiss G, Theurl I, Eder S, Koppelstaetter C, Kurz K, *et al.* Serum hepcidin concentration in chronic haemodialysis patients: associations and effects of dialysis, iron and erythropoietin therapy. Eur J Clin Invest 2009; 39(10): 883-90
(69) Zaritsky J, Young B, Wang HJ, Westerman M, Olbina G, *et al.* Hepcidin--a potential novel biomarker for iron status in chronic kidney disease. Clin J Am Soc Nephrol 2009; 4(6): 1051-6.
(70) Valenti L, Girelli D, Valenti GF, Castagna A, Como G, *et al*. HFE mutations modulate the effect of iron on serum hepcidin-25 in chronic hemodialysis patients. Clin J Am Soc Nephrol 2009; 4(8): 1331-7
(71) Malyszko J, Malyszko JS, Mysliwiec M. Serum prohepcidin and hepcidin in hemodialyzed patients undergoing iron therapy. Kidney Blood Press Res 2009; 32(4): 235-8
(72) Malyszko J, Malyszko JS, Mysliwiec M. Hyporesponsiveness to erythropoietin therapy in hemodialyzed patients: potential role of prohepcidin, hepcidin, and inflammation. Ren Fail 2009; 31(7): 544-8.
(73) Robach P, Recalcati S, Girelli D, Gelfi C, Aachmann-Andersen NJ, *et al.* Alterations of systemic and muscle iron metabolism in human subjects treated with low-dose recombinant erythropoietin. Blood 2009; 113(26): 6707-15.
(74) Costa E, Swinkels DW, Laarakkers CM, Rocha-Pereira P, Rocha S, *et al.* Hepcidin serum levels and resistance to recombinant human erythropoietin therapy in haemodialysis patients. Acta Haematol 2009; 122(4): 226-9
(75) Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? Nephrol Dial Transplant 2008; 23(8): 2450-3
(76) De Falco L, Totaro F, Nai A, Pagani A, Girelli D, *et al.* Novel TMPRSS6 mutations associated with iron-refractory iron deficiency anemia (IRIDA). Hum Mutat 2010 Mar 15. [*Epub ahead of print*]
(78) Beutler E, Van Geet C, te Loo DM, Gelbart T, Crain K, *et al*. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. Blood Cells Mol Dis 2010; 44(1): 16-21
(79) Swinkels DW, Janssen MCH, Bergmans J, Marx JJM. Hereditary hemochromatosis: Genetic complexicity and new diagnostic approaches. Clin Chem 2006; 52(6): 950-68
(80) Piperno A, Girelli D, Nemeth E, Trombini P, Bozzini C, *et al.* Blunted hepcidin response to oral iron challenge in HFE-related hemochromatosis. Blood 2007; 110(12): 4096-100.
(81) Bozzini C, Campostrini N, Trombini P, Nemeth E, Castagna A, *et al.* Measurement of urinary hepcidin levels by SELDI-TOF-MS in HFE-hemochromatosis. Blood Cells Mol Dis 2008; 40(3): 347-52.
(82) van Dijk BA, Laarakkers CM, Klaver SM, Jacobs EM, van Tits LJ, *et al.* Serum hepcidin levels are innately low in HFE-related haemochromatosis but differ between C282Y-homozygotes with elevated and normal ferritin levels. Br J Haematol 2008; 142(6): 979-85

3) Reference Works, Handbooks and Databases

(11) Burtis C, Ashwood E, Bruns D. Tietz textbook of clinical chemistry and molecular diagnostics. Elsevier Inc. 2006; 4th edition: pp 562-563 and pp 1186-1191
(13) Kliegman R, Jenson H, Behrman R, Stanton F. Nelson essentials of pediatrics. Saunders Elsevier Inc. 2008; 18th edition, chapter 455.

I. The analytical aspects implicated in hepcidin determination.

1.1 Hepcidin versus pro-hepcidin

The human hepcidin gene HAMP encodes a precursor protein of 84 amino acids, pre-prohepcidin, which undergoes enzymatic cleavage to prohepcidin (7). Prohepcidin itself has no biological activity, it is unable to degrade the iron exporter ferroportin in *in vitro* experiments (17) and unable to induce hypoferraemia in mice (18) unless maturated by a furin-dependant cleaving, generating hepcidin-25.
Many studies measure prohepcidin as a surrogate marker for hepcidin, as a specific ELISA for hepcidin-25 was not available until recently (*see 1.2, analytical techniques*). This raises the question if prohepcidin levels are physiologically relevant, reflecting mature hepcidin levels and/or interpretable in a clinical/ diagnostic setting. Attachment 5 gives an overview of studies assessing the correlation of prohepcidin levels with hepcidin levels, iron or anemia parameters or clinical data (19-36).
All studies that directly compared prohepcidin and hepcidin levels found no correlation between both (23,24,27-29,33,35). In healthy subjects, no correlations to serum iron, ferritin or transferrin saturation were described. One study however, reported an association of prohepcidin concentration with sTfR1, but not with ferritin in 45 healthy post-menopauzal women, while hepcidin correlated with ferritin but not with sTfR1 (24). No correlation of prohepcidin levels with iron absorption during supplementation can be found (20,25-27). The situation in patients with known iron disorders is less clear. Kemna *et al.* (23) described no differences in prohepcidin levels between healthy controls and patients with hemochromatosis, iron-deficiency anemia, thalassemia major or an acute phase reaction (in contrast to hepcidin). In contrast Kulaksiz *et al.* (30) reported significantly lower prohepcidin in hemochromatosis patients than healthy controls and significantly higher levels in renal insufficiency. Levels decreased in renal insufficiency with renal anemia, compared with renal insufficiency without concomitant anemia. However, defining a cut-off to differentiate these subpopulations would be problematic (see detail of results in attachment 6).
In conclusion, it is doubtful that prohepcidin is a physiologically relevant parameter. As hepcidin is the actually active hormone, direct measurement of this mature 25 amino acid peptide is clearly recommended.

1.2 Analytical techniques

Hepcidin is a small peptide, with a compact structure because of four disulfide bonds, implicating that only few antigenic epitopes are present. This, along with a high degree of conservation among species, makes the induction of antibodies in animals very difficult (42,55). As a result, no specific immunochemical detection method was available until recently. The former DRG 'hepcidin' assay actually measured prohepcidin as it recognized an epitope outside the amino acid sequence of hepcidin-25. Many of the current knowledge about hepcidin was obtained with a semiquantitative immunodot assay, a laborious method based on extraction by cation-exchange chromatography and subsequent chemiluminescence quantification with rabbit antibodies. Secondly, mass spectrometry (MS) techniques emerged as an alternative, mainly SELDI-TOF/MS (Surface-Enhanced Laser Desorption/Ionization Time Of Flight) and LC-MS/MS. But as these methods lack an internal standard, this results can be expressed only in semiquantitative arbitrary units (3).
In 2007, Murphy *et al.* (50) described the first quantitative method (LC MS/MS), but it's internal standard (calcitonin gene-related peptide) was far from ideal as it differed too much from hepcidin. Very recently, substantial advances in quantitative determination of hepcidin were achieved (3). In 2008, Ganz *et al.* (44) were the first to successfully create sufficient amounts of specific anti-hepcidin-Ab to design a competitive ELISA. Since then, several *in-house* RIA assays were described, including one recently reported assay with a very low detection limit and low imprecision (45). In 2009, a hepcidin-25 ELISA (DRG) became commercially available. "Second generation" SELDI-TOF MS and LC MS/MS now contain good internal standards, mostly isotope labeled hepcidin ($^{13}C/^{15}N$ on various positions), but also synthetic hepcidin-24 (lacking the aminoterminal Asp) or recombinant 25-hepcidin-His. Attachment 7 gives an overview of all quantitative techniques described (37-52).
Currently, there is no reference method for hepcidin-25 measurement. As a first step toward method harmonization, several (eight) hepcidin assays were recently compared (53). Spearman correlations between methods were generally high and the between-sample variation and the analytical variation of the methods are comparable. The analytical variation as percentage of total variance is low, indicating the suitability of all methods to distinguish hepcidin levels of different samples. However, hepcidin concentrations in urine and serum differed widely between all methods. These differences are suggested to be caused by (i) the use of different calibrators, (ii) possible hepcidin aggregation in the sample or the standard solution, (iii) measuring only free, only bound or both fractions of hepcidin, as hepcidin is shown to be 90% α2-macroglobulin bound in plasma, or (iv) by interference of hepcidin isoforms hepcidin-22 and hepcidin-20. Not only it is unknown what fraction (bound, unbound, isoforms) these different methods actually measure, but it also raises the question what fraction would be biologically relevant to measure.

1.3 Matrix: urine or blood?

Mainly for technical reasons, most of the initial studies of hepcidin used urinary hepcidin assays. This is an indirect measurement of the circulating hormone, normalized to creatinine (44). Multiple confounding factors exist concerning determination of hepcidin in urine (table 1). Only few quantitative studies directly compared urine and serum concentrations. Good correlations (r=0.82) between urinary and serum levels were found in 24 healthy controls (44). Kemna *et al.* (55) also reported a strong association of urinary and serum levels. In another trial (24 healthy controls), however, only a weak correlation of urinary and serum levels was described (r=0.55), no correlation of urinary levels with ferritin and a high relative contribution of (pre)analytical variability of 47% to the total variance - in contrast to serum, where the between-subject variability was largest (57%). This indicates a poor reliability of the urinary hepcidin results compared to serum (58). However, this was not confirmed in a round robin of analytical methods, where the relative analytical variance for urinary hepcidin was not higher than for plasma, higher between-sample CV's for urinary hepcidin than plasma hepcidin. Other (semiquantitative) studies (55-57) described a good relationship of urinary hepcidin with clinical iron disorders, hepcidin mRNA expression in liver cells or ferritin. Discrepancies between studies are possibly method dependent, as the matrix protein content can interfere with surface-binding in SELDI-TOF MS, isoforms are differently measured etc. In conclusion, the usefulness of urine as matrix for hepcidin determination remains unclear. Large trials comparing urine and serum or plasma are needed to determine whether the serum concentration or the urinary excretion is the most accurate biomarker of iron metabolism (59). Serum is likely to be more sensitive than urine for monitoring short-term kinetics of body hepcidin concentrations (55).

Table I: Confounding factors in urinary hepcidin determination and their implications		***Reference***
Isoforms hepcidin-20 and hepcidin-22 in urine*	- Unknown physiological relevance - Attributes to differences between methods	*53*
Altered characteristics of hepcidin-25 in urine? **	- Unknown physiological relevance - Unknown interference with determinations	*43*
Unknown renal reabsorption and excretion kinetics	- Interpretation in kidney disease unclear	*44*
Prohepcidin production in the kidney tubular system with possible renal iron regulatory role	- Relative contribution, regulation and function of this synthesis unknown	*18*
More affected by multiple freeze-thaw cycles and storage conditions than serum	- Less stable than serum	*55*
Discrepancies in studies assessing correlation of urine and serum levels in healthy individuals	- Correlation with serum hepcidin levels still unclear	*44, 58*
Different diurnal patterns of urinary and serum hepcidin	- Different protocols needed for urinary or serum determination	*58*
Higher between-sample CV for urinary hepcidin than serum hepcidin, in six different quantification methods	- Possibly not due to the method, but due to a biological mechanism, interpretation unclear. - Discrepancy with another study***.	*53, 58*

** hepcidin-20 is also detected in serum. ** In an assay measuring biologically active hepcidin (43), hepcidin could not be detected in urine while this assay could readily quantitate hepcidin when added to urine (for method, see attachment 7) *** cfr. description in text*

1.4 Pre-analytical aspects of serum hepcidin-25 quantitation

Table 2 gives an overview of described pre-analytical factors implicating hepcidin-25 determination. All these aspects indicate the need for careful handling and robustly standardized pre-analytical procedures. Firstly, standardized sampling time is needed. The 8 am fasting hepcidin concentrations appeared to be most consistent. Hepcidin is stable for 8 months at -80°C. Serum levels vary substantially between healthy subjects, the difference between men and women is significant implicating the need for different reference intervals.

Table 2: Pre-analytical aspects in quantification of hepcidin-25		***Reference***
Diurnal variation (intraindividual variation)	Hepcidin levels increase during the day. Circadian rhythm correlates inversely with daily variation in serum iron. Unknown whether this variation depends on the innate rhythm of hepatic production or by dietary factors.	*44, 55, 58*
Freeze-thaw, storage conditions	Decreases in serum levels due to multiple freeze-thaw cycles is of minor importance. Hepcidin is only stable when stored on -80°C compared to -20°C. Addition of protease inhibitor had no influence.	*55*
Hepcidin aggregation	Hepcidin-25 aggregates as the concentration is increased. Sedimentation studies gave unusual results consistent with hepcidin-25 aggregating to the point of precipitation as the spinning speed was increased.	*60*
Plastic laboratory material	Hepcidin in high concentrations can adhere to plasticware	*52*
Interindividual variation	Serum levels vary substantially between healthy subjects. Significant differences between men and women. Trend for age-related increase in hepcidin? (further confirmation needed). Potential role of body weight (obesity). Influence of exercise, even up to 24h after recovery from high-intensity exercise.	*44, 58, 60, 61, 62*

2. Role of hepcidin in the diagnosis of iron deficiency anemia in adults and children.

Serum hepcidin levels correlate well with serum ferritin in healthy subjects and significantly lower hepcidin levels are found in iron deficiency (44). Hepcidin is also a type II acute phase protein and increased hepcidin levels correlate with increased ferritin levels in chronic inflammatory conditions (28,56). This raises the question if hepcidin has additional value in the differential diagnosis of iron disorders, compared with the traditional parameters, especially with ferritin (63). Ferritin is a useful marker of iron stores, but changes in hepcidin concentrations are frequently *the cause* of iron disorders. An inflammation-dependant increase in hepcidin explains the reduced availability of iron in anemia of chronic disease. Hepcidin measurement should therefore give more information about the etiology of iron disorders (44) and could allow detection before irons stores are depleted. The potential advantageous aspects of hepcidin measurement and its evidence for diagnostic use in conditions that still propose a diagnostic challenge (cfr. *Clinical and diagnostic scenario*) is summarized below.

2.1 Hepcidin "time to positivity"

In the acute phase reaction following lipopolysaccharide (LPS) injection in healthy subjects, hepcidin levels in urine are shown to respond much faster than serum ferritin levels, as hepcidin peaked 22 hours after LPS administration, while ferritin stayed within normal reference intervals up to 22 hours (28). Serum hepcidin levels have the potential of responding even faster than urinary excretion. This creates the hope that hepcidin does not only respond earlier to acute phase, but also more readily to other iron disequilibrium states than ferritin or other markers, making diagnosis more sensitive. A possible application would be the screening of infants for iron deficiency before anemia develops (63). However, this advantage of hepcidin is still strictly hypothetical.

2.2 Anemia of chronic disease with/without iron deficiency anemia

Patients with iron-deficiency have significantly lower hepcidin levels than healthy controls (44,47). In anemia of chronic disease (ACD) hepcidin is increased due to inflammation. Only three studies determined hepcidin levels in ACD patients with and without concomitant ID(A) to evaluate if hepcidin could be a useful marker to detect underlying IDA in ACD (table 3). In these studies, deficient iron stores seems to be the dominant regulator of hepcidin, as hepcidin levels are low in the presence of IDA, independently of inflammation. This creates the potential of establishing a hepcidin cut-off for the differentiation of ACD with ACD+IDA. Lasocki *et al.* (64) defined a cut-off for ID in ICU-patients, but only 5 patients in their study had ID (only 3 with concomitant inflammation) and in 1 patient the presence of ID was unclear. Although a good specificity was reached, sensitivity of this cut-off was only 50%. There are currently no studies that have prospectively validated a cut-off for the diagnosis of IDA. Further studies are needed, first to confirm the distinct difference in hepcidin levels between "clear cases" of ACD and ACD+IDA and later to assess applicability of hepcidin in patients in the 'grey zone' in which the presence of IDA is unclear, as these are exactly the cases in which hepcidin is hoped to clarify differential diagnosis.

Table 3: Evidence for the clinical application of hepcidin quantitation in ACD with or without IDA

Study	Study population (+ definitions used)	Results
Theurl *et al.* (18)	**Control (n=26)** Not anemic, normal iron status, no sign of inflammation **IDA (n=12)** Hb ♀<12 g/dL, ♂ <13 g/dL; no sign of inflammation; ferritin <30 ng/mL **ACD (n=15)** Hb ♀<12 g/dL, ♂ <13 g/dL; chronic infection or autoimmune disease; ferritin >100 ng/mL OR 30-100 ng/mL with sTfR/log ferritin ratio <1 **ACD+IDA (n=14)** Hb ♀<12 g/dL, ♂ <13 g/dL; chronic infection or autoimmune disease; ferritin 30-100 ng/mL with sTfR/log ferritin ratio >2	ACD subjects had significantly higher serum hepcidin levels and IDA significantly lower levels than controls. ACD had significant higher levels than ACD/IDA patients. ACD/IDA did not differ significantly from IDA patients. *Note: detailed results in attachment 8*
Cherian *et al.* (65)	Pediatric refugees: African, <16years, n=147 of which **ID (n=35)** (≥2 abnormal age-corrected iron parameters: iron, TS, transferrin, ferritin) **IDA (n=25)** (ID definition + anemia). **High incidence of infections** in this population (helminth infection in 42%; *P. falciparum* in 8.8%; ; *H. pylori* in 81.8%)	Children with ID and IDA had significant lower urinary hepcidin levels. There was no influence of co-morbid infections (hepcidin was not significantly different in children with or without malaria, helminth infection or *H. pylori* infection) and no correlation with inflammatory cytokines as IL-6.
Lasocki *et al.*(64)	**ICU patients (n=48)** with Hb < 100 g/L and ICU stay > 1week 32/48 patients had >1 hepcidin measurement (weekly) 'ID' diagnosis (n=5/48) relied on consensus opinion of three experts in iron metabolism	Patients who developed a profile of ID during ICU stay had a progressive decline in hepcidin levels, despite elevated CRP. A cut-off for ID in these patients was defined at 129.5 µg/L (sensitivity 50%; specificity 85%)

2.3 Iron deficiency in chronic kidney disease and erythropoiesis-stimulating agents (ESA).

Hepcidin may be relevant in CKD and explain the frequent imbalance in iron metabolism and the resistance to ESA. As such, hepcidin could become an important tool in predicting responsiveness and guiding ESA therapy or IV iron. Most patients with an insufficient response to ESA have not an absolute but a functional iron deficit which cannot be identified with ferritin or TSAT. It is hypothesized that these patients have higher hepcidin levels, predicting hyporesponsiveness and a potential good response to IV iron. Increased hepcidin levels have been described in CKD (increasing gradually with CKD stage) and hemodialysis (39,44,48,69,70,74). However, many factors influence hepcidin levels in CKD: not only iron status or anemia but also inflammation, hypoxia and (endogenous and exogenous) erytropoietin (66). In addition, hepcidin correlates inversely with the estimated glomerular filtration rate (39), suggesting a contribution of decreased renal excretion to hepcidin increase. However, one study did not find a significant relationship of hepcidin-25 with eGFR, although total hepcidin (hepcidin-25, -20 and -22) did correlate inversely with eGFR in this study (67). These results need confirmation in a larger trial. Weiss *et al* (68) described clearance of hepcidin with the dialysis procedure, but levels returned to pre-dialysis levels before the next dialysis session. This clearance is variable, probably due to differences in membranes or residual kidney function.

Clinical studies assessing the usefulness of hepcidin determination to distinguish ESA responders and hypo-responders are sparse. Kato *et al.* (32) found no difference in the intensities of serum hepcidin between rHuEPO-hyporesponsive and responsive patients. In the study of Ashby et al. (39) hepcidin correlated with anemia in dialysis patients, consistent with a potential role for hepcidin excess in renal anemia, but, an inverse correlation of hepcidin and ESA dose was also described, limiting the diagnostic potential of hepcidin to predict increased rHuEPO requirements. Costa et al. (74) observed a trend for lower hepcidin levels among non-responders (not significant), but this results are possibly biased, since non-responders received much higher rHuEPO doses in comparison to responders. Some authors suggest to monitor the initial change in serum hepcidin after a first dose of IV iron and/or ESA as it seems unlikely that a single value before the start of anemia treatment will predict responsiveness (75). However, no studies are conducted yet that assess this kinetic change. In conclusion, more studies are needed to clarify the diagnostic utility of hepcidin determination in CKD.

Table 4*: Evidence for the diagnostic usefulness of hepcidin quantitation in CKD or rHuEPO therapy

Study	Study population	Results
Ashby *et al.* (39)	- CKD group (n=44) Stable, without dialysis (no rHuEPO or IV iron therapy) - Hemodialysis (n=94) - Controls (n=64)	Hepcidin levels are significantly elevated in CKD and correlated positively with ferritin and inversely with GFR Hepcidin is elevated in hemodialysis, but does not correlate with IL-6. Erythropoietin dose correlated inversely with hepcidin Higher hepcidin levels do not predict increased rHuEPO requirements
Zaritsky *et al.* (69)	- Pediatric CKD (n=48) with stage 2-4 CKD - Adult CKD (n=32) with stage 2-4 CKD - Pediatric CKD (n=26) with stage 5 CKD (on peritoneal dialysis) - Control (n=20): Healthy children	Hepcidin increased gradually and significantly across different stages of CKD.
Valenti *et al.* (70)	- Hemodialysis patients (n=65) - Controls (n=57)	Hepcidin is increased in hemodialysis patients, regulated by iron stores and inflammation and relatively reduced in subjects carrying HFE mutations
Li *et al.*(48)	- CKD (n=50) - Healthy controls (n=60)	Hepcidin mean concentration was <10 ng/mL in controls. CKD patients had significantly higher levels (mean serum concentration 99 ng/mL)
Malyszko *et al.* (71)	12 Hemodialysis patients, before and after IV iron	Serum hepcidin increased significantly after IV iron
Malyszko *et al.*(72)	Hemodialysis patients (n=98)	Not-significant tendency toward higher values of hepcidin in rHuEPO hyporesponsive patients in comparison to responsive patients. Hyporesponsiveness it is mainly associated with subclinical inflammation.
Robach *et al.*(73)	Healthy volunteers (n=8) receiving 4 weeks of rHuEPO	Hepcidin was rapidly suppressed from day 2 of rHuEPO administration.
Kato *et al.*(32)	Hemodialysis patients (n=75; 15 patients were rHuEPO hyporesponsive, 9 patients responsive)	No difference in the intensities of serum hepcidin between rHuEPO-hyporesponsive and responsive patients: hepcidin had no predictive value
Costa *et al.*(74)	- Hemodialysis patients under rHuEPO therapy (n=33) (16 rHuEPO responsive, 17 non-responsive) - Healty controls (n=17)	HD patients had higher hepcidin levels than controls. Trend for lower hepcidin levels among non-responders in comparison with responders (not significant). The results can possibly be explained by rHuEPO doses, since non-responders received much higher doses in comparison to responders.
Weiss *et al.*(68)	Hemodialysis patients (n=20) of which 5 rHuEPO therapy, 9 IV iron only, 6 IV iron + rHuEPO. (Patients with significant inflammatory co-morbidity were excluded)	Serum hepcidin levels were significantly reduced during the dialysis procedure and returned to pre-dialysis values before the next dialysis session. rHuEPO therapy, but not iron, significantly reduced hepcidin levels. Negative association of hepcidin with reticulocytosis.

* Table partly based on Castagna *et al.* (54)

2.4 Iron deficiency anemia in infants

Hepcidin could have the potential of detecting iron deficiency before anemia develops, making it a possible screening tool for ID in infants. However, no studies assessed this use of hepcidin yet. Studies concerning *pro*hepcidin in infants showed no correlation with iron status (19,22,31) (*cfr. attachment 5*). As infants are unable to regulate gut iron absorption, it is possible that hepcidin (as it is the "iron absorption regulator") will have no diagnostic value. Studies are needed to clarify this topic.
Iron-refractory iron deficiency anemia (IRIDA) is an familial disorder of IDA unresponsive to oral iron treatment. Mutations in IRIDA patients are heterogeneous and include frame-shift, splicing, missense and nonsense mutations in TMPRSS6, a gene encoding for matriptase-2. Matriptase-2 normally represses hepcidin expression by cleaving membrane-bound hemojuvelin (*cfr. attachment 2*). Both in humans or in animal models, these mutations lead to inappropriately high hepcidin levels, making gut absorption of iron impossible (76-78). Early identification is needed to implement parenteral iron supplementation. In iron-deficiency patients with inappropriately high hepcidin levels without inflammation, IRIDA can be suspected. No studies exist that use hepcidin as a diagnostic or screening tool for this condition in iron-deficiency.

3. Hepcidin in hemochromatosis

Diagnosis of hemochromatosis relies on genotyping. The only potential use of hepcidin in diagnosis is to direct the protocol of different genetic tests, as hepcidin (or hepcidin/ferritin ratio) can be high or low depending on the underlying genetic mutation (79) (Table 5). Rational gene targeting is based on information on clinical presentation, hemoglobin (low in secondary forms of iron overload and in some cases with ferroportin disease), family history (hereditary disease), concomitant diseases (e.g., hepatitis and alcohol abuse), age at presentation. Hepcidin could be added to this list to stratify molecular testing. Only one study suggested hepcidin determination for this purpose (79). Their protocol is given in attachment 9. Future studies are needed to validate this flow chart.

Table 5: Characteristics of the hereditary forms of iron overload

	Classic	Juvenile		TfR2-related	ferroportin disease
		HJV-related	Hepcidin-related		
gene	HFE	HJV	HAMP	TfR2	SLC40A1
OMIM type	1	2a	2b	3	4
interaction	TfR1?	BMP-R	ferroportin	transferrin	hepcidin
frequency	frequent	rare	very rare	very rare	rare
hepcidin	(↓)	↓↓	↓↓	↓	↑
severity	++	++++	++++	+++	+

Table adopted from Swinkels *et al.* 2006 (79)

A more important application of hepcidin in HH is situated in a therapeutic setting, namely to monitor (the need for) phlebotomy in HH. At first sight, hepcidin does not seem suitable for this goal, as abnormal hepcidin levels cause the disease, not sensing iron stores. Indeed, despite continued phlebotomy, persistently low hepcidin translates into persistently increased gut iron absorption. However, in a limited set of patients with a new HFE-related HH diagnosis (C282Y homozygotes) (80), low hepcidin levels in urine even further decreased with iron depletion through phlebotomy (80,81) and this decrease correlated with the decrease in transferrin saturation (80). The same trend was confirmed in serum (82). These data suggest that an 'overshooting' with phlebotomy could have a negative impact by further decreasing hepcidin levels, thus increasing gut iron absorption. Based on their results, Piperno *et al.* (80) suggest an upward revision of the current ferritin target (now <50 µg/L) to search for an optimal balance of low ferritin and minimal decrease of hepcidin. Hepcidin measurements should be evaluated in this setting in further clinical trials. It is unclear whether defining a hepcidin level or hepcidin/ferritin ratio as target for phlebotomy would be possible in C282Y homozygotes. No data exist concerning therapy monitoring in other HH mutations.

TO DO/ACTIONS

1) Evaluation of the commercial Hepcidin ELISA (DRG) for the diagnosis of anemia
A study protocol (accepted by the Ethical Committee UZ Leuven) has been drawn up, in which we will first test a limited number of healthy controls and adult patients with clear IDA or ACD to determine whether there is a significant difference in hepcidin levels between these groups. If true, we will prospectively validate a cut-off for serum hepcidin in patients with anemia.

2) Discuss findings of this CAT and the evaluation with adult and pediatric hematologists

Attachment 1: Key role of hepcidin in iron uptake and recycling.

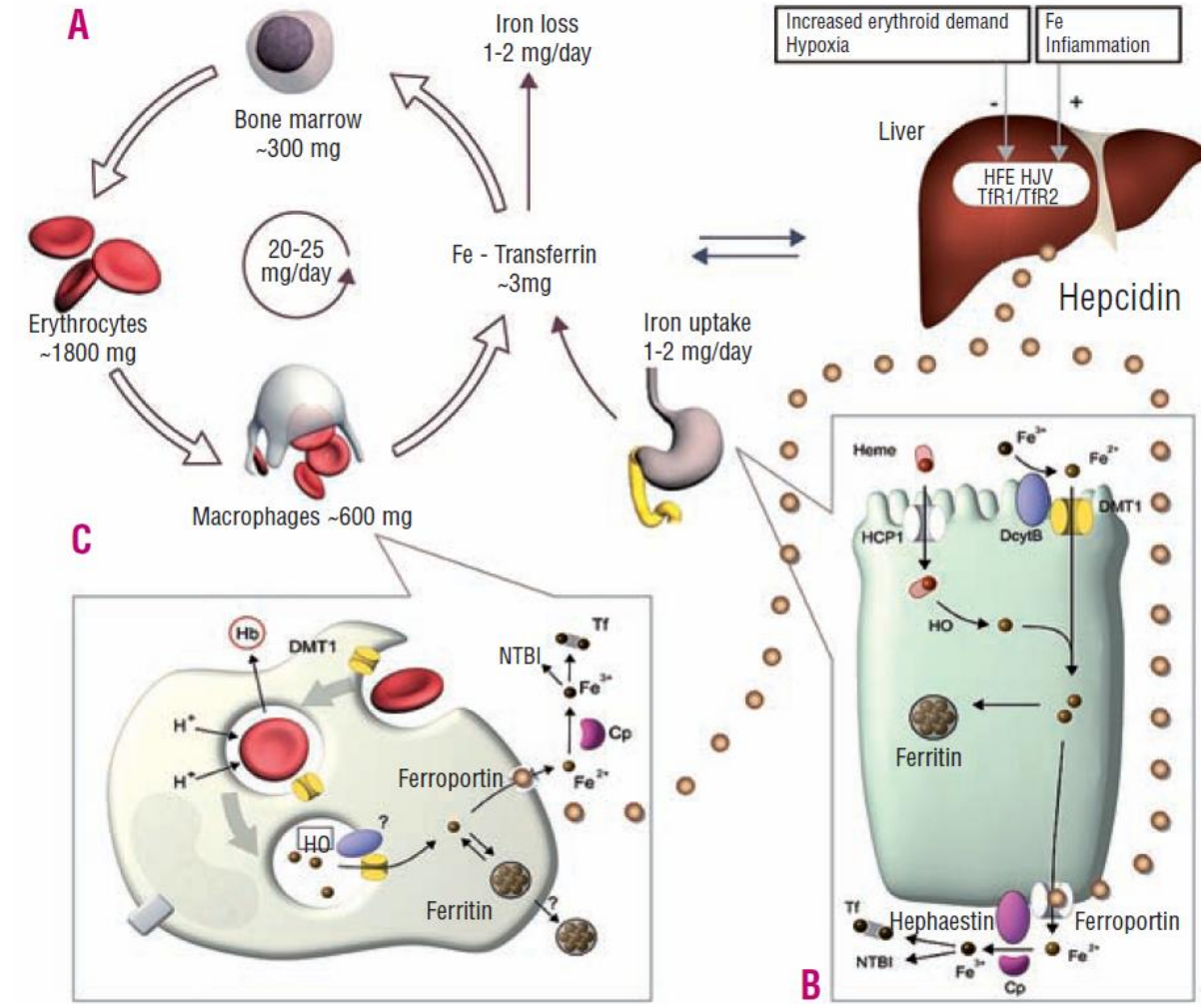


Figure and text adopted from Kemna et al. (7):

A. Most of the utilized body iron is recycled from senescent erythrocytes by macrophages and returned to the bone marrow for incorporation in erytroid precursors. The liver and reticuloendothelial macrophages function as iron stores. Only duodenal absorption is regulated by transporters such as DMT1 and HCP1, whereas iron loss occurs passively. **B.** The liver-produced peptide hepcidin controls the plasma iron concentration by inhibiting iron export by ferroportin from enterocytes and **C.** macrophages. This means that an increased hepcidin production leads to a decrease in plasma iron concentrations. Hepcidin induces internalization and degradation of ferroportin, diminishing the number of iron exporters and suppressing iron uptake and release respectively.

Abbreviations: DMT1: divalent metal transporter 1; Hb: hemoglobin; HO: heme oxygenase; NTBI: non-transferrin bound iron; Tf: transferrin; Cp: ceruloplasmin; HCP1: heme carrier protein 1; DcytB: duodenal cytochrome B. (7)

Attachment 2: Overview of regulating mechanisms of hepcidin production

Regulator	Mechanism	Signalling pathway	Effect	Human disease
Iron stores				
HFE	Part of a "hepatocyte iron sensing complex" through interactions with TfR1, TfR2, hepatocyte membrane.	BMPs/SMAD4	Sufficient iron stores induce HFE-mediated signal to upregulate hepcidin	Defective HFE is shown in hemochromatosis
TfR2	Fe^{2+}-bound transferrin levels increase the stability of TfR2 – hypothesized to be a serum iron sensor	?	Tfr2 induces hepcidin synthesis	
HJV	BMP co-receptor existing in a soluble (s) and membrane-bound (m) form. s-HJV can bind to BMP and function as a negative antagonist of m-HJV	BMPs/SMAD	Hypoxia and ID are suggested to release s-HJV and reduce m-HJV, inhibiting hepcidin production.	Hemochromatosis
TMPRSS6	TMPRSS6 participates in a transmembrane signaling pathway triggered by iron deficiency and independent to the known *Hamp* activation pathways.	?	TMPRSS6-mediated *Hamp* suppression is the predominant pathway, determinant for acquiring dietary iron uptake.	Mutation leads to iron deficiency (IRIDA); dysregulation or overexpression can lead to iron overload
GDF15	Growth factor secreted by erythroid precursors, Recently sensitive upregulation of GDF15 was shown in response to iron depletion	BMP/SMAD?	GDF15 is a potential interacting component between iron and oxygen sensing (further study needed)	In thalassemia, expanded erytroid compartment leads to GDF15 overexpression, contributing to iron overload. High levels observed in CDA I.
Inflammation				
IL-6, IL-1α	IL-6 and other cytokines induce *Hamp* transcription by activating the hepcidin gene promoter. The inactivation of HFE does not affect the increase of hepcidin expression and hypoferremia induced by inflammation.	STAT3 (requires SMAD4 to modulate gene expression)	Upregulation of hepcidin. Mechanism possibly dominant to the other regulatory pathways.	Anemia of chronic disease
Hypoxia				
HIF-1, ROS	In hypoxia these proteins are stable and function as transcription factors. In normal conditions: hydroxylation and degradation.	HIF-1 Oxygenases inhibition	In hypoxia downregulating hepcidin expression and upregulating EPO and ferroportin, needing adequate ROS levels.	
ROS	ROS levels are needed for inhibition of HIF-1 hydroxylation. ROS is induced in oxidative stress.	C/EBPα; STAT3 block ; oxygenases inhibition; HDAC activation	Downregulation of hepcidin	Hepatitis C viral and alcoholic liver disease
Oxygenases	Oxygenases are needed to cause HIF hydroxylation and maintaining high hepcidin expression	?	Maintaining high hepcidin expression in non-hypoxia conditions	
HJV	BMP co-receptor existing in a soluble (s) and membrane-bound (m) form. s-HJV can bind to BMP and function as a negative antagonist of m-HJV	BMPs/SMAD	Hypoxia and ID are suggested to release s-HJV and reduce m-HJV, inhibiting hepcidin production.	Hemochromatosis Iron-refractory iron-deficiency anemia (IRIDA)
Erytropoiesis				
EPO	At a high concentration able to inhibit hepcidin expression in vitro	EPO receptor and C/EPBα	Unclear in vivo. Effect in vitro, not seen in murine models.	
GDF15	Growth factor secreted by erythroid precursors. GDF15 is responsive to hypoxia and involved in hepcidin downregulation	BMP/SMAD?	GDF15 is a potential interacting component between iron and oxygen sensing (further study needed)	Thalassemia CDA I *(cfr supra)*

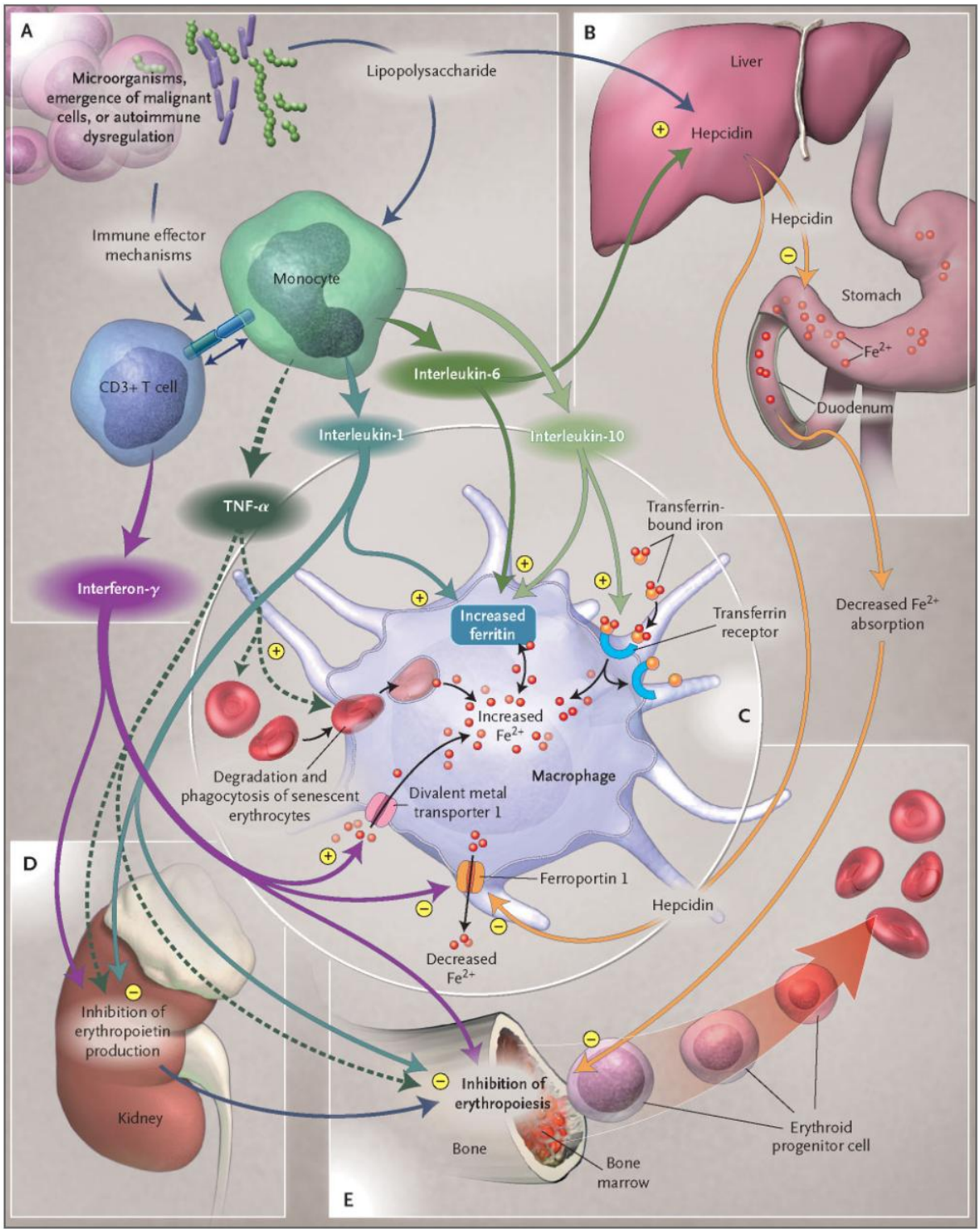
Note: The mandatory signalling pathway for expression of hepcidin is BMP/SMAD mediated. Specific BMPs (bone morphogenetic proteins) are shown to activate HAMP transcription through phosphorylation of receptor-SMADs (R-SMADs) within the cytosol of hepatocytes. This phosphorylated product forms a complex with SMAD4 which activates HAMP transcription. SMAD-4 knock out mice had massive iron overload through the abrogation of hepcidin synthesis. Most regulators act through a modulation of this BMP/SMAD signal transduction.

Attachment 3: Causes of iron deficiency

Neonates/Infants	Children	Adolescents	Adults
↓ intake			
> 6 mo exclusively breast-milk fed 9-18 mo fed with cow's milk or formula with low iron content	Inadequate caloric intake	Anorexia/ Inadequate caloric intake (esp. in student athletes)	Anorexia/ inadequate caloric intake
↑demand			
Prematurity: less iron reserve built up (esp. low birth weight) EPO administration for anemia of prematurity Excessive phlebotomy Fetal-maternal hemorrhage and other perinatal hemorrhagic events Chronic infection/ inflammation	GI Blood loss, esp. due to: - peptic ulcer - Meckel diverticulum - hemangioma - polyp - H. pylori gastritis - Drug-induced (aspirin, NSAID) - Parasitosis Trauma Excessive phlebotomy Pulmonary hemosiderosis Chronic infection/ inflammation	Polymenorrea GI blood loss, esp. due to; - refluxesophagitis - peptic ulcer - IBD - Drug-induced (aspirin, NSAID) Trauma Pulmonary hemosiderosis Chronic infection/ inflammation	Genitourinary blood loss: - Polymenorrea - Tumor - Chronic infection GI blood loss: - IBD - tumor - varices - peptic ulcer - diverticulosis - hemorrhoids Pulmonary blood loss - pulmonary hemosiderosis - infection Trauma Large vascular malformation EPO administration Chronic infection/ inflammation
↓ absorption			
Milk induced enteropathy of early childhood	Coeliakie iron malabsorption secondary to H. pylori gastritis, atrophic gastritis Parasitosis	IBD Iron malabsorption secondary to celiac disease, H. pylori gastritisand atrophic gastritis	Bowel resection IBD Antacid therapy or high gastric pH Excess dietary bran, tannins, starch or phytates

Data derived from (13-15)

Attachment 4 Pathophysiological mechanisms underlying ACD**.** Adapted from Weiss et al. (8)



A. Microorganisms, malignant cells, or autoimmune dysregulation leads to activation of CD3+ and monocytes. These cells induce immune effector mechanisms, producing cytokines such as interferon*γ* (IFγ, from T cells), tumor necrosis factor (TNF*α*) and interleukins IL-1, IL-6, IL-10 (from monocytes or macrophages). **B.** IL-6 and lipopolysaccharide (LPS) stimulate the hepatic expression of hepcidin, which inhibits duodenal absorption of iron. **C.** IF*γ*, LPS, or both increase the expression of divalent metal transporter 1 (DMT-I) on macrophages and stimulate the uptake of ferrous iron (Fe2+). The anti-inflammatory cytokine IL-10 upregulates transferrin receptor expression and increases transferrin-receptor–mediated uptake of transferrin bound iron into monocytes. In addition, activated macrophages phagocytose senescent erythrocytes for the recycling of iron, a process that is further induced by TNF*α* through damaging of erythrocyte membranes and stimulation of phagocytosis. IF*γ* and LPS downregulate the expression of macrophage ferroportin 1, thus inhibiting iron export from macrophages, a process that is affected by hepcidin. At the same time, TNF*α*, IL-1, IL-6, and IL-10 induce ferritin expression and stimulate the storage and retention of iron within macrophages. In summary, these mechanisms lead to a decreased iron concentration in the circulation and thus to a limited availability of iron for erythroid cells. **D.** TNF*α* and IFγ inhibit the production of EPO in the kidney. **E.** TNF*α*, IF*γ*, and IL-1 directly inhibit the differentiation and proliferation of erythroid progenitor cells. In addition, the limited availability of iron and the decreased biologic activity of EPO lead to inhibition of erythropoiesis and the development of anemia.

Attachment 5: Prohepcidin does not correlate with iron markers or anemia: overview of conducted studies

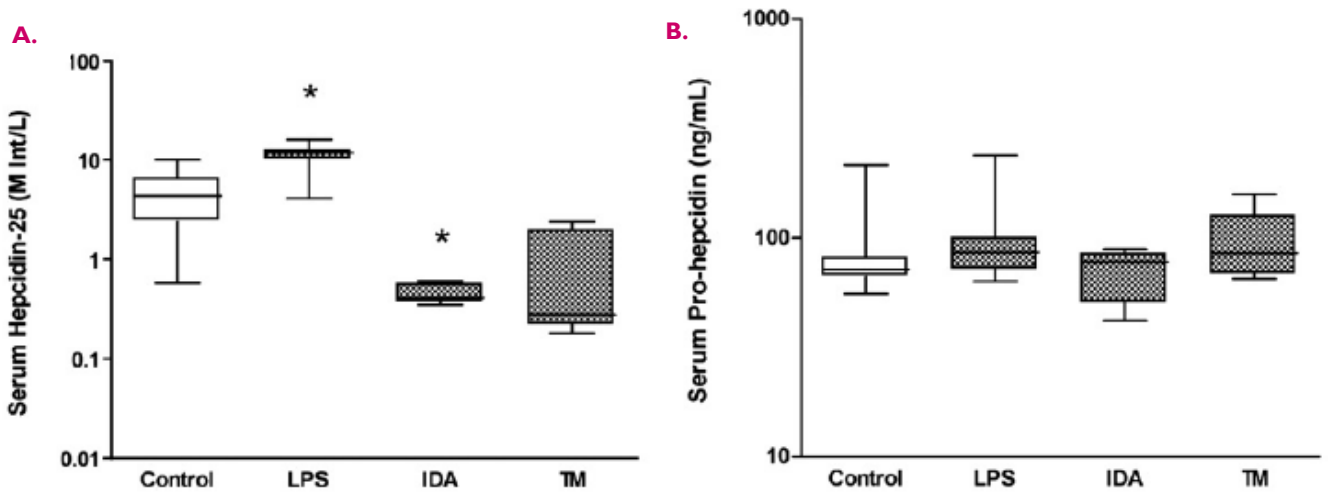
Study	Study design	Correlation with hepcidin	Prohepcdidin levels: correlation with iron/ anemia markers	Other findings[1]
Prohepcidin evaluation[2] in healthy patients				
Tiker *et al.* 2006 (19)	26 healthy preterm (gestational age <37 weeks) and 16 healthy, fullterm,appropriate-for-gestational age babies	/	No significant correlations between serum pro-hepcidin level and serum iron, serum ferritin, or transferrin in the preterm or term newborns.	No correlation with gestational age in the preterm group. Healthy preterm and term newborns have high prohepcidin levels.
Roe *et al.* 2007 (20)	138 adults (91 wild-type, 47 C282Y heterozygote)	/	No association between serum prohepcidin and indices of iron status (Hb, ferritin, sTfR, serum iron, TSAT)	No association of prohepcidin with C282Y genotype
Balogh *et al.* 2007 (21)	Cord blood and postnatal venous blood samples were taken from 20 healthy neonates (term)	/	No association with any of the investigated iron homeostasis or red blood cell parameters, but a correlation was detected with MCHC, in cord blood only.	Prohepcidin levels increased postnatally in 13/20, decreased in 7/20 Infants with detectable NPBI values in cord blood presented with lower prohepcidin levels than those with undetectable values
Ulukol *et al.* 2007 (22)	74 healthy non-anaemic infants aged 4–12 months	/	No significant correlations between the levels of prohepcidin and serum ferritin	Extensive physiologic variations Significantly lower levels in infants 7–12 mo (with iron supplementation) than the younger group.
Kemna *et al.* 2008 (23)	20 adults	No	No correlations with sTfR, TSAT, iron, NTBI, ferritin	No significant difference between all groups tested (HC, LPS, HH, TM, IDA) in contrast to hepcidin.
Huang *et al.* 2008 (24)	45 healthy post-menopausal women over 1-year period	No	Correlated with sTfR1 levels, but not with ferritin	Serum hepcidin levels are associated with ferritin but not with sTfR1 levels
Prohepcidin evaluation[2] in iron absorption studies				
Hadley *et al.* 2006 (25)	28 healthy women aged 22–51 y with normal Hb during iron supplementation	/	No correlation of serum or urinary with iron absorption. Serum levels correlated directly with ferritin	Serum levels were relatively consistent during supplementation, in contrast with urinary prohepcidin.
Roe *et al.* 2007 (20)	Iron absorption in 30 HC (15 wild-type, 15 C282Y heterozygote).	/	No significant association between iron absorption and serum prohepcidin concentration	
Luukkonen *et al.* 2006 (26)	53 HC and 18 HC receiving a 100-mg single oral dose of iron (ferrous sulphate) with sampling before and at 2,4,6, 8 and 24 h	/	No systematic change after supplementation in males (n=9), but increase in 9/10 females with rather low ferritin (mean 36 mg/L), along with an increase in serum iron	Extensive physiological variations in serum pro-hepcidin between HC. The responses of serum prohepcidin to a 100 mg oral dose of iron showed considerable inter-individual variation.
Young *et al.* 2009 (27)	18 healthy women aged 18-32y, with supplemental non-heme-iron	No	No relation with iron absorption or indicators of iron status (iron, Hb, sTfR).	In contrast, serum hepcidin-25 was inversely associated with iron absorption.
Prohepcidin evaluation[2] in acute phase reaction				
Kemna *et al.* 2005 (28)	Time-course analysis of 10 HC with LPS	No	No significant change in prohepcidin in the study period.	Change in urinary hepcidin preceded decrease in serum iron.
Kemna *et al.* 2008 (23)	16 HC with LPS	No	No correlations with any iron marker (sTfR, TSAT, iron, NTBI, ferritin)	No significant difference between all groups tested (HC, LPS, HH, TM, IDA) in contrast to hepcidin. LPS showed a tendency to the overall highest values.
Hoppe *et al.* 2009 (29)	5 male patients, before and after cardiac surgery	No	No correlations (with iron, TSAT, ferritin, hemoglobin, TIBC)	Serum prohepcidin decreases following acute-phase, after 22-48h. Significant correlations on day 2 postoperatively between serum hepcidin and CRP or TIBC
Prohepcidin evaluation[2] in iron disorders				
Kulaksiz *et al.* 2004 (30)	26 HC, 35 patients with HH, 59 patients with renal insufficiency(RI) undergoing chronic haemodialysis	/	No significant correlation (serum from HH, CRI, and RA) with iron, ferritin, or TSAT	Prohepcidin is significantly lower in HH than HC and significantly higher in RI. Levels significantly decreased in RI with renal anemia, compared with RI.
Roe *et al.* 2007 (20)	6 HH	/	No correlation with serum ferritin or transferrin saturation	
Orhon *et al.* 2007 (31)	16 infants 4–6 mo with IDA, not receiving any iron; 54 healthy infants 4-6 mo	/	Prohepcidin levels of infants with IDA were statistically similar with those of the HC.	Wide physiological variations in serum pro-hepcidin concentrations among the infants with IDA
Kemna *et al.* 2008 (23)	20 HC, 6 IDA, 5 TM, 16 HC after IV LPS	No	No correlations with any iron marker (sTfR, TSAT, iron, NTBI, ferritin)	No significant difference between all groups tested (HC, LPS, HH, TM, IDA) in contrast to hepcidin. IDA patients showed tendency to the overall lowest values

Prohepcidin evaluation[2] in other conditions				
Roe *et al.* 2007 (20)	13 pregnant women	/	Not associated with any of the iron status measures, week of gestation or iron supplementation.	The interaction term (supplement group:week of gestation) was significantly associated with all iron status indicators but not prohepcidin
Kato *et al.* 2007 (32)	75 hemodialysis patients (54 rHuEPO treated; 12 patients receiving iron supplementation)	/	No correlation with serum iron, ferritin and TSAT No difference with /without rHuEPO Positively correlated with rHuEPO dose and rHuEPO/Hb ratio (but not in patients with iron supplements)	No relation to age, time on HD, log-transformed CRP, intact PTH, and serum creatinine, albumin and total cholesterol Patients with iron supplements had lower prohepcidin levels
Beirão *et al.* 2008 (33)	24 patients with familial amyloidosis (FAP-I) (of which12/24 anemic); 33 HC	No	Significantly lower in anemic FAP patients than in non-anemic FAP. Correlation with hct and Hb in FAP but not in HC. No correlation with TSAT, ferritin.	Significantly lower in FAP than HC
Boinska *et al.* 2009 (34)	132 multi-time male blood donors and 25 healthy male volunteers (nondonors)	/	No differences in pro-hepcidin levels between any group of donors and HC. No correlation with iron markers in HC or donors with <4 donations/ 12 mo. Positive correlation with Hb and negative with sTfR in donors >4 donations/ 12 mo	Tendency to the lowest values in donors with least donations, increasing with the frequency of donations. Significant increase after 4–5 donations per year
Malyszko *et al.* 2009 (35)	130 kidney allograft recipients	No	Independently related only to ferritin, no relation with other iron markers.	Correlation with creatinine, GFR, albumin, time after transplantation, hsCRP, IL-6, and TNF-α. Hepcidin-25 correlated with serum iron, ferritin, hsCRP, IL-6, Hb, TSAT, creatinine, GFR.
Olmez *et al.* 2009 (37)	62 chronic viral hepatitis patients	/	No association with iron parameters (iron, NTBI, ferritin) in HC or hepatitis group.	Significantly lower prohepcidin levels in patients with chronic hepatitis B.

[1] Regarding prohepcidin levels, unless otherwise specified. [2] *Serum* prohepcdidin concentrations, unless otherwise specified. Studies in grey ☐ directly compared hepcidin-25 and prohepcidin levels. Studies testing multiple patient groups can be included in each subset of the table if correlations with iron markers were available for the different groups. *Abbreviations*: HC: healthy controls; HH: hereditary hemochromatosis; LPS: administration of 2ng/kg body weight *Eschericia coli* O:113 lipopolysaccharide (inflammation activator); TSAT: transferrin saturation; Hb: hemoglobin; NTBI: non-transferrin bound iron

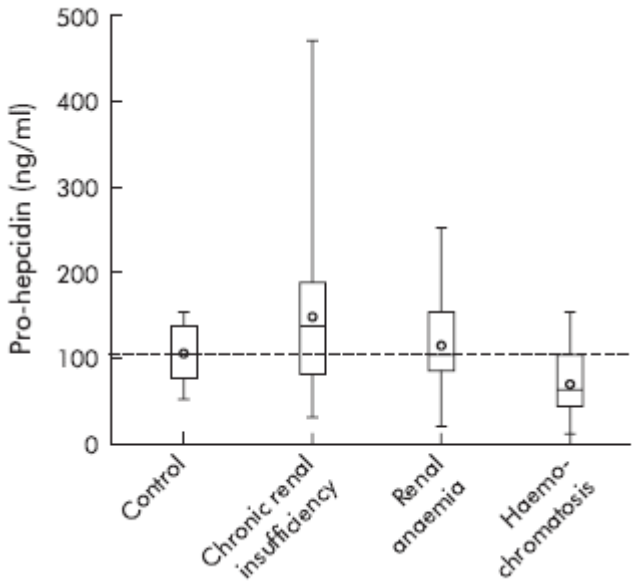
Attachment 6: Prohepcidin relevance as a biological marker: conflicting results of two trials

Kemna et al. 2008 (23): Prohepcidin does not allow differentiation between controls, IDA, inflammation or thalassemia major patients



Box plot of values in 20 healthy controls, 6 iron-defeciency anemia patients (IDA, based on low hemoglobin, MCV and ferritin), 5 thalassemia major (TM), 16 healthy individuals after IV lipopolysaccharid administration. Box plots show 25 and 75 percentile with median, and error bars represent minimum and maximum values. * Indicates significant difference from control group (P<0.05; non-parametric one-way ANOVAwith Dunn's post hoc test).
LPS: healthy volunteers injected with LPS; IDA: iron deficiency patients (); TM: thalassemia major patients treated with blood transfusions and iron chelators.
In contrast to hepcidin (**A**), no significant differences in prohepcidin levels existed in all groups tested (**B**). Furthermore, prohepcidin did not correlate to serum hepcidin or any other parameter tested (transferrin saturation, sTfR, CRP). (23)

Kulaksiz et al. 2004 (30): Prohepcidin is significantly lower in hemochromatosis than in healthy controls, and significantly higher in renal insufficiency. Renal insufficiency with renal anemia gives lower results than renal insufficiency without concomitant anemia.



Box plot of values for venous serum pro-hepcidinconcentrations in 26 healthy volunteers (control), 40 patients withchronic renal insufficiency, 19 patients with chronic renal insufficiency and renal anaemia, and 35 patients with hereditary haemochromatosis. The line within the box indicates the median, the circle indicates the mean. The lower and upper edges of the box indicates the first and third quartiles, and the whiskers the minimum and maximum values. The broken line marks the mean level of the control group for circulating immunoreactive prohepcidin (106.16 ng/ml). (30)

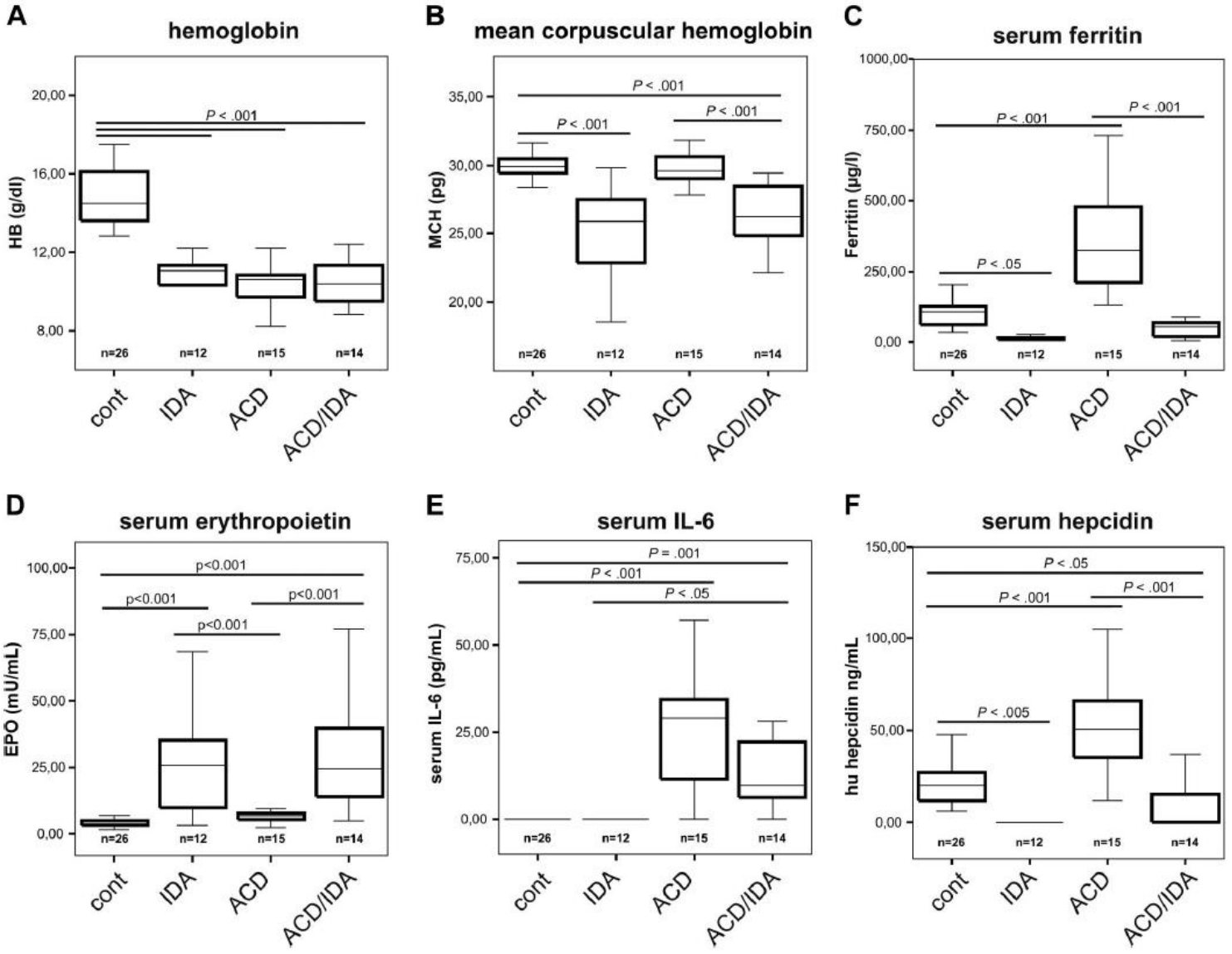
Attachment 7: Quantitative analytical methods in the determination of hepcidin-25

Method	Standard (I, E)*	Normal range (n)	Intra-assay precision (CV%)	LLOD	Matrix	Advantages/ Disadvantages	Reference
MALDI-TOF MS	I: stable isotope-labelled hepcidin	Not reported	12.1	2.5 nM (LLOQ)	U	Only in urine. Good correlation with immunoassay.	Anderson *et al.* 2010 (37)
Competitive RIA	I: not applicable	mean 15.3 ng/mL (n=25)	2.7-7.2	0.6 ng/mL	S	No cross-reactivity with similar structured proteins	Arnold *et al.* (38)
Competitive RIA	I: not applicable E: synthetic hepcidin-25	2-55 ng/mL (n=64)	5.8-7.2	0.6 ng/mL	P	No cross-reactivity with similar structured proteins, prohepcidin , some (9.6%) with hepcidin-20	Ashby *et al.* 2009 (39)
LC MS/MS	I: stable isotope-labelled hepcidin	Not reported	8.7-12.5	n.a.	U	Only in urine.	Bansal *et al.* 2008 (40)
MALDI-TOF MS	I: stable isotope-labelled hepcidin	average 10.1 +/- 2.9 nmol/ mmol creatinine	<21%	5 nmol/L	U	Only in urine. Good correlation with LC-MS/MS on same data set	Bansal *et al.* 2009 (41)
Competitive RIA	I: not applicable	1.1-55 ng/mL (n=47)	5.8-7.2	0.6 ng/mL	P	No cross-reactivity with similar structured proteins	Busbridge *et al.* 2009 (42)
Competitive HBD-binding test*	I: not applicable	39-88 ng/mL (n=40)	<5	n.r	S,U**	Measurement of biologically active hepcidin Further validation needed	De Domenico *et al.* 2008 (43)
Competitive ELISA	I: not applicable E: synthetic hepcidin-25	♂29-254 ng/mL; ♀17-286 ng/mL(n=114)	5-19	5 ng/mL	U,S	Simple Applicable for large series of patients	Ganz *et al.* 2008 (44)
Competitive RIA	I: not applicable E: synthetic hepcidin-24	♂ 0.37-29.15 ng/mL; ♀ 0.25-12.56 ng/mL (n=64)	4.4-6.2	0.02 µg/L	S	Very low detection limit Good correlation with quantitative SELDI-TOF	Grebenchtchikov *et al.* 2009 (45)
Micro HPLC MS/MS	I: stable isotope-labelled hepcidin	♂1-19.5 ng/mL; ♀1-6.5 ng/mL (n=10)	4.8-21	1 ng/mL	U,S		Kobold *et al.* 2008 (46)
Competitive ELISA	I: not applicable E: rHepcidin-25-His	13.4 – 133.5 ng/mL (n=32)	8-15	5.4 ng/mL	S	Good correlation with quantitative SELDI-TOF	Koliaraki *et al.* (47)
LC MS/MS	I: stable isotope-labelled hepcidin in rabbit serum	Average <10 ng/mL (n=60, only 29>LLOD)	5.1-13.4	2.5 ng/mL	S	Fully FDA validated	Li *et al.* 2009 (48)
LC/ESI MS MS	I: stable isotope-labelled hepcidin	Not reported	0.4-5.8	5 ng/mL (LLOQ)	S		Murao *et al.* 2007 (49)
LC MS/MS	I: CGRP (calcitonin gene-related peptide) in rabbit serum	medians: plasma 4.9 ng/mL serum 3.6 ng/mL (n=10 males)	11.0-15.3	1 ng/mL	S,P	CGRP no ideal internal standard (differences from hepcidin in mass, hydrophobicity, p*I* and charge)	Murphy *et al.* 2007 (50)
SELDI-TOF MS	I: synthetic hepcidin-24	♂9-45 ng/mL; ♀7-15 ng/mL (n=23)	5.7-11.7	3 ng/mL	U,S		Swinkels *et al.* 2008 (51)
SELDI-TOF MS	I: stable isotope-labelled hepcidin	♀50 ng/mL average (n=24)	8-9	10 ng/mL	U,S		Ward *et al.* 2008 (52)

Standards: I: Internal standard, E: external standard. Matrix: U: urine, S: serum, P: plasma. Normal range: n= numer of control subjects. LLOD: lower limit of detection

* Competitive assay of hepcidin with radioactively-labeled hepcidin for HBD (hepcidin binding domain: 19 amino acid long synthetic peptide of the binding domain on ferroportin); ** No hepcidin was detected in urine, but the assay could readily measure hepcidin when added to urine

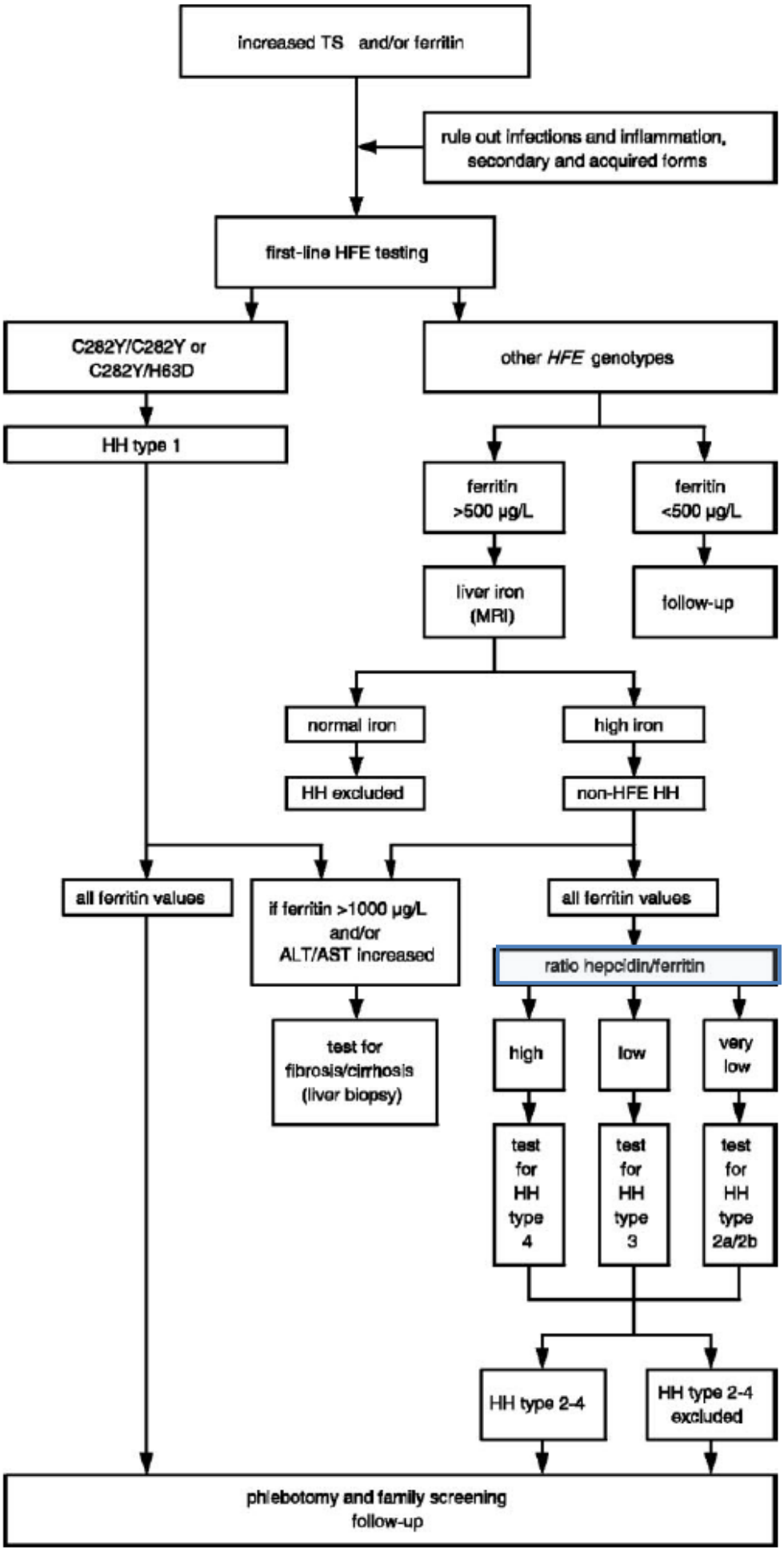
Attachment 8: Hepcidin in anemia of chronic disease and iron deficiency
Detailed results of Theurl *et al.* (18)



As the log ferritin/sTfR ratio was used to categorize patients as having either ACD or ACD/IDA, this ratio is not shown. Data are depicted as lower quartile, median, and upper quartile (boxes) and minimum/maximum ranges (whiskers). Calculations for statistical differences between the various groups were carried out by analysis of variance technique and Bonferroni correction for multiple tests.

A. Anemic patients had lower hemoglobin levels than control subjects. **B.** ACD/IDA patients had lower mean corpuscular hemoglobin (P< .00) and lower mean corpuscular volume (P < .05) than the ACD group. **C.** Serum ferritin levels were significantly elevated in ACD patients compared with controls (P< .001) and significantly lower in ACD/IDA patients than in ACD patients (P < .001). **D.** Serum EPO levels were significantly elevated in IDA (P < .001) and ACD/IDA (P< .001) patients but not in ACD patients compared with controls. **E.** IL-6 and C-reactive protein levels were significantly higher in ACD (P< .001) and ACD/IDA (P<.001) patients than in controls, whereas no difference was found between ACD and ACD/IDA patients. **F.** We found low hepcidin levels in IDA patients (P<.005) but increased hepcidin concentrations in ACD subjects (P<.001) compared with controls. Importantly,ACD subjects had significantly higher serum hepcidin levels thanACD/IDA patients (P<.001), and the latter group was not different from IDA patients. (18)

Attachment 9: Flow chart proposed for the diagnosis of the various forms of HH (79).



The diagram includes innovative molecular, hepcidin, and magnetic resonance imaging (*MRI*) tests.

Future studies are needed to collect evidence to validate this flow chart. Except for the information included in this diagram, rational gene targeting is also based on information on clinical presentation, hemoglobin (low in secondary forms of iron overload and in some cases with ferroportin disease), family history (hereditary disease), concomitant diseases (e.g., hepatitis and alcohol abuse), and age at presentation (young age in juvenile forms of HH). *HH types 1–4* refer to the OMIM classification). (79)