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THE HAPTOGLOBIN POLYMORPHISM IN HEREDITARY HEMOCHROMATOSIS AND CHRONIC HEPATITIS C.

Het haptoglobine polymorfisme in erfelijke hemochromatose
en in chronische hepatitis C virus infectie.

Proefschrift ter verkrijging van het
Doctoraat in de Medische Wetenschappen

door

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VOORWOORD

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Neem de tijd om te werken,
Dat is de prijs van het succes.

Neem de tijd om te denken,
Dat is de bron van de macht.

Neem de tijd om te spelen,
Dat is het geheim van de eeuwige jeugd.

Neem de tijd om te lezen,
Dat is de grondslag van de wijsheid.

Neem de tijd om aardig te zijn,
Dat is de weg naar geluk.

Neem de tijd om te dromen,
Dat is de manier om hoog te mikken.

Neem de tijd om te beminnen en bemind te worden,
Dat is het voorrecht van de goden.

Neem de tijd eens om, om u heen te kijken,
De dag is te kort om zelflustig te zijn.

Neem de tijd om te lachen,
Dat is de muziek van je ziel.

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1. Physiology of iron transport

Iron is an essential element for all living organisms (1). Most of the body iron is found intracellularly; in ferritin, haemosiderin or haem present in haemoglobin, myoglobin and cytochromes. The iron which is found in extracellular body fluid is bound to high affinity iron-binding glycoproteins: transferrin in serum and lymph, and another related protein called lactoferrin in external secretions and milk (2).

The distribution of iron in tissue is shown in Figure 1 (3).

Figure 1.

Distribution of iron in adults (N Eng J Med 1999;341(26):1986-1995).

More than two thirds of the body's iron is incorporated into haemoglobin in developing erythroid precursors and mature red cells. Uptake of erythroid iron is highly dependent on receptor-mediated endocytosis of diferric transferrin bound to transferrin receptors (3). Each erythrocyte contains a billion atoms of iron; at normal rates of turnover, this concentration corresponds to the incorporation of $2x10^{20}$ atoms of iron a day (4). Most of the remaining iron is found in hepatocytes and reticuloendothelial macrophages, which serve as storage depots. The liver has first-pass access to dietary nutrients and can readily take up an amount of circulating iron that exceeds the binding capacity of plasma transferrin. Reticuloendothelial macrophages ingest senescent red cells, catabolise haemoglobin to scavenge iron, and load the iron onto transferrin for reuse (3).

Although the amount of iron extracted from the diet is small, the regulation of the intestinal absorption of iron is critical because humans have no physiologic way of excreting iron. Duodenal crypt cells sense the iron requirements of the body and are programmed as they mature into mature enterocytes. Enterocytes lining the absorptive villi close to the gastroduodenal junction are responsible for iron absorption. Iron must pass from the gut lumen through the apical and basolateral membrane of the enterocyte to reach the plasma (Figure 2).

Figure 2.

Iron transport across the intestinal epithelium (N Eng J Med 1999;341(26): 1986-1995).

The low pH of gastric effluent facilitates enzymatic reduction of ferric (Fe^{3+}) to its ferrous form (Fe^{2+}) by brush border ferric reductase (5). Divalent metal transporter 1 (DMT1 or Nramp2 or DCT1) is a protein that transfers iron across the apical membrane and into the cell through a proton-coupled process (6,7). DMT1 is not specific to iron, it can transport a wide variety of divalent metal ions: manganese, cobalt, copper, zinc, cadmium and lead (7). Haeme iron is taken up by a separate process that is not well characterised.

Inside the absorptive enterocyte, iron has two possible fates: it may be stored as ferritin, or it may be transferred across the basolateral membrane to reach the plasma. Iron that remains in the form of ferritin in the enterocyte will be

sloughed with the senescent cell and will leave the body through the gastrointestinal tract. This process represents an important mechanism of iron loss (3). Normally, the total amount of iron in the body is well controlled. There is no mechanism for iron excretion, and normally iron exits the body in tiny obligatory amounts (approximately 1 mg per day) during the loss of desquamated epithelial cells. In women, menstrual loss accounts for an additional 15-20 mg monthly (3).

The basolateral enterocyte receptor for iron has not been identified, but a recently described protein, Ireg1, is a likely candidate (8). Genetic studies in mice have shown that the basolateral transporter requires an accessory protein, a multicopper protein called hephaestin (9). Another candidate could be ferroportin1, which has been identified as a conserved vertebrate iron exporter (10).

The absorption of intestinal iron is regulated in several ways. It can be modulated by the amount of iron recently consumed in the diet, by the requirements for erythropoiesis or it can be regulated by the total body iron. Acute hypoxia increases iron absorption. It is not known whether the hypoxic signal is transduced through one of the regulatory pathways discussed above or through an independent mechanism (3,11,12).

Transport of iron from one organ to another, is accomplished by a plasma protein called transferrin (13). Transferrin is the major iron-carrying protein in plasma and is believed to regulate iron-fluxes between sites of absorption, storage and utilisation (2). Transferrin has a high binding affinity for iron (14). Transferrin is normally partially saturated, to the extent of 30-40 % (15). As a result there is virtually no freely available iron in normal body fluids (16).

Transferrin receptors expressed by iron-requiring cells mediate the uptake of iron from transferrin by these cells (17,18). Transferrin receptors bind diferric transferrin and the receptor-transferrin complex is internalised into an endosome, where the iron is transferred to the cytosol.

The iron depleted transferrin, apotransferrin, recycles to the cell surface, where it dissociates from the transferrin receptor. Expression of cellular transferrin receptors is determined by the iron requirements of the cell (19,20).

Iron is transported by transferrin to iron-requiring cells for subsequent incorporation into haem and many other iron-requiring cytoplasmic enzymes and proteins (2). Surplus iron is stored as ferritin and its derivative haemosiderin, in both parenchymal cells and mononuclear phagocytic system (21). Ferritin is the major iron storage protein and although it is found in almost all cells of the body, it is present in greatest concentration in the liver, spleen and bone marrow (22). The total body iron content is 3-4 g and of which nearly two thirds is present in haemoglobin in erythrocytes where it is required for oxygen transport (23).

2. Hereditary hemochromatosis

A. Definition and clinical pattern

Hereditary hemochromatosis (HH) is an autosomal recessive inherited disorder of iron metabolism that affects 1 in 200 to 1 in 400 persons of Northern European descent and is characterized by increased gastrointestinal iron deposition (24-26). It was first described in 1865 as a clinical triad of glycosuria, cirrhosis, and hyperpigmentation of the skin (27). Von Recklinghausen later established that these clinical features were due to iron deposition and coined the term hemochromatosis (28). The majority of patients with HH are descended from a common Celtic ancestor who lived 60 to 70 generations ago (29). The Celts initially colonised Central, South-Western, and South-Central Europe around 1000 BC. Settlements than dispersed to Belgium, the United Kingdom, Ireland, Spain, Scandinavia and Eastern Europe (Figure 3) (30).

Figure 3

The Celtic settlement and migration pattern during the Iron Age (Hepatology 1997;25:1439-1446).

Patients with HH chronically absorb a small excess of iron, and middle-aged homozygotes frequently have 10 times normal body iron stores. As iron stores exceed the body's capacity for effective chelation, free iron accumulates (31). Unbound iron is highly toxic, owing to its participation in the generation of free radicals and reactive oxygen intermediates. These molecules provoke peroxidation of membrane lipids leading to cellular injury, ultimately resulting in severe damage to the liver, heart, joints and endocrine organs. Liver damage may be exacerbated by alcohol consumption or viral hepatitis. The onset of the disease is insidious, and the initial manifestations are often non-specific symptoms, including fatigue and arthropathy. Before the detection of the genetic mutation, the diagnosis of hereditary hemochromatosis has been based on the documentation of increased iron stores, namely increased hepatic iron indices (of more than 1.9 μmol/g dry liver tissue per year of life) associated with elevated serum ferritin levels. In the majority of the patients iron saturation is elevated (a cut-off level of 52 % in the female and 62 % in the male population has been proposed) (32).

If untreated, hemochromatosis invariably progresses and is ultimately fatal. However, if diagnosed before end stage organ damage, phlebotomy is an inexpensive, effective and life-saving treatment. Red blood cells are removed at regular intervals to deplete iron stores, and bone marrow erythropoiesis compensates effectively (31).

B. Genetics of hemochromatosis

The HH mutation has been known to be linked to the HLA region on the short arm of chromosome 6 since the mid 1970s (31,33). Twenty years later, in 1996, a positional cloning approach was used to detect a candidate gene for HH, now known as *HFE*. *HFE* codes for a novel MHC class I-like molecule that requires interaction with β_2-microglobulin for normal

presentation on the cell surface (34). Structural homology with other MHC class I proteins and X-ray crystallographic studies indicate that the HFE protein has a large extracellular domain, a single transmembrane region, and a short cytoplasmatic tail (Figure 4) (33).

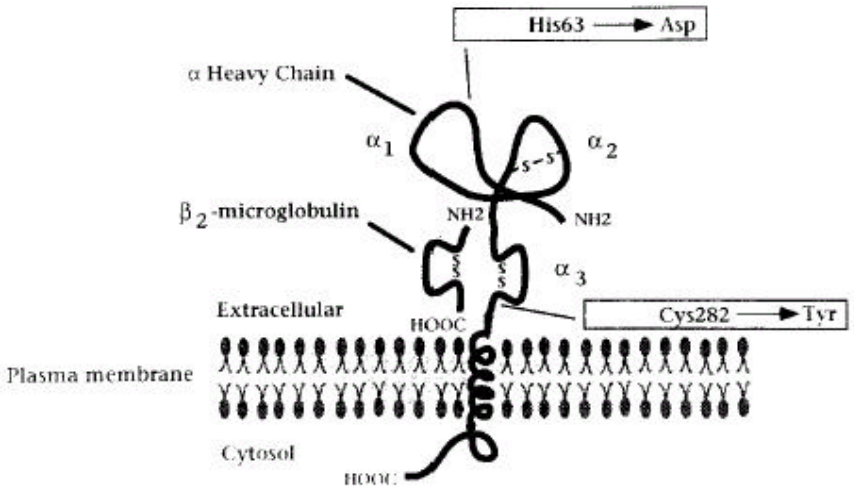

Figure 4.
Model of the HFE protein (Gastroenterology 2001;120:718-725).

The large extracellular domain consists of 3 α domains, and α_3 binds to β_2-microglobulin. HFE protein lacks a functional peptide-binding groove needed for antigen presentation (35).
Two missense mutations have recently been identified in *HFE*; one results in a change of cysteine at position 282 to tyrosine (Cys282Tyr); the second results in a change of histidine at position 63 to aspartate (His63Asp) (34).

In the original American study by Feder et al (34), 83 % of typical phenotypic HH patients were homozygous for the Cys282Tyr mutation, an additional 8 patients (5 %) were compound heterozygote, with one allele

containing the Cys282Tyr mutation and the other allele containing the His63Asp mutation. Afterwards, homozygosity for the Cys282Tyr missense mutation was confirmed in 64 % - 100 % of patients with typical phenotypic HH from the United States, France, Italy, Canada and Australia (25).

Other mutations (eg. Ser65Cys) have recently been identified (36), but their frequency seems low, and thus their clinical impact may be limited.

Recently, a pedigree from Italy was described with typical phenotypic HH but no mutations in the *HFE* gene, identifying a new inherited abnormality of iron overload (37). Mutations in the gene for transferrin receptor 2 can also result in iron overload (38).

C. Pathophysiology of HFE

Numerous studies have been performed to elucidate the pathophysiologic mechanisms of normal and dysregulated iron absorption. The creation of *HFE*-knockout mice and Cys282Tyr homozygous mice with a similar phenotype to that found in humans with HH, confirms that *HFE* is the gene responsible for HH (39-41). HFE protein is found in the crypt cells of the duodenum associated with β_2-microglobulin and transferrin receptor (42,43). It has been hypothesised that HFE protein may facilitate transferrin receptor-dependent iron uptake into crypt cells and that mutant HFE protein may lose this ability, leading to a 'relative' iron deficiency in duodenal crypt cells (Figure 5) (42-44). In turn, this may result in an increased expression of DMT-1 (3).

Figure 5.

Critical steps in mammalian iron transport (Blood 1998;92(6):1845-1851) (31).

Up-regulation of DMT-1 (Nramp2) expression has been confirmed in the *HFE*-knockout mice (45) and in humans with HH (46), providing supportive evidence for this pathophysiological mechanism. A similar mechanism may cause a status of a 'relative' iron deficiency in monocytes-macrophages of patients with HH (47).

D. Genotypic/phenotypic correlations in HH

The phenotypic expression of HH (homozygous for the Cys282Tyr missense mutation) may vary from a mere laboratory abnormality, namely an increased serum iron saturation to a fully penetrant clinical syndrome characterised by bronze pigmentation, cirrhosis, arthritis, endocrinopathy and cardiomyopathy (48).

In a recent study (49), Crawford et al have demonstrated that 17.3 % of the subjects homozygous for the Cys282Tyr mutation do not fulfil the current diagnostic criteria of hemochromatosis. In another trial Olynyk et al have shown that only 50 % of the population homozygous for the Cys282Tyr mutation expressed clinical features of hemochromatosis, whereas one quarter has serum ferritin levels within the normal range during a four-year period (50).

It is generally accepted that women and younger people have less severe phenotypic presentation of the disease. Iron overload develops at a slower rate in women with hereditary hemochromatosis than in men with the disorder, because in women iron stores are depleted by menstruation and pregnancy (50). Since hemochromatosis is a disease that progresses with time, younger people have milder disease than older people (50).

Recent reports have suggested that the phenotypic heterogeneity of hemochromatosis disease could be related to additional non-genetic causes, additional mutations or additional genes (51), and that one or more of these genes may be MHC-linked (52).

Prior to the identification of the hemochromatosis gene, clinical variation in hemochromatosis has been primarily ascribed to genetic factors; haplotype/ phenotype analysis in several populations indicated that subjects with the predominant ancestral haplotype presented with a more severe clinical phenotype.

A recent study by Pratiwi et al (52) has demonstrated that in male Cys282Tyr homozygous patients also homozygous for this predominant ancestral haplotype (D6S265 to D6S2236 covering a regio of approximately 5 Mb) significantly higher hepatic iron indices are found than in patients heterozygous or nullizygous for this gene. Analogous data are observed by others (53,54).

Alcohol intake is known to increase hepatic iron stores (55) and could be a non-genetic variable that influences the iron overload in C282Y homozygous individuals.

E. The immunological system in hemochromatosis

Various perturbations in the immune status of HH patients have been reported. Porto et al (56,57) found high CD4+/CD8+ ratios in peripheral blood T lymphocytes in these patients. High ratios were positively correlated with the number of phlebotomies required to deplete iron stores and were not corrected after achieving normal iron status. The high ratios were due to a reduction in CD8+ cells (58). Within the decreased CD8+ compartment, an increased percentage of CD8+CD28- T cells, with a corresponding reduction in the percentage of CD8+CD28+T cells was also present in HH patients carrying the HLA-A3 allele. CD28 is a T-cell membrane protein which interacts with the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) expressed by antigen presenting cells. CD28 expression was normal on CD4+ cells. CD8+CD28+ T cells from HH patients were capable of responding to exogenous mitogens. However, phytohaemagglutinin stimulation of peripheral blood mononuclear cells caused a decrease in CD8+CD28- T cells in healthy individuals but not in patients with hereditary haemochromatosis. CD8+ cytotoxic T lymphocytes also exhibited diminished cytotoxic activity (59).

Clearly, whether these immunological changes are directly tied to the absence of cell-surface molecules or are the secondary result of HH associated pathophysiological changes remains unclear. In a recent animal model, no difference in CD4 and CD8 subset T lymphocytes could be found between mice carrying the *HFE* missense mutation and those not carrying this missense mutation. This suggests that the described differences are rather post therapeutic alterations or secondary manifestations of severe iron overload (41).

3. Non transferrin bound iron (NTBI) in disorders of iron metabolism

A. The origin of NTBI

NTBI comprises all forms of iron in the plasma that are bound to ligands other than transferrin (60). Upon binding to transferrin, iron becomes effectively shielded from reactions leading to redox cycling and formation of reactive oxygen species. The main sources of plasma iron are absorption via the gastrointestinal tract and recycling of aged erythrocytes by macrophages. Since plasma iron is normally maintained at levels far below transferrin-binding capacity, its delivery to the plasma is believed to be tightly regulated (60). Thus when NTBI appears in the plasma, its is assumed to result from an imbalance in iron metabolism. It is observed in situations where transferrin becomes fully saturated, resulting in diminished plasma iron-binding capacity. Although paradoxical, NTBI has also been observed in situations where transferrin was not fully saturated, such as in patients with (early) HH (61-64) and dialysis patients (64). In these situations iron must be entering the circulation in a form that is inaccessible to transferrin.

There is sparse information about the biochemical nature of NTBI, but one may speculate that it is composed of a heterogeneous mixture of complexes whose combination might vary with the degree and type of iron-overload. Analysis of NTBI in sera of patients with HH by HPLC (high-performance liquid chromatography) and high-resolution nuclear magnetic resonance indicated the presence of citrate-iron and ternary citrate-acetate-iron complexes (65).

Albumin is another candidate ligand, and has been shown to form complexes with Fe^{3+} and with Fe^{2+}-citrate in vitro. Although the affinity of albumin for trivalent metals is rather low, this might be compensated by its high concentration in serum (66).

The likelihood that serum ferritin contributes to NTBI is low (63). The ferritin found in plasma tends to be iron-poor and the direct mobilization of iron from ferritin by various chelating and mobilizing agents is slow and inefficient unless reducing agents are used (67,68).

B. Occurrence of NTBI

It is generally accepted that NTBI is a pathological manifestation and is never found in healthy individuals. In principle, any condition or treatment that produces a short- or long-term iron load can give rise to NTBI. So NTBI is observed in 90 % of patients with haemolytic anaemia's (69-72), in 69-90 % of the patients with hereditary hemochromatosis (61-64,73), in 22 % of patients on haemodialysis (64), in patients on chemotherapy (79-97 %) (74-76), during cardiopulmonary bypass (13 %) (77-79), in (premature) neonates (21-50 %) (80,81) and in patients with African iron overload disease (82). It is even observed in individuals heterozygous for the Cys282Tyr missense mutation (83).

C. Detection of NTBI

There are presently 3 main approaches to determine NTBI in biologic fluids. One approach uses the antibiotic bleomycin, which combines with NTBI, but apparently not with iron bound to transferrin or other proteins, to form highly reactive complexes that are quantified by the amount of DNA cleavage products that they generate (84). A second approach is based on mobilisation of NTBI with an anionic ligand (EDTA, citrate or nitrilotriacetate) followed by ultrafiltration and quantitation of the filterable iron by a colorimetric method, a method based on inductive plasma spectroscopy or by HPLC (64). The sensitivity and reproducibility of the colorimetric method are acceptable compared with the other two methods and would be more convenient as a routine screening assay for NTBI.

However, the limitations of this method are such that it can not be used in situations were desferrioxamine as an iron chelator therapy, is used (63). A third approach operates in a 96-well enzyme-linked immunosorbent assay format. A weak ligand, oxalic acid, mobilizes the NTBI and mediates its transfer to the iron chelator deferoxamine immobilised in the plate. The amount of deferoxamine-bound iron, originating from NTBI, is quantitatively revealed in a fluorescence plate reader by the fluorescent metallosensor calcein. This last method has the advantage of being technically simple and having a low labour intensity for an identical sensitivity and specificity (64).

D. Is NTBI clinically important ?

The transferrin molecule is specifically designed to transport iron in a readily available, soluble form and at the same time prevent its participation in redox reactions that could give rise to reactive oxygen species (85,86). Conversely, NTBI in the circulation could in principle be available for such reactions and lead to oxidative damage. There is much evidence correlating elevated tissue and plasma iron levels with depletion of antioxidants and various pathological conditions (87,88), such as chronic hepatitis C (89). Since the effects of iron overload are multifactorial, NTBI is likely to be only one of several contributing factors to the observed overall oxidative stress. There have been claims that elevated body iron stores may increase the risk of cardiovascular disease (90) and may catalyse the oxidation of low density lipoproteins, which is thought to be one of the triggers of atherosclerosis (91,92). In addition, the uptake of NTBI is not feedback-regulated (93), unlike the receptor-mediated uptake of transferrin bound iron (94). Conceivably, excess iron in the form of NTBI could be internalised by cells, mobilised within lysosomes and ultimately give rise to cellular iron overload (60).

The presence of NTBI inhibits lymphocyte proliferation; this effect is more striking on Th1 CD4+ T cells and CD8+ CTL. These data support the hypothesis that NTBI has an immunoregulatory role in cell-mediated immunity (95,96).

4. Hepatitis C virus infection (HCV)

HCV is a RNA virus that belongs to the family of the flaviviruses (97). The natural targets of HCV are hepatocytes and possibly B lymphocytes (98,99). HCV encodes a single polyprotein of 3011 amino acids, which is processed into 10 mature structural and regulatory proteins (Figure 6) (100).

Figure 6.

The HCV genome (N Eng J Med 2001;345(1):41-52).

Structural components include the core protein and two envelope proteins: E1 and E2. Two regions of the E2 protein, designated hypervariable regions 1 and 2, have an extremely high rate of mutation, believed to be the result of selective pressure by virus-specific antibodies (100). E2 also contains the binding site for CD81, which is thought to act as a cellular receptor or coreceptor for the virus on hepatocytes and B lymphocytes (101). HCV also encodes a virus-specific helicase, protease and polymerase (100).

Six distinct but related HCV genotypes and multiple subtypes have been identified. In the United States and Western Europe genotypes 1a and 1b are most common, followed by genotypes 2 and 3 (100).

The prevalence of HCV infection varies throughout the world, with the highest number of infections reported in Egypt (102). In the Unites States 1.8 % of the population is positive for HCV antibodies (103). In Belgium the number of persons positive for HCV antibodies is estimated to be 0.9 % of the population (104).

The factors most strongly associated with infection are injection-drug use and receipt of blood transfusion before 1990 (103), but in some cases no risk factor can be identified (105,106). Maternal-fetal transmission occurs but is infrequent (107). Sexual transmission of the virus is also uncommon (108).

After infection with the virus, only a minority of the patients spontaneously clears the virus. Sixty to 80 % of the patients with an acute HCV infection evolves towards chronicity. After 20 years, cirrhosis develops in 20 % of them. In those having cirrhosis, the annual risk of developing a hepatocellular carcinoma is 5-7 % (109,110).

The immune response to viral antigens is thought to be responsible as well for viral clearance as for disease pathogenesis during chronic HCV infection. In chronically infected patients, the T cell response to the HCV is polyclonal and multispecific, although it is not as strong as the response in acutely infected patients who develop a more vigorous T-cell response. Thus the dominant cause of viral persistence during HCV infection may be the weak antiviral immune response to the viral antigens, and the ensuing inability to eradicate infected cells (111).

The diagnosis of hepatitis C virus infection is made by detection of HCV antibodies. The presence of circulating virus is demonstrated by HCV PCR. Qualitative and quantitative HCV RNA assays as well as HCV genotyping assays are available. Alanine aminotransferase (ALT) is an important non-specific laboratory test to identify hepatic disease.

However, in persons with HCV infection ALT levels may be normal or fluctuate, and therefore a single normal value does not rule out active infection, progressive liver disease, or even cirrhosis (112).

Histological evaluation of a liver-biopsy specimen remains the gold standard for determining the activity of HCV-related liver disease, and histological staging remains the only reliable predictor of disease progression (113).

Monotherapy for HCV infection with interferon alfa is associated with initial response rates of 40 percent, but the rates of sustained virological response are less than 20 percent (114,115). This is especially true for persons infected with HCV genotype 1a or 1b. Two large, prospective trials demonstrated that the combination of interferon alfa (3 MU thrice weekly subcutaneous) and ribavirin (1000-1200 mg per day, orally) during a period of 6 to 12 months, increased the percentage of sustained virological response in previously untreated patients from 16 to 40 percent (114,115). Both studies showed that in patients infected with HCV genotype 2 or 3 and in those with low viral loads (less than 2 million copies of virus/ml) before treatment, the response was maximal after 24 weeks of treatment. Patients infected with genotype 1 and those with a high viral load (higher than 2 million copies of virus/ml) before treatment required a course of 48 weeks for an optimal outcome. Therefore the EASL (European Association for the Study of the Liver) recommended the above-described regimes for treatment of untreated patients (116).

The chemical coupling of polyethylene glycol to interferon alfa (peginterferon alfa) extends the biological half-life and duration of activity of interferon alfa. In contrast to interferon alfa, which is given 3 times a week, peginterferon alfa is given only once a week. Treatment with peginterferon alfa results in a higher rate of sustained response than does conventional monotherapy with interferon alfa (39 % versus 19 %) (117).

Combining peginterferon alfa with ribavirin also increases the frequency of sustained response, when compared with peginterferon in monotherapy (118,119).

The side effects of the treatment are those related to (peg)interferon alfa and ribavirin. (Peg)interferon alfa mainly induces influenza-like symptoms, headache, fatigue, fever, myalgia, anorexia, irritability, thrombocytopenia, leukocytopenia, thyroid dysfunction …

Ribavirin is a water soluble synthetic guanosine analogue that exerts antiviral activity against DNA and RNA viruses after intracellular phosphorylation (120). Ribavirin also has immunomodulating capacities: it diminishes Th2 cytokine production while preserving Th1 cytokine production (121). The major side effect of ribavirin treatment is the occurrence of a reversible haemolytic anaemia in a substantial portion of treated patients (122). The underlying mechanism is unknown. Studies on steady-state pharmacokinetics of ribavirin have shown that the erythrocyte concentration of ribavirin greatly ex ceeds plasma concentrations (123) and that ribavirin is a transported permeant for a nucleoside transporter in human erythrocytes (124). Ribavirin is converted intracellularly to its corresponding triphosphate, producing a relative adenosine triphosphate deficiency in erythrocytes (125-127). The lowering of the intracellular adenosine triphosphate level may indirectly affect and lower the antioxidant defence mechanisms. The pro-oxidant phenomenon may induce a premature erythrocyte senescence and phagocytic removal (120). Haemolytic anaemia is the main side effect of ribavirin. Ribavirin also causes nausea, nasal congestion and seldomly gout (100).

5. Iron and chronic hepatitis C virus infection

It is generally accepted that iron increases the formation of hydroxyl radicals and other highly reactive oxidizing molecules in biological systems (128). Secondary reactions and indirect effects lead to lipid peroxidation, oxidative damage to proteins and nucleic acids, and to a net increase in collagen and ground substance formation. In iron-overloaded livers, such changes produce defects in organelle function (lysosomes, mitochondria, endoplasmic reticulum) and chronic deposition of scar tissue and eventual hepatocellular carcinoma (129,130).

Although in viral hepatitis the role of the host's immune system in causing hepatic damage is much more important than in the case of pure iron overload, changes attributed to iron also occur in chronic viral hepatitis. Evidence of increased lipid peroxidation has been identified consistently in plasma (131) and liver biopsies (132) of patients with chronic hepatitis C, compared with healthy controls or patients with chronic hepatitis B. If the stored iron seen in the liver of patients with chronic hepatitis C is the cause of these changes or only a consequence of hepatic inflammation caused by the virus remains speculative.

In chronic hepatitis C virus infection, serum iron concentration is elevated in 27-36 % of the patients, transferrin saturation in 18-24 % and serum ferritin concentration in 22-35 % (133,134). A strong correlation is seen between serum ferritin levels and AST levels, suggesting that release of tissue ferritin from damaged hepatocytes is an important mechanism for the elevations in serum iron studies (134). Increased serum iron studies are more common in chronic hepatitis C virus infection than in patients with chronic hepatitis B virus infection (135).

Hepatic iron concentration (HIC) is also elevated in patients with chronic hepatitis C, but less frequently than serum transferrin saturation or ferritin. The frequency of elevated HIC ranges from 10 to 36 % in patients with chronic hepatitis C (134,136,137). The elevation in HIC and HII (hepatic iron index, which is the HIC divided by the age in years of the patient) is usually mild, with a mean HII of 0.4-0.6 µmol/g/age (137,138). HIC does not appear to correlate with HCV RNA levels (139) and the effect of different genotypes is controversial (140,141). The presence of stainable hepatic iron has been shown to correlate with inflammation and fibrosis. This correlation could be a threshold effect: once inflammation or fibrosis is present, increases in iron appear to have no further linear correlation with increased inflammation or fibrosis (142).

In end-stage liver disease, even in the absence of the *HFE* missense mutation, iron accumulation can be significant and reaching HII values of greater than 1.9 µmol/g dry liver tissue per year of life; values which were in the past (before the *HFE* mutation era) pathognomonic for hereditary hemochromatosis (143-146).

Unlike hepatic iron accumulation in hereditary hemochromatosis, the mild hepatic iron overload often seen in patients with compensated liver disease due to hepatitis C does not appear to progressively increase in time, despite the worsening of the hepatic inflammation in a certain amount of patients. These findings suggest that hepatic iron accumulation is multifactorial and not solely related to the degree of inflammation or fibrosis (147).

The hepatic iron accumulation in patients with chronic hepatitis C is more often seen in the Kuppfer cells than in patients with hereditary hemochromatosis where iron accumulates in the parenchyma (148).

After a treatment with interferon alfa, a decrease in HIC is observed in both virologic responders and nonresponders. Histologically, the decrease in stainable iron is predominantly seen in mesenchymal cells as opposed to hepatocytes. These findings suggest that the elevations observed in chronic hepatitis C are primarily due to phagocytosis of necrotic hepatocytes and accumulation of iron in phagocytic cells. The decrease of HIC seen in nonresponders may reflect a reduction in inflammation despite failure to clear viremia (148).

After treatment with ribavirin and highly plausible due to the ribavirin induced haemolysis and related iron release, stainable hepatic iron significantly increased. This was associated with an increase of HIC. After treatment with ribavirin, iron is deposited mainly in the hepatocytes (149,150).

6. Haptoglobin polymorphism

Haptoglobin (Hp) is an α_2-sialoglycoprotein with haemoglobin (Hb)-binding capacity (151,152). The best-known biological function of Hp is capture of Hb to prevent both iron loss and kidney damage during haemolysis (153). Hp is also an acute-phase protein and is characterized by a molecular heterogeneity with three major phenotypes: Hp 1-1, Hp 2-1 and Hp 2-2 (151-154). Using starch gel electrophoresis, the three major phenotypes can be identified (155). These phenotypes are genetically determined by two alleles: Hp^1 and Hp^2 (151,152). The homozygote Hp^1/Hp^1 shows a single fast-migrating Hp1-1 protein band on starch gel electrophoresis. The homozygote Hp^2/Hp^2 has a series of slower migrating bands. The heterozygote Hp^1/Hp^2 displays another series of slow bands and a weak Hp 1-1 band. Hp consists of two different polypeptide chains, the α-chain and the β-chain (151,152). The β-chain (40 kDa) is heavier than the α-chain and is identical in all Hp types. The α-chain shows three major forms: α^{1S}, α^{1F} (s=slower, f=faster) and the slow migrating α^2-chain. The Hp 1-1 phenotype expresses only α^1-chains, α^2-chains are present in Hp 2-1 and Hp 2-2 (151,152). The loci involved for the Hp synthesis are located on chromosome 16q22. The Hp 1-1 is a small molecule (86kDa) with formula $(\alpha^1\beta)_2$. Heterozygote Hp 2-1, $(\alpha^1\beta)_2 + (\alpha^2\beta)_n$ (n=0,1,2,...), is characterized by polymerisation. Hp 2-2 comprises higher molecular mass forms (>200 kDa) with formula $(\alpha^2\beta)_n$ (n=3,4,5,...) (154,156).

The synthesis of Hp is considerably lower in fetal than in adult liver (151). The hepatic synthesis of Hp is induced by cytokines such as interleukin-6, interleukin-1 and tumor necrosis factor (151,157).

The haptoglobin concentration is Hp phenotype dependent. The reference range of the haptoglobin concentration is lower in individuals carrying the Hp 2-2 phenotype than individuals carrying the Hp 1-1 and Hp 2-1 phenotype (158).

The haptoglobin phenotype distribution differs according to geographical localisation of the population studied (159). In Belgium the phenotype distribution in a healthy population is: Hp 1-1: 16 %, Hp 2-1: 48 % and Hp 2-2: 36 % (160).

The haptoglobin protein has different functional properties

a. *Binding haemoglobin*

Hp forms a soluble complex with Hb. The binding of Hp with Hb is characterised by a high affinity (161). After destruction of erythrocytes, free Hb in the circulation passes through the glomerular filter and renal damage may occur. Hp reduces the loss of Hb and iron, because the Hb-Hp complex is not filtered through the glomeruli but is transported to the liver (153). Hb binding depends not only on serum concentration of Hp but also on Hp phenotype (162). The haemoglobin binding capacity is lowest in persons with the Hp 2-2 phenotype and highest in Hp 1-1 individuals (162).

b. *Protection against free radicals*

Free Hb promotes the accumulation of hydroxyl radicals (163). Haeme iron catalyses the oxidation of low-density lipoproteins, which can damage vascular endothelial cells (164). These dangers are reduced by the phenotype dependent Hb-binding capacity of Hp (162,165). Breakdown of erythrocytes in the interstitial fluid results in Hb-mediated $^{\cdot}OH$ formation. The distribution of highly polymeric Hp 2-2 proteins in extravascular fluids is restricted by their molecular mass (154). Consequently, the antioxidative capacity of body fluids is less effective in Hp 2-2 individuals (154).

c. *Inhibition of prostaglandin synthesis*

Hp is an endogenous inhibitor of prostaglandin synthesis (166,167). The inhibitory effects of Hp 2-2 and Hp 2-1 are less pronounced than that of Hp 1-1 (154).

d. *Bacteriostatic effect*

As a consequence of the capture of free Hb by Hp, haeme iron is unavailable for bacterial growth (168). An iron-restrictive environment established by Hp is part of the non-specific defence against bacterial infection.

e. *Angiogenesis*

Hp has been identified as one of the serum angiogenic factors required for proliferation and differentiation of endothelial cells in the formation of new blood vessels (169,170). Hp 2-2 is more angiogenic than the other phenotypes (169).

f. *Immunological properties*

Human serum from Hp 2-2 and Hp 2-1 individuals agglutinates the Streptococcus pyogenes group A, carrying the T4 antigen. The Hp 2-2 serum has higher agglutination titres than the Hp 2-1 serum. In contrast, Hp 1-1 has no agglutination effect. Hp is not a true antibody because it does not possess the highly variable antigen-binding sites characteristic for the Fab moiety of immunoglobulins. The agglutination is probably mediated via binding with lectin-like structures (171,172).

Comparison of reference values for lymphocyte subsets in peripheral blood and bone marrow show significant differences between haptoglobin phenotypes. Hp 2-2 is associated with higher peripheral B-lymphocyte counts and CD4+ T-lymphocytes counts. In contrast, in bone marrow, CD4+ T-cell percentages are high but B-cell percentages low in Hp 2-2 type. Flow cytometric analysis demonstrates that Hp binds to human B-lymphocytes via the CD22 receptor. Although the affinity of the binding is the same for the three phenotypes, the number of free CD22 binding sites is estimated to be higher in Hp 2-2 blood. No significant binding is detected for T-cells and NK-cells (173).

g. *Influence on iron status*

In males but not in females, the Hp 2-2 phenotype is associated with higher serum iron, transferrin saturation and ferritin concentrations than in the Hp 1-1 and Hp 2-1 phenotype, whereas soluble transferrin concentrations are lower. Serum ferritin correlated with monocyte L-ferritin content which is also highest in the male Hp 2-2 subgroup (174). CD163 has been identified as the monocyte-macrophage receptor binding the Hb-Hp complex. CD163 binds only haemoglobin and haptoglobin in complex, which indicates the exposure of a receptor-binding neoepitope. Complexes of haemoglobin and multimeric Hp 2-2 haptoglobin exhibit higher functional affinity for CD163 than do complexes of haemoglobin and dimeric Hp 1-1 haptoglobin (175).

Considering the different properties of the haptoglobin phenotype, the polymorphism has demonstrated to have an effect on different clinical entities (159). The higher immune reactivity of the Hp 2-2 phenotype results in an overrepresentation of this phenotype in autoimmune diseases (176,177) and an underrepresentation in malignancies (178-181). Depending on the nature of the antigen, differences in antibody titre after vaccination are seen between the Hp phenotypes (182-184).

Associations between Hp phenotypes and atherosclerotic disorders have been demonstrated. However, it remains unclear whether the role of Hp in development of atheromatous lesions can be attributed to inhibition of local oxidative stress or to modulation of immune and inflammatory reactions (185-187).

HIV patients carrying the Hp 2-2 show a worse prognosis. This is attributed to a less effective protection against iron-driven oxidative stress (188).

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AIMS OF THE WORK

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Since the *HFE* mutation and the haptoglobin polymorphism influence the iron storage capacity (1,2) and are related with different immunological properties (3,4), we studied the effects of these gene polymorphisms on two diseases associated with iron accumulation.

Hereditary hemochromatosis is the most frequent genetically influenced iron storage disease in Western Europe (5). Before the discovery of the *HFE* mutation in 1996, clinicians had to rely on the phenotypic presentation and the hereditary character of the disease to establish the diagnosis. After the recognition of the *HFE* mutation (6), it became clear that not all individuals with the Cys282Tyr missense mutation behaved identically and expressed the phenotype of hereditary hemochromatosis (7).

Due to its effect on iron metabolism, the haptoglobin polymorphism could alter the clinical presentation, prognosis and perhaps treatment of patients with hereditary hemochromatosis.

The first part of this work investigated:

a) Whether Flemish patients which were classified as having hereditary hemochromatosis before the recognition of the *HFE* missense mutation, carried the Cys282Tyr and/or the His63Asp missense mutation.
b) Whether the haptoglobin polymorphism had influence on the phenotypic expression of Cys282Tyr homozygous hereditary hemochromatosis.
c) Whether the percentage of CD163 monocyte-macrophage receptors differed between the different haptoglobin phenotypes in healthy males and whether the number of CD163 receptors differed in patients with hereditary hemochromatosis.

Chronic hepatitis C is a second disease associated with a largely unexplained iron overload and higher oxidative stress (8,9). Moreover, ribavirin, which has become a standard part of the treatment of chronic hepatitis C, causes higher serum and liver iron levels. Probably this phenomenon is due to the haemolytic anaemia induced by ribavirin treatment (10).

Since both the *HFE* missense mutation and the haptoglobin polymorphism could alter, through their effect on iron metabolism and differences in immunological response, the progression to chronicity, iron status and response to treatment in patients with chronic hepatitis C infection, its is interesting to evaluate the influence of these two variables in these patients.

The second part of this work investigated:

a) Whether the frequency of the *HFE* missense mutation and the distribution of the haptoglobin phenotypes differed between chronic hepatitis C patients and a healthy control population.
b) Whether these two factors (*HFE* missense mutation and haptoglobin polymorphism) were associated with each other and had influence upon the clinical presentation of chronic hepatitis C virus infection.
c) Which pre-treatment factors influenced the degree of ribavirin-induced haemolysis.
d) Whether NTBI was present in patients with chronic hepatitis C and whether treatment with ribavirin might induce the appearance of NTBI.

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CHAPTER I

PREVALENCE OF THE Cys282Tyr AND His63Asp MUTATION IN FLEMISH PATIENTS WITH HEREDITARY HEMOCHROMATOSIS

Prevalence of the Cys282Tyr and His63Asp mutation in flemish patients with hereditary hemochromatosis

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Abstract

Recently Feder *et al.* have identified the gene responsible for hereditary hemochromatosis ; it is located 3 Mbp telomeric of the MHC region on chromosome 6p and is called the HFE gene. The majority of the patients with hemochromatosis harbour the same missense mutation, Cys282Tyr. A second missense mutation (His63Asp) of which the significance is less clear, has also been described. To our knowledge the percentage of these two missense mutations in Flemish hemochromatosis patients is not known.

***Materials and methods :* Forty nine (49) unrelated patients with the clinical diagnosis of hemochromatosis were screened for the two missense mutations. The missense mutations were diagnosed with a PCR technique.**

***Results :* Of the 49 patients, 46 patients were homozygous for the Cys282Tyr mutation (94%), 2 were heterozygous (4%) and 1 carried two normal alleles (2%). Of the 3 patients not homozygous for the Cys282Tyr mutation, 3 were heterozygous for the His63Asp mutation (2 patients were 'compound heterozygotes').**

***Discussion :* The percentage of homozygotes (Cys282Tyr) in a Flemish hemochromatotic population is comparable with the figures published in the literature. The second missense mutation (His63Asp) could be of importance in association with the Cys282Tyr missense mutation.** (Acta gastroenterol. belg., **2000**, 63, **250-253**).

Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive genetic disorder of iron metabolism, which predominantly affects Caucasians. The disease has a prevalence of 2-5 per 1000 (1). HH is characterized by an increased intestinal iron absorption, resulting in progressive iron overload of parenchymal organs, leading in midlife to the onset of clinical complications such as cirrhosis of the liver, diabetes mellitus, cardiopathy, endocrine dysfunctions and arthropathy. If iron excess is detected at a very early stage, these complications can be prevented.

On the basis of an increased frequency of the HLA-A3 allele in hemochromatosis patients, Simon *et al.* were able to map a putative hemochromatosis gene on the short arm of chromosome 6 (2). Further refinement of its localization resulted in the discovery by Feder *et al.* in 1996 (3) of a candidate gene for hemochromatosis.

The authors identified a missense mutation in 85% of the patients with hemochromatosis. The missense mutation is located in a HLA-like protein ; on amino acid position 282 of that protein the missense mutation results in replacement of the amino acid cysteine by tyrosine (Cys282Tyr). Eighty-two % of the patients were homozygous for this Cys282Tyr mutation. Thereafter other authors confirmed this high assocation (4,5,6) (table 1).

In the study of Feder, a second missense mutation was found on the remaining allele in 8 of the 9 heterozygotes. That missense mutation results in a replacement of histidine by aspartate on position 63 (His63Asp). The association of this second missense mutation with HH is less clear than the assocation with the Cys282Tyr mutation, since the prevalence of the His63Asp mutation is not different between controls and patients with hemochromatosis (9).

We studied the frequency of these two missense mutations in Flemish patients with HH.

Materials and methods

Patients

Forty-nine unrelated Flemish patients with unequivocal HH were examined for the Cys282Tyr mutation. The clinical diagnosis of HH was made between 1985-1997. Patients carrying the Cys282Tyr mutation only on one allele or patients not carrying this mutation on both alleles were further examined for the His63Asp mutation.

Table 1

	Cys282Tyr+/+	Cys282Tyr+/–	Cys282Tyr–/–
Feder *et al.* (3) (n = 178)	83%	4.5%	12.5%
Beutler *et al.* (4) (n = 147)	82%	6%	12%
Jouanolle *et al.* (5) (n = 65)	91%	5%	4%
Jaxwinska *et al.* (6) (n = 112)	100%	0%	0%
Our group (n = 49)	94%	4%	2%

Prevalence of the Cys282Tyr mutation in patients with hereditary hemochromatosis.

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Flandres is located in the northern part of Belgium. Although in the past this region was colonized by different cultures (Spain, France, Austria), the majority of the people are from Celtic ancestry.

The diagnosis of HH was made using the following criteria :

1) iron saturation of more than 52% in female patients, more than 62% in male patients ; 2) a ferritin level above 750 ng/ml (normal : < 250 ng /ml), with the majority of our patients with a level above 1500 ng/ml ; 3) histochemical staining of iron on liver biopsy was performed semi-quantitatively by a Perl's stain. At least 75% of the hepatocytes had stainable iron ; 4) more than 3 g iron removed by phlebotomy.

The control group consisted of 96 unrelated normal persons. Iron status in these patients was not known. In these persons the frequency of both missense mutations was examined.

Methods

After extraction of genomic DNA from peripheral lymphocytes (2-3 ml EDTA blood) or from buccal cells, DNA was amplified by PCR using primers as described by Feder *et al.* (3). The amplified framents were further examined by restriction enzyme digestion followed by agarosegel electrophoresis.

The missense mutation Cys282Tyr results in an additional *Rsa*I restriction site. The missense mutation His63Asp results in a loss of a *Mbo*I restriction site.

The DNA profiles obtained in the homozygous and heterozygous individuals and in the normal subjects are readily distinguishable (fig. 1).

When a patient was found to be homozygous for the Cys282Tyr mutation, his partner was screened for this missense mutation.

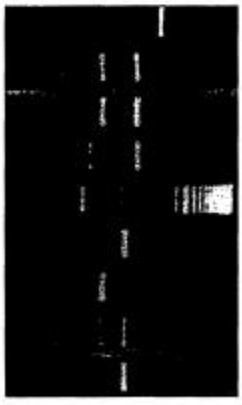

Fig. 1. — Visualisation of the PCR fragments after 2% agarosegel electrophoresis and digestion with *Rsa*I (Cys282Tyr) or *Mbo*I (His63Asp). N means a normal sequence ; M means that the mutation is present.

Stastistical analysis

Chi-square test was used to determine differences between control group and patient group. P value of less than 0.05 was considered significant.

Results

In the control group (table 2) 87.5% of the subjects were homozygous normal for the Cys282Tyr missense mutation ; 10.5% were heterozygous and 2% were Cys282Tyr+/+. These results are in accordance with the Hardy-Weinberg equilibrium.

Forty-six out of 49 patients (94%) were homozygous for the Cys282Tyr mutation (table 2). Two patients (4%) were heterozygous and one (2%) did not carry the missense mutation on either of both alleles (Chi-square versus control population : $p < 0.001$). In the latter 3 patients the His63Asp mutation was additionally examined (table 3). All were heterozygous for the His63Asp missense mutation, implying that two of these three patients were compound heterozygotes (Cys282Tyr+/–, His63Asp+/–).

The characteristics of these 3 patients are presented in table 4.

Table 2

Cys282Tyr	Patients	Controls
+ / –	2% (1/49)	87.5%(84/96)
+ / –	4% (2/49)	10.5%(10/96)
+ / +	94%(46/49)	2%(2/96)

Table 3

His63Asp	Patients	Controls
– / –	94% (46/49)	68% (65/96)
+ / –	6% (3/49)	31% (30/96)
+ / +	0% (0/49)	1% (1/96)

Table 4. — Clinical details of the patients not homozygous for the Cys282Tyr mutation

Patient	Cys282Tyr	His63Asp	Known family history	Age at onset
1	– / –	+ / –	no	32
2	+ / –	+ / –	yes	55
3	+ / –	+ / –	no	55

Patient	Iron removed	Liver histology	Iron saturation	Serum ferritin (ng/ml)
1	15 g	siderosis	65%	1200
2	3.5 g	siderosis	62%	795
3	7 g	cirrhosis	67%	4220

Patient 1 did not carry the Cys282Tyr mutation and is heterozygous for the His63Asp mutation. This male patients complained about diffuse arthritic pains. The diagnosis of hemochromatosis was made on iron studies and liver biopsy (Perl's staining was highly positive in hepatocytes and Kupffer cells). He had no risk factors for secondary iron overload : alcohol abuse, hemolytic anaemia, blood transfusion, chronic viral liver disease. Phlebotomy was started and the iron donation was 15 g before normalisation of the ferritin level could be achieved. These findings are very suggestive for hemochromatosis. However there was no family history of hereditary hemochromatosis and there was no HLA pattern as found in hereditary hemochromatosis (A1 A2 B8 B18).

Patient 2 consulted because two brothers had hereditary hemochromatosis. This male patient is a compound heterozygote for both mutations, but he has only mild iron overload (ferritin : 795 ng/ml, moderate iron staining on liver biopsy, and an iron donation of 3.5 g before normalisation of the ferritin level was achieved). He had no risk factors for secondary iron overload.

Patient 3 consulted for chronic fatigue. The diagnosis of hemochromatosis was made on iron studies and liver biopsy (highly positive Perl's staining in hepatocytes and Kupffer cells in a cirrhotic liver). The iron donation before normalisation of ferritin was 7 g. There was no family history and the patient had no risk factors for secondary iron overload.

This male patient was compound heterozygote.

Concerning the His63Asp missense mutation, 68% of our control population did not carry the missense mutation, 31% were heterozygous and 1% was homozygous for this missense mutation (table 3). These results are in accordance with the Hardy-Weinberg equilibrium.

Family screening was extremely easy using the cheek brush technique. In 11 patients the partner was checked for the Cys282Tyr mutation. The partner was heterozygous in 8 out of 11 marriages (72%), which is high when compared with our control population (10.5%).

Discussion

The present results are in accordance with the previously reached conclusion that mutations in the HFE gene are present in a high percentage of patients with HH. Our results are comparable with those of other authors (4,5,6) (table 1). Patients were homozygous for the Cys282Tyr mutation in 94% of the cases, whereas in the control group homozygosity for this mutation was found in only 2%(results in accordance with the Hardy-Weinberg equilibrium, HFE allele frequency in the control group of 0.07 which is comparable with data from other centers in the Western World, cfr. table 1).

The finding of this high percentage of the Cys282Tyr missense mutation in our and other groups of patients demonstrates that this missense mutation really contributes to the phenotypic presentation of the disease. Crawford et al could demonstrate a very high penetrance of this missense mutation (10).

However, a recent publication by Adams et al reported about homozygous individuals with no evidence of clinical iron overload (11). Determination of the iron status of the homozygous people in our control group, could give some additional information about the genotype/phenotype correlation. However we were not able yet to compare the homozygous patients and the control group with respect to clinical symptoms, biochemical signs of iron overload or pathological iron overload.

Whether this gene is really responsible for the disease or is only associated at a high frequency in the patients with HH as an 'innocent bystander', is unclear at present. There is however some evidence for the former hypothesis. In the presence of the Cys282Tyr mutation, a disulfide bridge is broken which alters the presentation of the HLA-H protein outside the cell. This disulfide bridge is necessary for the binding with beta2-microglobulin. Animal studies show that beta2-microglobulin knock-out mice have an iron accumulation pattern similar to patients with HH (7,12).

Feder *et al.* could demonstrate that in cell culture, the binding between the transferrin receptor and the wild-type HFE gene, decreases the affinity between the transferrin receptor and transferrin. Whereas in the presence of the mutant HFE gene this binding is completely inhibited. These data show that the HFE gene could be directly involved in the iron metabolism (13).

The significance of the His63Asp mutation is less clear. It is suggested that it is only of importance in the compound heterozygote setting (9). In our study two patients heterozygous for the Cys282Tyr missense mutation were also heterozygous for the His63Asp missense mutation (compound heterozygotes).

Patient 2 had a family history of hereditary hemochromatosis. Since the iron overload was only moderate in this patient (table 4) and since the age at presentation was 55 years, he had only a mild form of hereditary hemochromatosis. Determining the missense mutations and the degree of iron overload in the other affected family members, would give us the opportunity to investigate whether the presence of the His63Asp missense mutation modifies the clinical presentation of HH.

Patient 3 had substantial iron overload and already advanced liver disease. On biochemical and pathological grounds there was no doubt about the diagnosis of hemochromatosis. The iron donation was 7 g before ferritin normalisation could be achieved. Although the patient had no family history, the clinical picture is highly suggestive for hemochromatosis. In this patient it seems that being compound heterozygous, could be a risk factor for developing hemochromatosis. However in the study of Jouanolle (5) the prevalence of compound heterozygotes was not different in the control group than in the group of patients with HH. This frequency was about 2%.

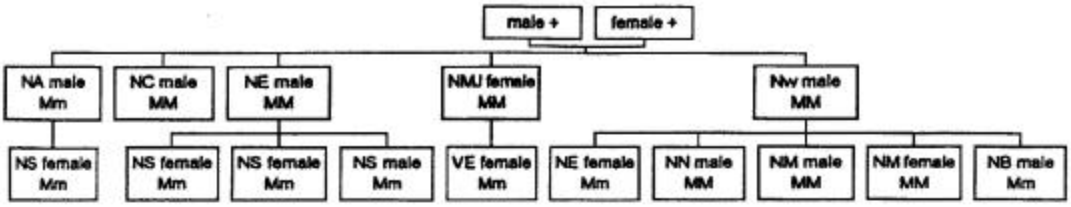

Fig. 2. — Family tree of an index patient with HH. MM means homozygous for the Cys282Tyr missense mutation. Mm means heterozygous for the Cys282Tyr missense mutation. + means deceased.

We found a frequency of about 30% of heterozygotes for the His63Asp mutation in our control population. As such marriages between Cys282Tyr homozygotes and His63Asp heterozygotes would have a 50% occurence risk of compound heterozygote offspring. If the His63Asp missense mutation is involved in the pathogenesis of HH, these children should develop hemochromatosis

This figure is higher than seen in clinical practice. These are indirect arguments that the His63Asp mutation is of only minor importance. However more longitudinal follow-up of compound heterozygous people and additional family tree studies are necessary to explain the real importance of the His63Asp missense mutation. However Rish could demonstrate that being compound heterozygote results only in a minor risk to develop HH (15).

Patient 1 did not carry the Cys282Tyr or the His63Asp missense mutation. And although there was no family history of HH, the clinical presentation was very typical for HH. Another still not established mutation could be the explanation of this finding. So recently, Wallace et al demonstrated the presence of a new gene responsible for HH (14).

Screening the family of an index patient with HH becomes extremely easy using the cheek brush technique. Buccal cells prelevated by this technique can be examined in the same way as lymphocytes prelevated by blood sampling. Prelevation can be done at home by the patient and his family. In our experience this makes the screening method more acceptable.

It is more cost-effective to screen the partner of the patient for the Cys282Tys mutation first and to screen only the children if the partner is heterozygous for this mutation (8). Since the His63Asp missense mutation is of minor importance(15), one can restrict the screening to determining only the Cys282Tyr missense mutation.

We were able to screen 11 partners of patients with HH. Eight out of 11 were heterozygotes. Although this prevalence is higher than in the control population, it seems to us to be purely coincidental. However this high frequency illustrates the importance of family screening (fig. 2.)

We conclude that in our group of Flemish patients with HH, a high number (94%) of patients is homozygous for the Cys282Tyr mutation. The His63Asp mutation seems to be of minor importance, however in some patients who are compound heterozygotes, clinical expression of hereditary hemochromatosis can be present.

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CHAPTER II

HAPTOGLOBIN PHENOTYPE 2-2 OVERREPRESENTATION IN Cys282Tyr HEMOCHROMATOTIC PATIENTS

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Haptoglobin phenotype 2-2 overrepresentation in Cys282Tyr hemochromatotic patients

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***Background/Aims*: Patients with genotypic Cys282Tyr homozygous hemochromatosis differ largely in phenotypic presentation. The HFE mutation on itself does not explain the different manifestations of hemochromatosis. We hypothesized that the genetic haptoglobin (Hp) polymorphism, because of its effect on iron metabolism, could be a modifying factor that influences the clinical presentation of hereditary hemochromatosis.**

***Methods*: In 167 Cys282Tyr homozygous hemochromatotic patients, the frequencies of Hp types (1-1, 2-1 and 2-2) and alleles (Hp1, Hp2) were compared with those in 918 healthy subjects. Clinical and laboratory indices of iron overload were incorporated in the analysis.**

***Results*: The Hp 2-2 type was overrepresented in the patient group ($P < 0.01$). Male patients carrying Hp 2-2 had higher serum iron ($P = 0.003$) and ferritin levels ($P = 0.03$) than those with a Hp 1-1 or 2-1 type. The amount of iron removed with phlebotomy was also higher in Hp 2-2 patients ($P = 0.03$).**

***Conclusions*: The Hp 2-2 type is overrepresented among Cys282Tyr homozygous hemochromatotic patients. At diagnosis, iron overload was more pronounced in male patients carrying Hp 2-2. Our data suggest that Hp polymorphism affects iron metabolism in hereditary hemochromatosis.**

***Keywords*: Haptoglobin phenotype; Hemochromatosis**

1. Introduction

Hereditary hemochromatosis is an autosomal recessive genetic disorder of iron metabolism, which affects 0.5% of Caucasians [1]. The disease is usually due to a mutation in the HLA-linked hemochromatosis gene (HFE) on chromosome 6, that leads to a change from cysteine to tyrosine at position 282 in the HFE protein (C282Y) [2,3]. The phenotypic expression may vary from a fully penetrant clinical syndrome characterized by bronze pigmentation, cirrhosis, arthritis, endocrinopathy and cardiomyopathy to a mere laboratory abnormality, namely an increased serum iron saturation [4]. Recent reports have suggested that the phenotypic heterogeneity of hemochromatosis could be related to additional non-genetic causes, additional mutations or additional genes (linked or not linked to the MHC complex) [5–7].

Haptoglobin (Hp) might be one of the modifier genes in the phenotypic expression of HLA-linked hemochromatosis. Hp is a haemoglobin-binding plasma protein and allows the hepatic recycling of haemoglobin (Hb) iron. Following intravascular haemolysis Hb–Hp complexes are formed which are rapidly delivered to the hepatic parenchymal cells by receptor-mediated endocytosis. In humans, Hp is characterized by a genetic polymorphism with three major

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types (Hp 1-1, Hp 2-1 and Hp 2-2) which results from the expression of two different alleles (Hp1 and Hp2) located on chromosome 16q22. The Hp types show an important molecular heterogeneity; Hp 1-1 is a small molecule (86 kDa) of well defined structure, whereas Hp 2-1 is characterized by heteropolymers (86–300 kDa), and Hp 2-2 forms large macro molecular complexes (170–1000 kDa) [8].

Functional differences between the various Hp types have important biological and clinical consequences [8]. In healthy men carrying the Hp 2-2 type, a fraction of Hb–Hp complexes is delocalised into monocyte-macrophages, resulting in some degree of iron retention [9,10,12].

The recently identified Hb scavenger receptor CD163 on monocyte-macrophages was demonstrated to have a higher affinity for the multimeric Hb–Hp 2-2 complex than for the monomeric Hb–Hp 1-1 complex [11]. In healthy men, the Hp 2-2 type is associated with higher serum iron and ferritin levels than the Hp 2-1 and Hp 1-1 type [12].

In this study, we aimed to test the hypothesis that Hp polymorphism might influence the phenotypic expression of iron overload in hereditary hemochromatosis. For this purpose, we investigated Hp polymorphism in hemochromatotic patients homozygous for the Cys282Tyr missense mutation. Clinical and laboratory findings of iron overload were compared between the Hp types.

2. Materials and methods

2.1. Patients

One hundred and sixty-seven unrelated patients with hereditary hemochromatosis were included in this study. They were examined in the outpatient clinics of six Belgian hospitals (Ghent University Hospital, Clinique St. Luc Brussels, UZA Antwerp, Virga Jesse Hasselt, General Hospital Brugge and Jolimont Hospital). All patients were homozygous for the Cys282Tyr missense mutation determined according to the method of Feder et al. [2].

The patients visited the clinics for symptoms that ranged from fatigue, arthralgia, breathlessness to symptoms of decompensated liver disease ($n = 2$). Some were referred to the clinic because of abnormal liver function test, abnormal glucose tolerance tests or increased serum iron and ferritin levels. The control population for the Hp phenotype consisted of 918 healthy Caucasian blood donors (451 male donors, 467 female donors).

2.2. HFE mutation and haptoglobin phenotype

The Cys282Tyr missense mutation of the HFE gene was determined by polymerase chain reaction and restriction enzyme digestion according to the method of Feder et al. [2]. The Hp phenotype of all subjects was determined using starch gel electrophoresis of haemoglobin-supplemented serum, followed by peroxidase staining [13].

2.3. Biochemical investigations

Fasting blood samples were obtained, allowed to clot and centrifuged. The supernatant serum was collected for analysis. Serum iron concentration and total iron binding capacity (TIBC) were determined using commercial agents (Roche), and transferrin saturation was calculated by the formula: 100 × (serum iron/TIBC). Serum ferritin levels were measured using fixed-time immunonephelometry (Dade Behring).

Serum alanine aminotransferase (ALT) activity and glucose concentrations were assayed using commercial agents (Roche).

2.4. Liver biopsy and phlebotomy

Liver biopsy was performed at diagnosis. Fibrosis was defined histologically as bridging fibrosis with or without cirrhosis. In 20 patients, liver iron concentration was measured on liver biopsy and hepatic iron index was expressed as hepatic iron concentration/age.

After phlebotomy, the amount of iron removed was determined as the number of liters of blood removed multiplied by 0.5 g to achieve iron depletion (defined as serum ferritin level <50 μg/l).

2.5. Statistics

At diagnosis the following data were recorded: 1) gender and age; 2) medical history, including alcohol consumption; 3) serum iron level, transferrin saturation and serum ferritin level; 4) serum ALT activity; 5) fasting serum glucose level; 6) INR (international normalised ratio) 7) liver histology; 8) hepatic iron index; 9) amount of iron removed by phlebotomy to achieve iron depletion.

Statistics were performed using the Medcalc program version 5.00.002 [14]. Normality of the distribution of the different variables was tested using the Kolmogorov–Smirnov test. Variables with a normal distribution are presented as mean ± standard deviation. Variables with a non-Gaussian distribution are presented as median and interquartile range.

Parametric (Student t-test) and non-parametric (Wilcoxon) tests were used according the distribution of the variable. Multiple regression analysis was performed with serum iron, ferritin and iron donation as dependent variables.

The Chi-square test was used to evaluate the Hardy–Weinberg equilibrium and to compare the distribution of the Hp types between patients and controls. Relative risk was calculated using odds ratio and 95% confidence interval. P-values of less than 0.05 were considered significant.

3. Results

3.1. Patient population

The patient population is presented in Table 1. The majority of the patients is male (70%). Liver fibrosis is present in 35% of the patients. Male patients were characterized by higher prevalence of fibrosis, higher serum ferritin concentrations, younger age at diagnosis and higher amount of iron removed to achieve iron depletion.

3.2. Haptoglobin phenotype distribution

Hp types and alleles are presented in Table 2. The distribution of Hp types among patients and healthy subjects were all in accordance with the Hardy–Weinberg equilibrium. A low Hp1 allele frequency was found in hereditary hemochromatosis patients and was attributable to an over-representation of the Hp 2-2 type ($P < 0.01$). Hp 2-2 was associated with a relative risk of 1.73 (95% CI 1.14–2.62) for hemochromatosis. Sex distribution was comparable between Hp types. The under-representation of the Hp1 allele was only observed in the male patients ($P < 0.01$) but was not significant in the female patients ($P = 0.3$).

Table 1
Characteristics of the hemochromatosis patients at diagnosis

	Total ($n = 167$)	Male patients ($n = 115$)	Female patients ($n = 52$)	P-value between males and females
Age (years)	50 ± 13	48 ± 13	55 ± 14	0.02
Serum iron level (μmol/l)	38 ± 8	39 ± 8	35 ± 9	NS
Transferrin saturation (%)	90 (78–97)	92 (82–99)	85 (69–95)	0.02
Serum ferritin level (μg/l)	1025 (670–1726)	1300 (767–2077)	670 (449–1203)	0.0003
ALT (U/l)	42 (27–62)	45 (28–64)	35 (20–59)	NS
Glucose level (mmol/l)	5.3 (4.9–5.9)	5.0 (4.9–5.9)	5.5 (5.1–6.3)	NS
INR	1.01 ± 0.09	1.01 ± 0.10	1.01 ± 0.08	NS
Presence of fibrosis (%)	35	45	8	0.04
Hepatic iron index ($n = 20$)[a]	1.23 (0.76–2.35)	1.26 (0.94–2.06)	1.07 (0.68–4.09)	NS
Iron donation, g	6.0 (3.4–11.2)	8.0 (4.0–13.9)	4.2 (2.2–6.7)	0.02

[a] Seventeen males, three females. NS, not significant.

3.3. Indices of iron overload

In the overall hemochromatotic population, serum iron and ferritin concentrations at diagnosis were higher in hemochromatosis patients carrying the Hp 2-2 type (Table 3). These observations, however, were only attributable to Hp type-related differences in serum iron and ferritin levels in the male subgroup (Table 4). The amount of iron removed by phlebotomy to achieve iron depletion was also higher in Hp 2-2 patients (Table 3).

Higher ferritin levels were not only seen in patients with the Hp 2-2 phenotype but also in male patients ($P < 0.001$), patients with a higher age at diagnosis ($P = 0.001$) and in patients with a higher ALT level ($P < 0.01$). After multiple regression analysis, the Hp 2-2 phenotype remained an independent variable influencing the serum ferritin level. Similar results were obtained for serum iron concentration and iron donation in multivariate analysis (Table 5).

4. Discussion

Hereditary hemochromatosis is one of the most frequent genetically caused disorders in Western Europe and in Belgium in particular. In Belgium, 10.5% of the population carry the missense mutation C282Y [3]; this results in a frequency of the disease of about one individual in 400 persons. Homozygosity for the C282Y mutation is found in 85 to 90% of patients of Northern European origin who have typical hereditary hemochromatosis [2,3,15,16]. Since the introduction of the genetic diagnosis by Feder et al. [2] in 1996, it became obvious that the phenotypic expression of the disease is highly variable. This is illustrated in our cohort by the wide distribution of the iron removal data and the hepatic iron index (Table 1). In a recent study [17], Crawford et al. have demonstrated that 17.3% of the subjects homozygous for the C282Y mutation do not express iron overload that meets the current diagnostic criteria of hemochromatosis. In another trial Olynyk et al. have shown that only 50% of the population homozygous for the C282Y mutation expressed clinical features of hemochromatosis, whereas one quarter had serum ferritin levels within the normal range during a four-year period [18].

It is generally accepted that women and younger people have less severe phenotypic presentation of the disease. Iron overload develops at a slower rate in women with hereditary hemochromatosis than in men with the disorder, because in women iron stores are depleted by menstruation and pregnancy [18]. This is also observed in our cohort where men more often display hepatic fibrosis and have higher serum ferritin levels than women. Since hemochromatosis is a disease that progresses with time, younger people more often have milder disease than older people [18].

Alcohol intake is known to increase hepatic iron stores [19] and could be a non-genetic variable that influences the iron overload in C282Y homozygous individuals. In our

Table 2
Haptoglobin phenotype distribution in patients and controls

Hp phenotype	Patients ($n = 167$)	Male patients ($n = 115$)	Female patients ($n = 52$)	Controls ($n = 918$)	Male controls ($n = 451$)	Female controls ($n = 467$)
Hp 1-1	21 (12.5%)	12 (10.5%)	9 (17.5%)	140 (15.0%)	71 (16%)	69 (15%)
Hp 2-1	65 (39.0%)	46 (40.0%)	19 (36.5%)	445 (48.5%)	223 (49%)	222 (47.5%)
Hp 2-2	81 (48.5%)	57 (49.5%)	24 (46.0%)	333 (36.5%)	157 (35%)	176 (37.5%)
Hp 1 allele frequency	0.320	0.304	0.356	0.395	0.405	0.385
P-value versus control population[a]	0.004	0.005	0.3			

[a] P-value: Chi-square test comparing the frequency of the Hp 2-2 phenotype between patients and controls.

Table 3
Patient characteristics at diagnosis according to haptoglobin phenotype

	Hp 2-2 ($n = 81$)	Hp 1-1 and Hp 2-1 ($n = 86$)	P-value
Age (years)	52 ± 13	48 ± 13	NS
Serum iron level (μmol/l)	41 ± 8	35 ± 8	0.0005
Transferrin saturation (%)	92 (82–97)	89 (69–97)	NS
Serum ferritin level (μg/l)	1300 (740–2156)	936 (507–1324)	0.01
ALT, (U/l)	42 (25–58)	42 (28–63)	NS
Glucose level (mmol/l)	5.3 (4.9–5.9)	5.2 (4.8–6.0)	NS
INR	1.01 ± 0.10	1.01 ± 0.09	NS
Presence of fibrosis (%)	30	29	NS
Hepatic iron index ($n = 20$)[a]	1.23 (0.63–2.77)	1.36 (1.01–1.96)	NS
Iron donation (g)	8.0 (3.7–12.2)	4.8 (3.2–10.1)	0.03

[a] Patients with Hp2-2: 13; patients with Hp 1-1 or 2-1: 7. NS, not significant.

group of patients (data not shown) only two subjects were abundant alcohol abusers, defined as an intake of ethanol exceeding more than 80 g per day. Therefore it seems unlikely that in the present study, this variable has played an important role in shaping the phenotypic expression of the disease.

Recent reports have suggested that the phenotypic heterogeneity of hemochromatosis disease could be related to additional non-genetic causes, additional mutations or additional genes [5], and that one or more of these genes may be MHC-linked [6]. A recent study by Pratiwi et al. [7] has demonstrated that in male patients homozygous for a predominant ancestral haplotype significantly higher hepatic iron indices are found than in patients heterozygous or nullizygous for this gene. Analogous data are observed by others [20,21].

Since the haptoglobin polymorphism causes differences in serum markers of iron status in healthy males [12], this polymorphism may alter the phenotypic presentation of patients with hereditary hemochromatosis. Indeed, Langlois et al. demonstrated that in healthy men the Hp 2-2 phenotype is associated with higher serum iron levels, higher transferrin saturation, higher serum ferritin levels and lower soluble transferrin receptor concentrations.

In our group of patients with hereditary hemochromatosis, the Hp 2-2 phenotype is highly overrepresented, particularly in male patients ($P < 0.01$). The lack of significance in females is possibly related to the lower number of female patients included. Although other factors (e.g. menstruation, pregnancy) could have a greater influence on the phenotypic expression of hereditary hemochromatosis in female patients.

As in a population of healthy subjects [12], the male patients with the Hp 2-2 phenotype have higher serum iron and ferritin levels than the male patients with the Hp 1-1 or Hp 2-1 phenotype. No difference could be seen in female patients. The higher iron overload in Hp 2-2 patients is also illustrated by the higher amount of iron that has to be removed to achieve iron depletion. The lack of difference in the hepatic iron index between the different phenotypes is possibly due to the low amount of patients ($n = 20$) in which the hepatic iron index was measured.

Although no haptoglobin type-related differences are found when considering age at diagnosis, hepatic iron index and occurrence of liver fibrosis, the higher serum iron and ferritin levels in Hp 2-2 hemochromatotic patients are striking. The higher levels of serum ferritin both in Hp 2-2 healthy men and Hp 2-2 hemochromatotic male patients could be explained by the Hp 2-2 specific delocalizing of Hb–Hp complexes into monocyte-macrophages [12]. In healthy subjects, this results in a certain degree of iron retention [12]. This Hb–Hp related iron uptake in monocyte-macrophages has recently been demonstrated to occur via the Hb scavenger receptor CD163 [11]. This receptor has a higher affinity for the multimeric Hb–Hp 2-2 complex than for the monomeric Hb–Hp 1-1 complex [11]. In Hp 2-2 individuals, this result in a higher intracellular monocyte ferritin level [12]. Monocyte L-ferritin

Table 4
Serum iron and ferritin in males and females according the haptoglobin phenotype polymorphism

	Hp 2-2 ($n = 81$)	Hp 1-1 and Hp 2-1 ($n = 86$)	P-value[a]
Serum iron (μmol/l)			
Males	43 ± 7	37 ± 7	0.003
Females	36 ± 8	38 ± 9	NS
Serum ferritin (μg/l)			
Males	1747 (750–2661)	1100 (843–1603)	0.03
Females	925 (529–1349)	960 (569–1999)	NS

[a] NS, not significant.

Table 5
Multiple regression analysis of the variables influencing serum ferritin level, serum iron level and blood donation[a]

	Serum ferritin level	Serum iron level	Iron donation
Hp 2-2 phenotype	<0.01	<0.001	<0.05
Age at diagnosis	<0.001	NS	NS
Gender	<0.001	NS	<0.01
ALT activity	<0.01	NS	NS

[a] NS, not significant.

content positively correlates with serum ferritin concentration and thus higher serum levels are found in Hp 2-2 subjects [12].

In conclusion, we have found a significant overrepresentation of the Hp 2-2 phenotype in a cohort of patients with hereditary hemochromatosis. This overrepresentation is only observed in the male part of the population. In addition, male hemochromatotic patients with the Hp 2-2 phenotype have higher serum iron and ferritin levels than Hp 1-1 and 2-1 patients. Our data suggest that the Hp phenotype polymorphism affects iron metabolism in hereditary hemochromatosis.

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CHAPTER III

LACK OF RELATIONSHIP BETWEEN THE RELATIVE NUMBER OF CD163+ MONOCYTES-MACROPHAGES AND IRON METABOLISM IN HEREDITARY OR SECONDARY HEMOCHROMATOSIS.

(submitted: Gastroenterology)

LACK OF RELATIONSHIP BETWEEN THE RELATIVE NUMBER OF CD163+ MONOCYTES-MACROPHAGES AND IRON METABOLISM IN HEREDITARY OR SECONDARY HEMOCHROMATOSIS.

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Keywords:
CD163, haptoglobin phenotype, hereditary hemochromatosis, secondary hemochromatosis

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ABSTRACT

Introduction

The monocyte-macrophage receptor CD163 has been shown to be the receptor of haemoglobin-haptoglobin (Hb-Hp) complexes. The binding of the Hb-Hp complexes is haptoglobin phenotype dependent, resulting in a higher macrophage iron content in Hp 2-2 individuals. We determined the percentage of CD163+CD33+ monocytes-macrophages in patients with different haptoglobin phenotypes and in patients with hereditary and secondary hemochromatosis.

Materials and methods

The percentage of CD163+ CD33+ peripheral blood monocytes was determined by flow cytometry. The percentage in 30 male volunteers with different haptoglobin phenotypes was determined and compared with the percentage in 16 patients with hereditary hemochromatosis and 5 patients with secondary iron overload.

Results

In the healthy volunteers there was no difference in the percentage of CD163+CD33+ monocytes-macrophages between the different haptoglobin phenotypes. No difference was found in the percentage of CD163+CD33+ monocytes-macrophages between patients with hereditary hemochromatosis (67 ± 23) or secondary hemochromatosis (58 ± 27) versus controls (54 ± 22).

Discussion

There is no haptoglobin related difference in the percentage of CD163+CD33+ monocytes-macrophages. The data in the patients with hereditary and secondary hemochromatosis argue against a relationship between the relative number of CD163+CD33+ circulating cells and iron metabolism.

Abstract electronic word count: 194

Introduction

CD163 is a member of the scavenger receptor cysteine-rich group B family of proteins. Members of this family contain highly conserved scavenger receptor (SRCR) domains, and include the type I scavenger receptor, CD5, CD6 and WC1 (found on bovine γ δ T cell) (1). SRCR proteins are typically associated with immune functions and are expressed on a number of different cells of the immune system (2). CD163 expression is restricted to cells of monocyte lineage and increases as monocytes mature into macrophages (3). The mRNA and protein levels of CD163 are tightly controlled by inflammatory regulators. Whereas proinflammatory stimuli like lipopolysaccharide or IFN-γ suppress its expression, anti-inflammatory molecules like glucocorticoids or IL-10 strongly induce CD163 on monocytes and macrophages (4,5).

Recently CD163 was demonstrated to act as a receptor that scavenges haemoglobin by mediating endocytosis of haemoglobin-haptoglobin complexes. Haemoglobin released into plasma is captured by the acute phase protein haptoglobin, which is depleted from plasma during elevated haemolysis. Efficient removal of free haemoglobin is essential for health because of the oxidative and toxic properties of iron-containing haem in haemoglobin. CD163 binds only haptoglobin and haemoglobin in complex, which indicates the exposure of a receptor-binding neoepitope (6).

In humans, three major haptoglobin (Hp) variants exist: Hp 1-1, Hp 2-2 and the heterozygous Hp 2-1 (7). Complexes of haemoglobin and the multimeric haptoglobin (Hp 2-2) exhibit higher functional affinity for CD163 than do complexes of haemoglobin and dimeric haptoglobin (Hp 1-1) (6). In healthy male Hp 2-2 subjects the serum ferritin and the L-ferritin concentration in monocytes-macrophages are higher than in Hp 1-1 subjects (8). This could be the consequence of the higher affinity of the Hp 2-2 – haemoglobin complex for the CD163 receptor.

Hereditary hemochromatosis is a very common autosomal recessive disease of iron metabolism. Recently the *HFE* mutation was found to be present in the majority of the patients with hereditary hemochromatosis. Despite intensive investigations, the cellular location of the defect is still controversial. In fact, the liver, duodenum and reticuloendothelial system have all been proposed as the primary site of the metabolic abnormality (9).

It is known that iron storage in macrophages differs depending on the cause of iron accumulation (10). In secondary iron overload, as a result of dyserythropoiesis, haemolysis or transfusions, macrophages are heavily loaded with iron. In contrast, in hereditary hemochromatosis, little iron is seen in the Kupffer cells and other macrophages, while hepatocytes already suffer from iron overload. In both forms of iron overload, low-molecular weight complexes of iron are observed in the plasma. Iron overload of macrophages in secondary hemochromatosis can be explained partially by the fact that these cells must process large quantities of (immature) erythrocytes, while the primary cause of iron overload in hereditary hemochromatosis is an inappropriately increased absorption of iron. However, defects in erythrophagocytosis or differences in cellular iron processing and iron release may contribute to the low amounts of iron in the macrophages of patients with hereditary hemochromatosis (10).

Considering the relative low iron content in monocytes-macrophages seen in patients with hereditary hemochromatosis, we examined if the percentage of CD163+CD33+ monocytes-macrophages differed in patients with hereditary hemochromatosis from normal control subjects and from patients with secondary hemochromatosis.

We also studied the relationship between the haptoglobin polymorphism and the percentage of CD163+CD33+ monocytes-macropaghes in healthy male individuals.

Materials and methods

Subjects

Thirty healthy male volunteers were recruited from the hospital staff of the Ghent University Hospital. Blood was sampled for CD163 expression, amino alanine transferase activity, serum iron, serum transferrin, serum ferritin, C-reactive protein, haptoglobin concentration and haptoglobin phenotype.

Considering the influence of inflammation on CD163 expression, only volunteers with a normal C-reactive protein (CRP < 1,2 mg/dl) were included. These volunteers were used as controls.

Sixteen patients (13 male, 3 female) with Cys282Tyr homozygous hereditary hemochromatosis were recruited from the out-patient clinic of the department of Gastroenterology, Ghent University Hospital. Informed consent was obtained. Blood was taken during a therapeutic phlebotomy session. Six patients were on a schedule of weekly phlebotomy to achieve serum ferritin levels of less than 50 µg/l, the other 10 received phlebotomy on an average schedule of 3 to 4 times a year to keep serum ferritin at a level of 50 µg/l.

Five patients with secondary iron overload as a result of multiple blood transfusions for the symptomatic treatment of myelodysplastic syndrome (n=4) or aplastic anaemia (n=1) were recruited from the department of Haematology.

Biochemical analysis

Blood samples were obtained, allowed to clot and centrifuged. The supernatant serum was collected for analysis. Serum iron concentration, total iron binding capacity (TIBC), serum alanine aminotransferase (ALT) activity and C-reactive protein were determined using commercial reagents (Roche).

Transferrin saturation was calculated by the formula:

$$100 \times (\text{serum iron}/\text{TIBC}).$$

Serum ferritin concentration and serum haptoglobin concentration were measured using fixed-time immunonephelometry (Dade Behring).

Determination of the haptoglobin phenotype

The Hp phenotypes were determined using starch gel electrophoresis of haemoglobin-supplemented serum followed by peroxidase staining (11).

HFE missense mutation

The Cys282Tyr missense mutation was searched for according to the method described by Feder et al (12). In short, after extraction of genomic DNA from peripheral blood mononuclear cells DNA was amplified by PCR technique. After amplification these fragments were further investigated by restriction enzyme digestion followed by agarose gel electrophoresis. The missense mutation Cys282Tyr resulted in an additional restriction site for the restriction enzyme *Rsa*I. Homozygotes, heterozygotes or normals were easily recognized. Recently, a polymorphism in intron 4 of HFE was described causing an overestimation of the Cys282Tyr homozygote prevalence in hemochromatosis. We examined all samples using a new antisense primer that excluded the site of the new polymorphism (13,14).

CD163 expression

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml heparinized blood by Ficoll-Paque 1077 gradient (Pharmacia, Upssala, Sweden) centrifugation. The PBMC were transferred into separate wells of a 24 well culture plate (Gibco BRL, Grand Island, USA) and incubated for 24 hours in RPMI 1640 medium (Gibco BRL) containing 10 % autologous plasma. The cultured cells were harvested, resuspended in phosphate buffered saline (PBS) and incubated for 30 min in the dark in the refrigerator with the following fluorochrome labelled monoclonal antibodies against cell surface markers: anti-CD33 APC (monocytes, macrophages, clone P67.6, Becton Dickinson, San Diego, USA) and anti-CD163 PE (clone GHI/61, Pharmingen, San Diego, USA).

Isotype and concentration matched control monoclonal antibodies with PE and APC were used as controls. Subsequently, the cells were washed with PBS, resuspended in PBS and analysed by flow cytometry using a FACSort (Becton Dickinson). Data were analysed using Cellquest software (Becton Dickinson). Monocytes were identified using a forward and side scatter gate in combination with a gate for CD33 positive cells. CD163 expression was determined as the percentage of CD33 positive monocytes (Figure 1).

Statistical analysis

Statistics were performed using the Medcalc program version 5.00.002 (15). Normality of the distribution of the different variables was tested using the Kolmogorov-Smirnov test. Variables with a normal distribution are presented as mean ± standard deviation. Variables with a non-Gaussian distribution are presented as median and interquartile range.

Parametric and non-parametric tests were used according the distribution of the variable. P-values of < 0.05 were considered significant.

Ethics

The protocol was approved by the Ethical Committee of the Ghent University Hospital.

Results

Healthy volunteers

All male volunteers have normal C-reactive protein levels (cf. selection criteria) and normal ALT activity. Serum iron studies, haptoglobin concentrations and percentage of CD163+CD33+ monocytes-macrophages are shown in Table 1. The mean age of the group is 33 years ± 8. The haptoglobin phenotypes distribution was: Hp 1-1: 6, Hp 2-1: 16 and Hp 2-2: 8. The percentage of CD163+CD33+ monocytes-macrophages, serum iron studies and haptoglobin concentration according the haptoglobin phenotype are shown in Table 2. There is no difference in the percentage of CD163+CD33+ monocytes-macrophages between the different haptoglobin phenotypes. Haptoglobin concentration is clearly haptoglobin phenotype dependent (Table 2).

No correlation is seen between the percentage of CD163+CD33+ monocytes-macrophages and age, serum iron, serum iron saturation and serum ferritin concentration.

Patients with hereditary hemochromatosis

The mean age of the 16 patients with hereditary hemochromatosis is 55 years ± 9. This is older than the group of healthy volunteers ($p<0.01$). Serum iron studies and percentage of CD163 cells are presented in Table 1.

Serum iron and iron saturation are higher in patients than in controls ($p<0.02$ and $p<0.01$). Serum ferritin concentration is not higher in patients than in the healthy controls, reflecting that the patients are adequately treated to achieve serum ferritin concentrations of lower than 50 µg/l. However the range in serum ferritin concentration is higher in the patient group than in the control group (Table 1). C-reactive protein concentration is normal in all 16 patients.

The percentage of CD163+CD33+ monocytes-macrophages is not significantly different in patients with HH than in controls.

In the patient group, there is no correlation between the percentage of CD163+CD33+ monocytes-macrophages and serum iron studies. In particular, the percentage of CD163+CD33+ monocytes-macrophages is not different between patients with elevated serum ferritin concentrations and those with normal serum ferritin concentrations (73±17 versus 57±30, p=0.2).

Patients with secondary hemochromatosis

The mean age of the 5 patients with secondary hemochromatosis is 63 years ± 16 ($p<0.001$ versus the control population, $p=0.3$ versus the patients with hereditary hemochromatosis). Serum iron studies and percentage of CD163+ cells are presented in table 1.

C-reactive protein concentration is higher than normal (5.6 mg/dl) in one patient; the percentage of CD163+CD33+ positive monocytes-macrophages is 30 % in this patient.

The percentage of CD163+CD33+ positive monocytes-macrophages is not different in patients with secondary hemochromatosis as compared to controls and patients with hereditary hemochromatosis. In patients with secondary hemochromatosis no correlation is found between the percentage of CD163+CD33+ monocytes-macrophages and serum iron studies.

Discussion

Recently the CD163 protein was shown to act as a receptor that scavenges haemoglobin-haptoglobin complexes and a higher affinity of the receptor for haemoglobin-Hp 2-2 complex than for the haemoglobin-Hp 1-1 complex was observed (6). In healthy males, it has been demonstrated that the subjects with the Hp 2-2 phenotype have higher serum ferritin and monocyte ferritin content than subjects carrying the Hp 1-1 and 2-1 phenotype. A good correlation has been demonstrated between serum ferritin concentration and monocyte L-ferritin content (8). In our control group of 30 male healthy volunteers there is neither a difference in the percentage of CD163+CD33+ monocytes-macrophages between the different haptoglobin phenotypes nor a correlation between the percentage of CD163+CD33+ monocytes-macrophages and serum iron indices. Thus the higher serum and monocyte ferritin content in Hp 2-2 subjects seems to be solely the consequence of the higher affinity of the haemoglobin-Hp 2-2 complex for the CD163 receptor, and not of a higher percentage of CD163+CD33+ monocytes-macrophages in the Hp 2-2 subjects.

In patients with hereditary hemochromatosis, the monocyte-macrophage has a relative iron-poor content in comparison with the abundant iron which is present in the body (10,16). This can be attributed to different mechanisms. It is known that patients with hereditary hemochromatosis have a significantly decreased ability to phagocytose red blood cells (17). It is also established that after erythrophagocytosis, monocytes of patients with hereditary hemochromatosis, release twice as much iron in a low molecular weight form as control cells (10). In macrophages of patients with hereditary hemochromatosis a significant increase in iron regulatory protein (IRP) activity is demonstrated (9). By preventing ferritin mRNA translation, high IRP activity in monocytes may represent a molecular mechanism contributing to the inadequate ferritin accumulation and insufficient macrophage iron storage in hereditary hemochromatosis.

IRP is a cytoplasmic sensor of the labile iron pool which is regarded as one of the main regulators of cellular iron homeostasis. The upregulation of IRP in hereditary hemochromatosis is monocyte-macrophage specific, since it is absent in other circulating blood cells. Moreover this abnormality seems characteristic of the genetic form of hemochromatosis, as a reduced IRP activity is found in patients with secondary hemochromatosis. However, after phlebotomy in patients with hereditary hemochromatosis, iron regulatory protein activity did not differ form that of control subjects (9).

Since the CD163 receptor could contribute, via the uptake of haeme-iron of the haemoglobin-haptoglobin complex, to the intracellular iron content of monocytes-macrophages, we have investigated if this mechanism plays an important role in the determination of the macrophage iron content. Since liver and spleen macrophages are not easily accessible in humans and since in vitro differentiation of peripheral blood monocytes has been extensively used as a model to investigate macrophage development (18), we have studied peripheral blood monocytes. We could not detect a difference in the percentage of CD163+CD33+ monocytes-macrophages between patients with hereditary hemochromatosis and controls or patients with secondary hemochromatosis. Since the majority of our patients have serum ferritin concentrations in the normal range, due to an adequate phlebotomy treatment, one can argue that the CD163 receptor could be only downregulated in situations where iron overload is present. However, no correlation between the percentage of CD163+CD33+ monocytes-macrophages and serum iron studies is seen. Moreover when comparing hereditary hemochromatosis patients with still elevated serum ferritin concentrations and patients with normal serum ferritin concentrations, still no difference in percentage of CD163+CD33+ monocytes-macrophages could be found. This, again, argues against the influence of elevated serum ferritin concentrations on the percentage of CD163+CD33+ monocytes-macrophages.

Moreover in the group of patients with secondary hemochromatosis, although serum ferritin concentrations are significantly higher than in controls and patients with hereditary hemochromatosis, no difference in the percentage of CD163+CD33+ monocytes-macrophages is observed.

In this subgroup of patients with secondary hemochromatosis, high serum ferritin concentrations could be the consequence of inflammation. But only one patient in this group has a C-reactive protein concentration higher than normal. Also we suspect, that in the presence of inflammation, a low percentage of CD163+CD33+ monocytes-macrophages should have been found, which is not the case in this patient subgroup.

Attributing high serum ferritin concentrations to tissue iron overload in the group of patients with secondary hemochromatosis, would ideally have required the measurement of hepatic iron concentration. We thought however that it was unethical to perform a liver biopsy in these patients. On the other hand, it has been illustrated that there is a good correlation between serum ferritin concentration and tissue iron overload (19).

We conclude that in healthy males, there is no difference in the percentage of CD163+CD33+ monocytes-macrophages between the different haptoglobin phenotypes. We could not find a difference in the percentage of CD163+CD33+ monocytes-macrophages in patients with hereditary or secondary hemochromatosis and controls. It seems unlikely that the CD163 receptor contributes to the low macrophage iron content seen in patients with hereditary hemochromatosis.

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Table 1.

Serum iron studies and percentage of CD163+CD33+ monocytes-macrophages for the total group of healthy male volunteers, patients with hereditary and secondary hemochromatosis.

	Healthy volunteers (n=30)	Hereditary hemochromatosis (n=16)	Secondary hemochromatosis (n=5)	Reference range
Serum iron (µmol/l)	21 ± 6	28 ± 14*	25 ± 7	7 - 22
Serum iron saturation (%)	37 (27-45)	57 (33-95)**	77 (69-85)$	Female <52 Male <62
Serum ferritin (µg/l)	131 (104-198)	162*** (68-370)	2965$$ (2325-4195)	36 - 262
Percentage CD163+CD33+ monocytes-macrophages	54 ± 22	67 ± 23	58 ± 27	

Variables with a normal distribution are presented as mean ± standard deviation. Variables with a non-Gaussian distribution are presented as median and interquartile range.

* $p<0.02$ versus control population

** $p<0.01$ versus control population

*** $p<0.01$ versus patients with secondary hemochromatosis

$ $p<0.01$ versus control population

$$ $p<0.01$ versus control population

Table 2.

Serum iron studies, haptoglobin concentration and percentage CD163+CD33+ monocytes-macrophages in healthy male volunteers according the haptoglobin phenotype.

	Hp 1-1 (n=6)	Hp 2-1 (n=16)	Hp 2-2 (n=8)	Reference Range
Serum iron (µmol/l)	18 ± 6	21 ± 6	20 ± 7	7 - 22
Serum iron Saturation (%)	32 (28-36)	40 (30-48)	36 (24-48)	Female <52 Male <62
Serum ferritin (µg/l)	133 (103-231)	163 (114-185)	153 (70-259)	36-262
Haptoglobin concentration (g/l)*	1.23 ± 0.25	0.94 ± 0.27	0.69 ± 0.21	0.38 - 2.27
Percentage CD163+CD33+ monocytes-macrophages	54 ± 27	53 ± 25	58 ± 18	

* $p<0.01$ (Kruskal-Wallis test).

Variables with a normal distribution are presented as mean ± standard deviation.

Variables with a non-Gaussian distribution are presented as median and interquartile range.

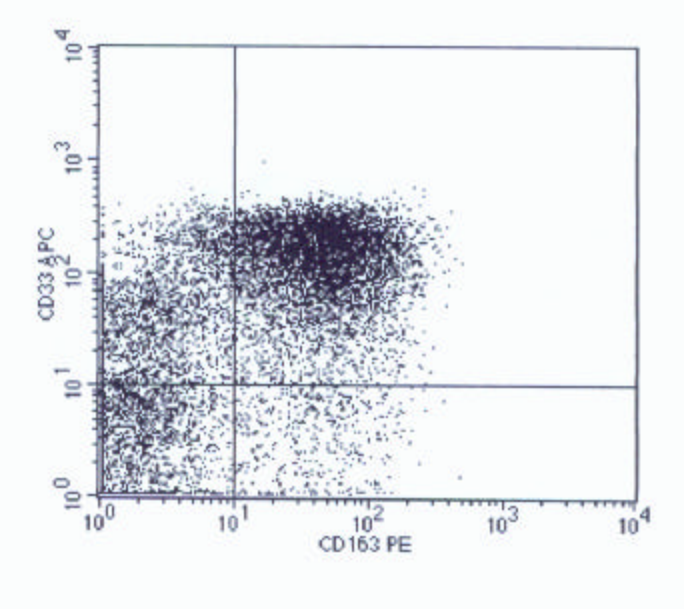

Figure 1.

FACS (fluorescent activated cell sorter) dot plot of CD163+ CD33+ monocytes-macrophages (right upper quadrant).

CHAPTER IV

ASSOCIATION BETWEEN Cys282Tyr MISSENSE MUTATION AND HAPTOGLOBIN PHENOTYPE POLYMORPHISM IN PATIENTS WITH CHRONIC HEPATITIS C

Association between Cys282Tyr missense mutation and haptoglobin phenotype polymorphism in patients with chronic hepatitis C

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Introduction In patients with chronic hepatitis C infection, the haptoglobin (Hp) 1-1 phenotype is overrepresented. Data regarding the occurrence of the Cys282Tyr missense mutation in these patients are less clear. We studied the prevalence of both variables in a cohort of patients with chronic hepatitis C and looked for interaction between the two variables.

Materials and methods The study group consisted of 142 patients chronically infected with the hepatitis C virus. All patients were examined for the occurrence of the Cys282Tyr missense mutation, and in 132 of them the Hp phenotype was determined.

The Cys282Tyr missense mutation was detected by restriction fragment length polymorphism (RFLP) using a standard polymerase chain reaction (PCR) technique and *Rsa*I digestion. Hp phenotypes were determined using starch gel electrophoresis of haemoglobin-supplemented serum followed by peroxidase staining.

Results A significant overrepresentation of the Hp 1-1 phenotype was found (36/132, 27%, $P < 0.01$ *v.* control population). This overrepresentation was observed only in the patients homozygous for the wild-type allele of the HFE gene. The Cys282Tyr allele was significantly overrepresented in hepatitis C patients (0.12 *v.* 0.07, $P < 0.05$) and principally in patients with the Hp 2-1 and 2-2 phenotypes.

Conclusion In patients with chronic hepatitis C infection, both the Hp 1-1 and the Cys282Tyr allele occur more frequently than in a control population. Remarkably, these genes seem to determine each other's occurrence, such that the overrepresentation of the Hp 1-1 phenotype is seen only in Cys282Tyr-negative subjects, while the overrepresentation of the Cys282Tyr allele is observed in Hp 1-1-negative subjects. Differences in immunomodulating and in oxidative stress-inducing capacities between the two genes may explain this finding. *Eur J Gastroenterol Hepatol* 13:1077–1081 © 2001 Lippincott Williams & Wilkins

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Keywords: Cys282Tyr missense mutation, haptoglobin phenotype, hepatitis C

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Introduction

The seroprevalence of chronic hepatitis C virus infection is approximately 1–3% of the population in Western Europe and the USA. Administration of blood and blood derivatives (before 1990) and intravenous drug abuse are the main routes of transmission [1]. After an acute infection, 60–80% of infected people evolve towards chronicity. Differences in viral factors (genotype, viral load) and/or host factors (immune response) seem to influence the outcome of a hepatitis C virus infection [2–4].

Several studies suggest an association between chronic hepatitis C and haemochromatosis; people with the haemochromatosis gene have a more severe course of the hepatitis C infection [5–9]. Inherited haemochromatosis, which is an inborn error of iron metabolism, affects 1 in 200–400 people living in Belgium.

Recently, two missense mutations in the HFE gene, responsible for haemochromatosis, have been described [10]. One mutation results in a substitution of tyrosine for cysteine, a highly conserved residue at position 282 of the HFE protein (Cys282Tyr). This mutation is responsible for 81–91% of hereditary haemochromatosis cases in the USA, Western and Central Europe, and Australia.

In Belgium, this mutation was found in 94% of patients with hereditary haemochromatosis [11]. The role of the second missense mutation resulting in a replacement of histidine at residue 63 by aspartic acid (His63Asp) is

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still controversial. It is unlikely that this second mutation alone is able to cause iron overload [12].

Haptoglobin (Hp) is an alpha 2 globulin (alpha 2-sialoglycoprotein) present in human plasma. Three different phenotypes can be distinguished: Hp 1-1, Hp 2-1 and Hp 2-2. Hp is capable of binding haemoglobin to prevent both iron loss and kidney damage during haemolysis [13]. Moreover, it is also known that individuals carrying the Hp 2-2 phenotype display a more vigorous immune response to different stimuli than subjects with the 1-1 phenotype [13]. Recently, we found an overrepresentation of subjects with the Hp 1-1 phenotype in a cohort of patients suffering from chronic hepatitis C [14], and suggested that the differences in the immunomodulating capacities of the Hp phenotypes influenced the clinical evolution of the hepatitis C infection.

The aim of our study is to examine whether there is an interaction between the Hp phenotype and the HFE genotype in patients with chronic hepatitis C infection.

Materials and methods

Study population

The studied patient population consisted of 142 consecutive patients of Belgian ancestry (68 women, 74 men) with untreated chronic hepatitis C with a mean age of 48 years (range 19–76). The diagnosis of hepatitis C was based on elevated alanine aminotransferase (ALT) levels, and the presence of hepatitis C antibodies and HCV RNA in the serum. The transmission mode was transfusion related in 79 patients, due to intravenous drug abuse in 30 patients, and unknown in 33 patients. The genotype of the virus was HCV 1a or 1b in 115 patients (81%). In 111 patients a liver biopsy was performed. Forty-eight (43%) patients had chronic persistent hepatitis, 63 (57%) patients had chronic active hepatitis, and 30 (27%) patients had evidence of cirrhosis. None of the patients had clinical evidence of porphyria cutanea tarda.

Patients with previously known hepatitis B infection, alcoholic liver disease, hereditary haemochromatosis, autoimmune liver disease, primary biliary cirrhosis and other forms of chronic liver disease were excluded. Patients with chronic alcohol abuse (> 70 g alcohol daily) were also excluded.

Control population

A total of 196 healthy, unrelated, Caucasian people of Belgian ancestry with unknown iron status served as controls for the determination of the prevalence of the Cys282Tyr missense mutation. Reference values for Hp phenotype distribution were obtained from a group of 1000 healthy Caucasian blood donors of Belgian ancestry [15].

Detection of the Cys282Tyr missense mutation

In 142 patients, the Cys282Tyr missense mutation was searched for according to the method described by Feder *et al.* [10]. In short, after extraction of genomic DNA from peripheral blood mononuclear cells or buccal cells, DNA was amplified by PCR technique. After amplification, these fragments were investigated further by restriction enzyme digestion followed by agarose gel electrophoresis. The missense mutation Cys282Tyr resulted in an additional restriction site for the restriction enzyme *Rsa*I. Homozygotes, heterozygotes or normals were recognized easily. Recently, a polymorphism in intron 4 of HFE was described causing an overestimation of the Cys282Tyr homozygote prevalence in haemochromatosis. We re-examined all samples using a new antisense primer that excluded the site of the new polymorphism [16,17].

Determination of the haptoglobin phenotype

In 132 patients, the Hp phenotypes were determined using starch gel electrophoresis of haemoglobin-supplemented serum followed by peroxidase staining [18]. In 127 patients, both the Cys282Tyr missense mutation and the Hp phenotype could be determined.

Statistics

The allele frequency of both genes (HFE and Hp) was calculated by the following formula:

$$\text{Allele frequency} = \frac{(2 \times \text{number of homozygotes}) + \text{number of heterozygotes}}{2 \times \text{total number of individuals}}$$

For the Cys282Tyr and the Hp phenotype distribution, the validity of the Hardy–Weinberg equilibrium was tested using the Chi-squared test. For dichotomous variables, the Chi-squared test was used. P values of < 0.05 were considered significant. When multiple comparisons were made, a correction of the P value was made [19]:

$$Pc = 1 - [(1 - P)n]$$

where Pc is the corrected P value, P is the uncorrected P value, and n is the number of comparisons.

Results

Cys282Tyr missense mutation

The results of the Cys282Tyr missense mutation for the patient and control groups are shown in Table 1. The results of the control group are in agreement with the Hardy–Weinberg equilibrium. There is no difference in results when the Feder technique is compared with the technique using the new antisense primer, which excluded the new polymorphism site. An overrepresentation of the Cys282Tyr allele is found in our

Table 1 Distribution of the Cys282Tyr missense mutation in patients and controls

Cys282Tyr	Patients ($n = 142$)	Controls ($n = 195$)
−/−	114 (80%)	171 (87%)
+/−	21 (15%)	23 (12%)
+/+	7 (5%)	2 (1%)
Cys282Tyr allele frequency	0.12*	0.07

* $P = 0.02$.

group of patients (allele frequency of 0.12 *v.* 0.07 in the control group; Chi-squared 5.21, $P = 0.02$). However, this overrepresentation is due completely to an excess of Cys282Tyr homozygous patients.

Haptoglobin polymorphism

The Hp phenotype distribution in the patient and control groups is shown in Table 2. The results in the control group are in agreement with the Hardy–Weinberg equilibrium. In the hepatitis C group, there is a significant overrepresentation of the Hp 1-1 phenotype (Chi-squared 9.578, $P < 0.01$; RR, 1.7, 95% CI 1.25–2.33). According to these data, the Hp 1 allele frequency is 0.48 (*v.* 0.40 in the control population; Chi-squared 6.0, $P < 0.05$).

Cys282Tyr missense mutation and haptoglobin phenotype interaction

When the distribution of the Hp 1-1 phenotype is compared amongst the different HFE genotypes, the overrepresentation of this phenotype is only present in the group of patients homozygous for the wild-type allele (Chi-squared 10.38, $P < 0.01$, $Pc < 0.01$ *v.* control population; Table 3). This results in an overrepresentation of the Hp 1 allele frequency in this group of 0.50 (*v.* expected: 0.40, Chi-squared 7.13, $P < 0.01$, $Pc = 0.01$ *v.* control population; Table 3). This overrepresentation is not found in the patients heterozygous or homozygous for the Cys282Tyr missense mutation. This may be due to the smaller number of patients included in these subgroups. However, when the data of these two groups are cumulated, Hp 1-1 is still not overrepresented (Table 3). When Cys282Tyr homozygous patients are left out of this analysis, the data remain comparable (Table 3).

On the other hand, when the distribution of the Cys282Tyr genotype is compared amongst the Hp phenotypes, the Cys282Tyr allele frequency in the group of patients with the Hp 1-1 phenotype is not different from the control group (0.085 *v.* 0.07, Chi-squared 0.06, P = NS). The overrepresentation of the Cys282Tyr allele only becomes significant in the Hp 2-1 and 2-2 phenotypes (Cys282Tyr allele frequency 0.13, Chi-squared 5.142, $P = 0.02$, $Pc = 0.04$ *v.* control population; Tables 1 and 4).

Table 2 Distribution of the haptoglobin (Hp) phenotypes in patients and controls

Hp phenotype	Patients ($n = 132$)	Controls ($n = 1000$)
1-1	36* (27%)	160 (16%)
2-1	55 (42%)	480 (48%)
2-2	41 (31%)	360 (36%)
Hp 1 allele frequency	0.48**	0.40
Hp 2 allele frequency	0.52	0.60

* $P < 0.01$; ** $P < 0.05$.

Table 3 Haptoglobin (Hp) 1 allele frequency and Hp 1-1 phenotype frequency in patients with chronic hepatitis C and with different HFE genotypes

Cys282Tyr genotype	Hp 1 frequency	Hp 1-1 frequency
Cys282Tyr −/−	103/206* (50%)	30/103**
Cys282Tyr +/− and +/+	19/48 (40%)	5/24
Cys282Tyr +/−	14/36 (39%)	4/18

* $P < 0.01$, $Pc = 0.01$ *v.* control population; ** $P < 0.01$, $Pc < 0.01$ *v.* control population.

Clinical correlation

There was no association between either Hp phenotype or Cys282Tyr missense mutation and histological severity of liver disease, biochemical abnormalities (ALT level, aspartate aminotransferase (AST) level) or abnormalities in serum iron levels (serum iron level, serum iron saturation, serum ferritin level) (data not shown).

Discussion

Patients infected with the hepatitis C virus have a 60–80% chance of developing chronic hepatitis. Several host and viral factors determine the evolution of the infection [2–4]. In particular, the quality of the virus-specific immune response seems to determine the outcome of the disease. Since the HFE genotype and the Hp phenotype are two genetic factors that directly and indirectly influence the immune system, we examined the presentation of, and interaction between, these two genes in patients with naive chronic hepatitis C virus infection.

In 142 patients suffering from chronic hepatitis C infection, we observed a significant overrepresentation of the Cys282Tyr missense mutation (allele frequency 0.12). The allele frequency of this mutation in our

Table 4 Frequency of the Cys282Tyr mutation in chronic hepatitis C patients with different haptoglobin (Hp) phenotypes

Hp phenotype	Cys282Tyr allele frequency
Hp 1-1	6/70 (8.5%)
Hp 2-1 and 2-2	24/184* (13%)

* $P = 0.02$, $Pc = 0.04$ *v.* control population.

control group was 0.07, which is in accordance with the prevalence of this allele in Western Europe [20]. However, the overrepresentation of the Cys282Tyr allele is largely the consequence of an overrepresentation of Cys282Tyr homozygous patients. This could be interpreted as a selection bias. Patients, however, were selected on HCV positivity, not on the presence or absence of the HFE mutation.

Hezode *et al.*, examining a group of patients chronically infected with the hepatitis C virus, did not see an overrepresentation of the Cys282Tyr allele in their study group, whereas the frequency of the mutation in their control group was comparable to our data [6,9]. However, in this French group of patients, only 9% had cirrhosis compared with 27% in our cohort. It is unlikely that this difference reflects a more aggressive course of the disease as a consequence of the HFE missense mutation, since in our group of patients cirrhosis was observed with the same frequency in the different HFE genotypes (data not shown). No differences in the occurrence of fibrosis in the different HFE genotypes were seen in an Austrian study [5] or the aforementioned French study [9]. However, in a British survey [8], the rate of fibrosis was higher in subjects carrying the Cys282Tyr allele. The small number of patients heterozygous for the Cys282Tyr missense mutation in this British trial may explain the discrepancy with our and other investigators' data.

The overrepresentation of the Cys282Tyr allele in patients with chronic hepatitis C could explain the differences in immunomodulating capacities predisposing an individual to a chronic infection. Indeed, multiple changes in immune response are described in genetic haemochromatosis. People with inherited haemochromatosis and high ferritin levels have an abnormally high CD4/CD8 ratio [21]. Less activated T-cells [22] and low CD8 cells (in peripheral blood and liver) [23] are correlated with high iron stores. Lower CD8+CD28+ cells are found in haemochromatosis patients who are more prone to develop cirrhosis [24]. The presence of the Cys282Tyr missense mutation may result in an inferior host immune response in patients infected with the hepatitis C virus [20].

We have also found an overrepresentation of the Hp 1-1 phenotype in our cohort of patients. This confirms our previous observation in a different patient group [14]. Hp occurs in three phenotypic forms: Hp 1-1, Hp 2-1 and Hp 2-2. Each of these phenotypes has different functional capacities. The beta chain of the Hp molecule has been demonstrated to bind to CD22, a B-cell adhesion glycoprotein. CD22 mediates B-cell interactions with erythrocytes, T-lymphocytes, monocytes, neutrophils and endothelial cells [25–30]. Flow-cytometric analysis shows that Hp 1-1, 2-1 and 2-2 bind the cell surface of human lymphocytes with equal affinity [31]. However, the saturation of the CD22 molecules depends on Hp type because of differences in molar concentrations required. Therefore, more free CD22 binding sites are estimated in Hp 2-2 blood [32]. This may result in better binding properties between B-cells and other immune cells. Hp also has antibody-like properties. Köhler and Prokop have demonstrated that *Streptococcus pyogenes* group A, carrying the T4 antigen, can be agglutinated by human serum from Hp 2-2 and Hp 2-1 individuals, the Hp 2-2 serum having higher agglutination titres than the Hp 2-1 serum. In contrast, Hp 1-1 has no agglutination effect [33,34]. Subjects carrying the Hp 1-1 phenotype have lower peripheral B-lymphocyte counts and CD4+ lymphocyte counts than subjects carrying the Hp 2-2 phenotype [33]. Therefore, we can postulate that people with the Hp 1-1 phenotype have a lower cellular and humoral immune response towards the hepatitis C virus when compared with the Hp 2-1 and 2-2 phenotype, resulting in a higher tendency to chronicity.

However, the properties of the Hp phenotype are clearly influenced by the presence or absence of the Cys282Tyr allele. Where both the Hp 1 allele frequency and the Cys282Tyr allele frequency are overrepresented in our group of patients, carrying both the Cys282Tyr allele and the Hp 1 allele does not result in a higher risk towards chronicity (Tables 3 and 4). Moreover, in the absence of the Cys282Tyr missense mutation, the Hp phenotype determines the risk to develop a chronic hepatitis C infection. Indeed, only in this group of patients is a significant overrepresentation of the Hp 1-1 phenotype found. This overrepresentation is not seen in patients carrying the Cys282Tyr missense mutation (Table 3). In addition, we observe a significantly higher Cys282Tyr allele frequency in the group of patients with the Hp 2-1 and 2-2 phenotype (Table 4). This overrepresentation is not found in the group of patients with the Hp 1-1 phenotype. We assume that the higher oxidative stress that is present both in individuals with the Cys282Tyr allele and the Hp 2-1 or 2-2 phenotype results in a higher risk in this subgroup of patients to develop a chronic hepatitis C infection. A linkage disequilibrium between the two variables is unlikely since both variables are located on different chromosomes.

In summary, we observed a significant overrepresentation of both the Hp 1-1 phenotype and the Cys282Tyr allele in a cohort of patients with untreated chronic hepatitis C infection. The overrepresentation of the Hp 1-1 phenotype is seen only in the HFE wild-type group. The overrepresentation of the Cys282Tyr allele is observed only in the Hp 2-1 and 2-2 phenotypes. Differences in immunological and oxidative stress capacities may explain the modifying character of the Cys282Tyr gene on the Hp phenotype.

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CHAPTER V

FACTORS INFLUENCING RIBAVIRIN-INDUCED HAEMOLYSIS

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Factors influencing ribavirin-induced hemolysis

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Background/Aims: **One of the major side effects of the combination therapy for chronic hepatitis C is ribavirin-induced hemolytic anemia. Little is known about variables influencing this anemia. Our study tried to search for these variables in a large group of patients with hepatitis C treated with the combination therapy.**

Methods: **Two hundred and forty-four patients chronically infected with the hepatitis C virus were treated either with induction treatment (daily dose of interferon) or with a standard treatment (interferon thrice weekly). Both groups received 1000–1200 mg of ribavirin from week 4 until the end of the treatment. The drop in hemoglobin level was defined as the difference between the pretreatment hemoglobin level and the hemoglobin level at week 8. Seventeen variables which could possibly influence this drop in hemoglobin level were examined.**

Results: **After multivariate analysis, the drop in hemoglobin level was only significant influenced by pretreatment platelet level, treatment and haptoglobin phenotype. The ribavirin dose did not influence the drop in hemoglobin level or the early virological response.**

Conclusions: **Ribavirin-induced hemolysis is influenced by the pretreatment platelet level, the administered amount of α-interferon and the haptoglobin phenotype. A careful search for the minimal dose of ribavirin needed in combination treatment is necessary.**

Keywords: **Hemolytic anemia; Ribavirin; Hepatitis C; Combination therapy**

1. Introduction

Today's treatment of patients with chronic hepatitis C consists of the combination of interferon-α and ribavirin. According to the genotype of the infecting virus, the presence or absence of liver cirrhosis and the pretreatment viral load, the duration of the treatment is either 6 or 12 months; the longer treatment is needed for the patients with genotype 1, cirrhosis and a high viral load (more than 2 million copies/ml) [1,2].When the combination therapy is compared with interferon monotherapy, the number of adverse events is increased and withdrawals reach 20% for the 12 months of combination therapy versus 14% for a 12-month monotherapy. Dose reduction is common, and is mainly necessitated by anemia that occurs in 7–9% of the combination-treated patients [1,2]. It is generally accepted that the anemia is provoked by the ribavirin intake [3–7].

Ribavirin is a synthetic oral nucleoside analogue which is not only used for the treatment of patients with chronic hepatitis C, but has also been evaluated for a broad spectrum of DNA and RNA viruses in man [8]. The major toxicity associated with the use of ribavirin is hemolytic anemia. This adverse effect has been ascribed to the accumulation of ribavirin triphosphate in the erythrocyte, which interferes with erythrocyte function [9].

Haptoglobin is an α2-sialoglycoprotein with hemoglobin-binding capacity. After destruction of erythrocytes, the released hemoglobin is immediately captured by haptoglobin and the hemoglobin–haptoglobin complexes are instantly cleared by the reticuloendothelial system (e.g. liver). Exhaustion of haptoglobin occurs rapidly leading to the appearance of free hemoglobin in the circulation. Free

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hemoglobin passes through the glomerular filter, and may cause renal damage. In physiological conditions, serum Hp is saturated when approximately 500–1500 mg/l free hemoglobin is present. Due to a genetic polymorphism three major phenotypes of Hp occur: Hp 1-1, Hp 2-1 and Hp 2-2. Hemoglobin binding not only depends upon the haptoglobin concentration but also on the Hp phenotype. The complex formation of hemoglobin is less effective in Hp 2-2 [10]. Since little is known about the host, viral and treatment factors that may influence ribavirin-induced anemia. We have examined the influence of several variables on this side effect in a large cohort of chronic hepatitis C patients treated with the combination therapy. Special attention has been paid to the impact of the different haptoglobin (Hp) phenotypes on this phenomenon.

2. Materials and methods

2.1. Patients

Two hundred and forty-four (244) patients with chronic hepatitis C, participating in a trial comparing induction therapy with standard treatment, were included. The diagnosis of chronic hepatitis C was made on the basis of elevated ALT activity (above the upper limit of normal) and the presence of HCV RNA in serum. Patients with cirrhosis belonged to the Child–Pugh A classification. Patients suffering from other chronic liver diseases and hepatocellular carcinoma were excluded. In the induction arm of the trial (group A), patients received a daily dose of interferon-α 2b (Intron A) of 5 MU s.c. for the total duration of 8 weeks. In the control arm of the trial (group B), patients received interferon-α 2b 5 MU s.c. thrice weekly for the total duration of 8 weeks. After week 8 both groups received interferon-α 2b 3 MU s.c. thrice weekly for the total duration of 12 months. After 4 weeks both groups were given ribavirin per os at a daily dose of 1000–1200 mg (patients weighing less than 75 kg received 1000 mg; patients weighing more than 75 kg received 1200 mg).

Ribavirin was not administered at week 0 because when we started the trial, no data were available about the cumulative toxicity of high doses of interferon-α 2b and ribavirin. Therefore it was considered safer to wait for 4 weeks before starting ribavirin administration. Patients with major toxicity from high doses of interferon-α 2b could be selected before ribavirin was administered. However, after terminating the trial, the side effects in the high-dose interferon group were similar when compared with the standard combination arm (data not shown).

The patient pretreatment characteristics are shown in Table 1.

Table 1
Pretreatment patient characteristics[a]

Total number of patients	244
Gender (male/female)	156/88 (64/36%)
Age	Median: 43 years (range: 36–53)
Weight	Median: 73 kg (IQR: 64–82)
ALT	Median: 90 U/l (IQR: 61–149)
Leukocyte count	Median: 6100/μl (IQR: 5100–7480)
Platelet count	208 000 ± 65 000/μl
Body mass index	25.0 ± 4.5 kg/m^2
% Cirrhosis	12%
Treatment A/Treatment B	124/120 (51/49%)
HCV genotype	1: 181 (74%) 2: 9 (3.8%) 3: 33 (13.4%) 4: 6 (2.5%) 5: 13 (5.4%) Mixed: 2 (0.8%)
HCV RNA viral load	Median: 498 000 copies/ml (IQR: 203 700– > 500 000)
Hemoglobin level pretreatment	Median: 14.7 g/dl (IQR: 13.3–15.5) Male: 14.7 g/dl Female: 13.2 g/dl
Haptoglobin level pretreatment	0.91 ± 0.51 g/l
Haptoglobin phenotype distribution	Hp 1-1: 41 (16.7%) Hp 2-1: 120 (49.4%) Hp 2-2: 83 (33.9%)
Ribavirin (in mg) administered/body weight	Median: 14.7 mg/kg (IQR: 13.7–15.9)

[a] Variables with a normal distribution are expressed as mean ± standard deviation. Variables with a non-Gaussian distribution are expressed as median and IQR (interquartile range).

2.2. Methods

Hemoglobin levels were measured on an automated hematological analyzer. Anemia at week 8 was defined as a hemoglobin level of less than 13.3 g/dl for male patients, and less than 11.7 g/dl for female patients.

Serum Hp concentrations were assayed using Fixed-time immunonephelometry with a BN nephelometer (Behringwerke, Marburg, Germany) [12], and expressed according to IFCC standards [13]. Hp phenotypes were determined using starch gel electrophoresis of hemoglobin-supplemented serum followed by peroxidase staining [11].

Qualitative and quantitative Amplicor HCV tests (Roche Molecular Systems, Pleasanton, OR, USA) were used to detect and quantitate HCV genomes in the circulation.

2.3. Statistics

We decided to compare the hemoglobin levels between weeks 0 and 8 and between weeks 4 and 8. At that moment the patients had received ribavirin for 4 weeks which is long enough to observe the ribavirin-induced hemolysis. After the time point of week 8, no further drop in hemoglobin level was observed (Fig. 1). Week 8 was also considered as a better time point than week 12 or 16, since the trial protocol did not foresee dose reductions for ribavirin before this time point. The drop in hemoglobin level between week 0 and 8 is further called ΔHb.

The influence of 17 variables on ΔHb was examined. These 17 variables are: gender, age, body weight, body mass index (BMI), treatment schedule, pretreatment hemoglobin concentration, pretreatment uric acid concentration, pretreatment haptoglobin concentration, haptoglobin phenotype, pretreatment leukocyte and platelet counts, pretreatment ALT level, hepatitis C virus genotype, pretreatment viral load, presence or absence of cirrhosis, ribavirin dose (absolute and per kg body weight).

Since in clinical practice, ribavirin is started at the same time as inter-

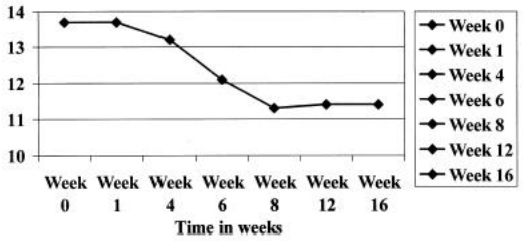

Fig. 1. The hemoglobin level (*x*-axis) before treatment and at different time points during treatment (*y*-axis).

feron-α2b, we also looked for the influence of these 17 variables on the drop in hemoglobin level between week 4 and 8.

We further examined whether there is any influence of the ΔHb on the early virological response (HCV RNA level at week 8; early response: undetectable HCV RNA level, no early response: detectable HCV RNA level).

Body mass index was calculated as follows: body weight (kg)/(length (m))^2.

Statistics were performed using Medcalc version 5.00.002 [14]. Normality of the distribution of the different variables was tested using the Kolmogorov–Smirnov test. Variables with a normal distribution are presented as mean ± standard deviation. Variables with a non-Gaussian distribution are presented as median and 25–75th interquartile range. Parametric or non-parametric tests were used, according the distribution of the variable. P values of less than 0.05 were considered significant. Multivariate analysis using multiple regression was performed on those variables that were statistically significant in monovariate analysis.

3. Results

3.1. Pretreatment characteristics of the patients (Table 1)

The haptoglobin phenotype distribution was in accordance with the Hardy–Weinberg equilibrium; this distribution was comparable with the distribution seen in a control group of 1000 Caucasian blood donors [15]. The pretreatment haptoglobin concentration was higher in patients with the Hp 1-1 and Hp 2-1 phenotype (Hp 1-1: 1.06 ± 0.27 g/l; Hp 2-1: 1.06 ± 0.64 g/l) and lower in the patients with the Hp 2-2 phenotype (0.82 ± 0.4 g/l). This findings are in accordance with a control population [10].

3.2. Variables influencing the ΔHb

Anemia was present in 67.4% of the patients (n = 164/244) This percentage was not significantly different between males and females (70 vs. 62%, P = 0.2) In the total group of patients the mean ΔHb is 2.5 ± 1.4 g/dl. Only six of the variables had a statistically significant association with ΔHb in monovariate analysis: gender, pretreatment hemoglobin level, pretreatment platelet count, pretreatment uric acid, treatment and haptoglobin phenotype (Table 2).

After multivariate analysis performed with multiple regression analysis only three variables remained statistically significant: treatment (induction or standard treatment), pretreatment platelet count and haptoglobin phenotype (Table 2). Since the pretreatment hemoglobin level was not independent of the ΔHb, it was not included in the multiple regression analysis.

Table 2
Variables influencing ΔHb[a]

Variable	P value in monovariate analysis	P value after multiple regression
Gender	0.003	NS
Platelet count	0.04	0.01
Treatment (A or B)	0.005	0.001
Pretreatment hemoglobin level	0.001	NA
Haptoglobin phenotype 1-1	0.01	0.01
Uric acid	0.04	NS

[a] NS, not significant; NA, not applicable.

3.2.1. Pretreatment hemoglobin level

Patients with a higher pretreatment hemoglobin level had a larger ΔHb (Spearman's coefficient of rank correlation = 0.385, $P < 0.001$). Although males had a greater pretreatment hemoglobin level than females (14.7 ± 2.2 g/dl vs. 13.2 ± 2.0 g/dl, $P < 0.0001$), a similar correlation was found in the two groups.

3.2.2. Treatment

Patients randomized to the induction arm of the trial (group A) suffered from a higher drop in hemoglobin level than the patients receiving the standard treatment (in group B) (2.7 ± 1.4 g/dl vs. 2.2 ± 1.3 g/dl, P = 0.005) (Fig. 2).

3.2.3. Pretreatment platelet count

Patients with an initially higher platelet count developed a lower drop in hemoglobin level after treatment. Patients with a pretreatment platelet count of less than 110 000/μl had a higher ΔHb than the patients with a pretreatment platelet count of more than 110 000/μl (3.2 ± 1.9 g/dl vs. 2.4 ± 1.4 g/dl, P = 0.04) (Fig. 3). A higher platelet count was also seen in patients younger than 50 years (P = 0.001) of female gender ($P < 0.001$) and without cirrhosis ($P < 0.001$).

3.2.4. Haptoglobin phenotype

The drop in hemoglobin level between weeks 0 and 8 was higher in the patients carrying the Hp 1-1 phenotype as compared to those not carrying this phenotype (3.1 ± 1.5 g/dl vs. 2.4 ± 1.4 g/dl, P = 0.01) (Fig. 4). There was no

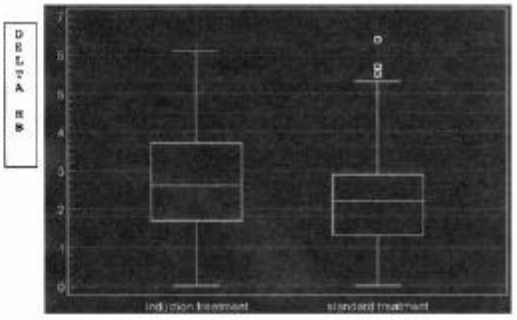

Fig. 2. Box-and-whisker plot comparing the ΔHb between induction treatment (left) and 'standard treatment' (right). In the Box-and-whisker plot, the central box represents the value from the lower to upper quartile (25th to 75th percentile). The middle line represents the median. The vertical line extends from the minimum to the maximum value, excluding 'outside' and 'far out' values which are displayed as separate points.

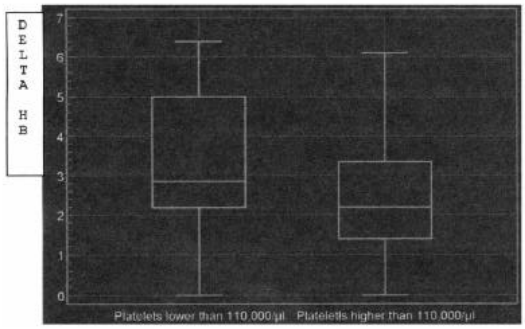

Fig. 3. Box-and-whisker plot comparing the ΔHb between patients with platelet counts lower or higher than 110 000/µl.

difference in the ΔHb between patients carrying the Hp 2-1 and Hp 2-2 phenotype (2.5 ± 1.4 g/dl vs. 2.2 ± 1.3 g/dl, $P = 0.3$).

3.2.5. Combination of risk factors

Only when haptoglobin phenotype and treatment schedule were combined were enough data available to perform statistical analysis. Subjects with the Hp 1-1 phenotype receiving the induction treatment turned out to have a higher ΔHb than subjects not carrying these risk factors (3.8 ± 1.4 g/dl vs. 2.4 ± 1.4 g/dl, $P < 0.001$) (Fig. 5).

3.2.6. Variables influencing the drop in hemoglobin level between weeks 4 and 8

Apart from gender, which is in this situation not a significant variable, the other variables influencing the drop in hemoglobin level between weeks 4 and 8 are the same as the variables influencing the ΔHb (difference between weeks 0 and 8).

3.2.7. Ribavirin dose and ΔHb

The lack of association between ribavirin dose (total dose and ribavirin dose/kg) is unexpected. There is no relation

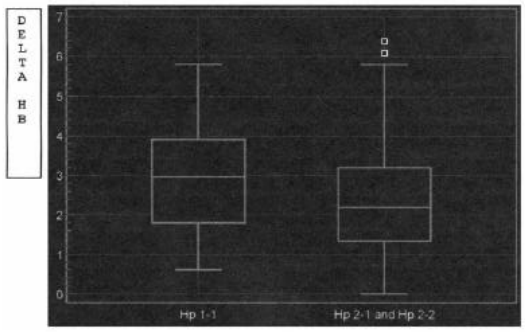

Fig. 4. Box-and-whisker plot comparing the ΔHb between patients carrying the Hp 1-1 phenotype (left) and patients not carrying this phenotype (right).

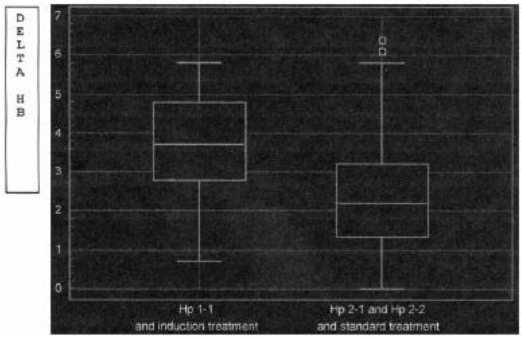

Fig. 5. Box-and-whisker plot comparing patients with the Hp 1-1 phenotype treated with the induction treatment and the patients with the Hp 2-1 and 2-2 phenotypes treated with standard treatment.

between the ΔHb, the difference in hemoglobin level between weeks 4 and 8 and the ribavirin dose/kg (Table 3).

3.3. ΔHb and early virological response

Early virological response was seen in 56% of the patients ($n = 136/244$). A higher rate of early response was seen in the induction arm (65 vs. 41% in the standard arm, $P < 0.01$). Neither ΔHb, total ribavirin dose nor ribavirin dose/kg of body weight had any influence upon the early virological response.

4. Discussion

Treatment with ribavirin, an oral nucleoside analogue, frequently induces hemolytic anemia and reticulocytosis [3–7]. Anemia is a frequent finding, occurring in 67% of our patients. Little is known about the risk factors for hemolytic anemia in patients treated for chronic hepatitis C. In a large group of patients with chronic hepatitis C, we examined which variables might influence the magnitude of this anemia and should induce special alertness when administering ribavirin to patients. Week 8 was considered an ideal time point, because (a) no further drop in hemoglobin level was observed after week 8; (b) according to trial protocol, no dose reductions in ribavirin were foreseen before that time point. A higher drop in hemoglobin is seen in patients with a higher pretreatment hemoglobin level, lower

Table 3
Drop in hemoglobin level between week 0 and week 8 (ΔHb) and between weeks 4 and 8 (ΔHb 4–8)

Ribavirin dose (mg/kg)	ΔHb (mean ± SD) (g/dl)	ΔHb 4–8 (mean ± SD) (g/dl)
< 13.5	2.57 ± 1.23	1.87 ± 1.22
13.5–14.6	2.64 ± 1.43	1.80 ± 1.57
14.6–15.8	2.51 ± 1.53	1.76 ± 1.36
> 15.8	2.46 ± 1.47	1.85 ± 1.33

pretreatment platelet counts, the Hp 1-1 phenotype and induction treatment. The correlation between the pretreatment hemoglobin level and the ΔHb seems logical and can be explained by the fact that the hemolysis associated with ribavirin breaks down a certain proportion of the available erythrocytes pool (approximately 17%). Higher absolute drops in the hemoglobin level will therefore be observed in subjects with the higher pretreatment hemoglobin levels. When the ΔHb is expressed as a percentage of the pretreatment hemoglobin levels, the decline is the same in both cases.

After multiple regression with exclusion of the dependent variable (pretreatment hemoglobin level), three variables remained to have a statistically significant present influence on the severity of the anemia (ΔHb). These were pretreatment platelet counts, treatment regimen and the haptoglobin phenotype.

Patients with lower platelet counts had a significantly higher drop in hemoglobin level than those with higher counts. Low platelet counts were also seen in particular in older male patients with cirrhosis. The low pretreatment platelet count can be considered as a hallmark of the presence of liver cirrhosis with hypersplenism. Although the histological diagnosis of cirrhosis does not seem to influence the drop in hemoglobin level, it appears that the clinical expression of cirrhosis with the associated hypersplenism, as expressed by a lower platelet count [16], is a risk factor to develop a more severe ribavirin-induced anemia. Patients receiving a higher dose of interferon-α (group A or the induction treatment group) also have a higher drop in hemoglobin level than those receiving lower doses. This again is not surprising, since interferon-α is known to be myelosuppressive and a cause of anemia when given in higher doses [17].

Somewhat surprising is the fact that the haptoglobin phenotype has an impact on the ribavirin-induced hemolysis. The three haptoglobin phenotypes have not only different properties concerning protection against free radicals, bacteriostatic effects, angiogenesis, inhibition of nitric oxide and prostaglandin synthesis [18], but the three phenotypes have also different hemoglobin binding capacities. In normal circumstances individuals with the Hp 1-1 phenotype are more effective in handling episodes of hemolysis. In healthy controls, hemoglobin-binding capacity was highest in Hp 1-1, intermediate in Hp 2-1 and lowest in Hp 2-2 [10]. In our group of patients treated with ribavirin, we find a higher drop in hemoglobin level in the Hp 1-1 phenotype. The explanation of this phenomenon is rather speculative: differences in uptake or competition in uptake of ribavirin between the different Hp phenotypes could be one explanation, although until this moment proof is lacking.

The combination of risk factors increases the risk to develop an important drop in hemoglobin level. Patients with the Hp 1-1 phenotype who are treated with induction treatment have a higher ΔHb than the patients not exposed to these risk factors. Since the haptoglobin phenotype is easy to perform (see Section 2) and is a non-expensive test (2.5 Euros per test), we think that before starting high-dose interferon treatment in patients with low platelet counts, this test should be performed. If the haptoglobin phenotype demonstrates an additional risk (e.g. Hp 1-1) to develop anemia, the clinician can decide to give a lower interferon dose. Also it would seem relevant that patients with the more sensitive haptoglobin phenotype are considered for lower doses of ribavirin. Further exploration of the clinical utility in this situation using higher and lower doses of ribavirin should be considered.

We could not examine the combined effect of the haptoglobin phenotype and the induction treatment with the pretreatment platelet count, because of the limited number of patients carrying these factors.

The ribavirin dose administered to treat chronic hepatitis C is rather arbitrary. The present finding that neither the total dose of ribavirin nor the dose of ribavirin per kg body weight has influence on the degree of hemolysis or on the early virological response should open the discussion to which amount of ribavirin is needed to obtain an optimal response in the combination treatment of patients with chronic hepatitis C.

To summarize, we can conclude that ribavirin-induced hemolysis in patients with chronic hepatitis C is influenced by the pretreatment platelet counts, the administered amount of α -interferon and the haptoglobin phenotype. In patients with the Hp 1-1 phenotype, induction treatment with interferon and ribavirin can result in a higher drop in hemoglobin level when compared with non Hp 1-1 patients treated with standard combination treatment. Since the drop in hemoglobin level and the early virological response are not influenced by the dose of ribavirin, a careful search for the minimal dose of ribavirin needed in combination therapy in patients with chronic hepatitis C is warranted.

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CHAPTER VI

NON-TRANSFERRIN-BOUND IRON IN UNTREATED AND RIBAVIRIN TREATED CHRONIC HEPATITIS C PATIENTS

(submitted: Alimentary Pharmacology and Therapeutics)

NON-TRANSFERRIN-BOUND IRON (NTBI) IN UNTREATED AND RIBAVIRIN TREATED CHRONIC HEPATITIS C PATIENTS

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Non-transferrin-bound iron, chronic hepatitis C virus infection.

Electronic word count: 4418

ABSTRACT

Introduction

In patients with chronic hepatitis C, elevations in serum iron levels, hepatic iron content and oxidative stress-related molecules have been reported. Treatment with ribavirin induces an increase in hepatic iron concentration. Since in situations of iron overload, non-transferrin bound iron (NTBI) can appear, we determined NTBI in untreated chronic hepatitis C patients and in patients during interferon-ribavirin treatment.

Materials and methods

In 10 untreated and in 19 interferon+ribavirin-treated chronic hepatitis C patients, we examined NTBI levels by a colorimetric method using nitrilotriacetic acid as a ligand and sodiumtriscarbonatecobalt (III) to block free iron binding sites on transferrin.

Results

Despite the presence of high serum iron saturation and ferritin levels, NTBI was absent in the majority of the HCV patients (25/29, 86 %). There was no difference in NTBI levels between untreated and treated patients. Four patients with high NTBI levels could be distinguished by higher serum iron levels. In two of these patients, hepatocytic iron was present on liver biopsy.

Discussion

In the majority of chronic hepatitis C patients, NTBI levels are normal. Treatment with ribavirin does not induce high NTBI levels.

NTBI levels are only higher than normal in hepatitis C patients with higher serum iron levels.

Abstract electronic word count: 198

Introduction

Several recent studies have explored the prevalence and significance of elevated serum iron levels in chronic hepatitis C virus infection (1). In patients with chronic hepatitis C, serum iron is elevated in 27-36 %, transferrin saturation is elevated in 18-24 % and serum ferritin is increased in 22-35 %. A strong correlation has been observed between serum ferritin and serum AST levels, suggesting that release of tissue ferritin from damaged hepatocytes causes the elevations in serum iron (2). Others did not find this correlation (3).

Elevated hepatic iron concentration have been observed in patients with chronic hepatitis C, but less frequently than suspected by elevated serum transferrin saturation or serum ferritin. The frequency of elevated hepatic iron concentration ranges from 10 to 36 % (2,4,5). The elevation in hepatic iron content associated with chronic hepatitis C infection is usually mild with a mean of hepatic iron index of 0.4-0.6 µmol/g dry liver tissue per year of life (5,6). Unlike hepatic iron accumulation in hereditary hemochromatosis, the mild hepatic iron overload seen in patients with compensated liver disease does not progressively increase over time (7). However, in end-stage liver disease and cirrhosis caused by hepatitis C, hepatic iron index values of more than 1.9 µmol/g dry liver tissue per life year can be found. Although in this case the hepatic iron index is in the range of patients with hereditary hemochromatosis, the Cys282Tyr missense mutation is absent (8).

After interferon treatment, the hepatic iron concentration decreases significantly in virological responders and nonresponders. The decrease in stainable liver iron is predominantly seen in mesenchymal cells as opposed to hepatocytes (9). This leads to the speculation that elevations in hepatic iron seen in chronic viral hepatitis are mainly due to phagocytosis of necrotic hepatocytes and accumulation of iron in phagocytic cells.

On the contrary, increases in hepatic iron concentration have been seen after monotherapy with ribavirin. In this case, excess of iron deposits are seen in hepatocytes and less in macrophages. Hepatic iron accumulation is well known to occur in association with haemolytic disorders. The ribavirin-induced haemolysis could be a causal factor in this excess of hepatic iron (10).

In hepatitis C patients, a pattern of free radical production and lipid peroxidation is observed. This correlates with the presence of steatosis and activation of gluthatione metabolism, which represents the liver's physiological scavenging response to peroxidative damage (3). The higher serum and tissue iron found in patients with chronic hepatitis C, could be the consequence of a local and systemic derangement of iron metabolism.

Non-transferrin-bound iron (NTBI) represents various forms of labile iron appearing in the plasma of patients with various pathological conditions (11). NTBI is most commonly found in patients whose transferrin iron binding capacity has been exceeded by iron overload. NTBI is capable of mediating tissue damage by stimulating free radical formation and lipid peroxidation (12). As its name indicates, NTBI comprises all forms of iron in the plasma that are bound to ligands other than transferrin (13). These ligands are citrate (14), albumin and perhaps ferritin (15). In a healthy individual the incoming iron becomes effectively shielded from reaction to redox cycling and formation of reactive oxygen species by the binding with transferrin (11). Thus, when NTBI appears in the plasma, it is believed to result from an imbalance of iron metabolism.

Increased NTBI values are seen in conditions such as: atransferrinemia (11), hereditary hemochromatosis (16), haemolytic anaemias (17), end stage renal disease on dialysis (18), after chemotherapy (19), during cardiopulmonary bypass (20) and in neonates (21).

In most of these conditions transferrin is completely saturated, however NTBI is also seen in situations where transferrin is not completely saturated (indivi duals heterozygous for the hemochromatosis gene (12), in patients on dialysis (18) ...). Since in patients with chronic hepatitis C higher serum and hepatic iron levels are present, we examined whether NTBI could be one of the factors contributing to the increased oxidative stress observed in chronic hepatitis C patients. Ribavirin-induced haemolytic anaemia causing a higher liver iron content in chronic hepatitis C patients, could also induce the appearance of NTBI.

Materials and methods

Patients

Ten untreated patients with chronic hepatitis C and 19 patients with chronic hepatitis C on treatment with interferon and ribavirin were included in this study. The diagnosis of chronic hepatitis C was made on the basis of elevated ALT activity (above the upper limit of normal) and the presence of HCV antibodies and HCV RNA in serum. Patients suffering from other chronic liver diseases and hepatocellular carcinoma were excluded. The patient characteristics are shown in Table 1.

The patients on treatment received interferon alfa at a dose of 3MU SC thrice weekly, while the ribavirin dose was chosen according to the body weight. Patients with a body weight less than 75 kg, received 1000 mg of ribavirin per day, orally, divided in two doses. Patients weighing more than 75 kg, received a dose of ribavirin 1200 mg per day, orally, divided in two doses.

In untreated patients blood sampling was done at diagnosis. In patients on treatment, blood sampling was performed during treatment when they had been taken ribavirin for at least 8 weeks. It has been reported that at this moment the haemolytic activity of ribavirin is maximal and stable for the rest of the treatment (22).

Controls

In 13 healthy volunteers blood was taken for NTBI measurement. These volunteers had no evidence of iron overload or hepatitis C. Informed consent was obtained.

Biochemical analysis

Fasting blood samples were obtained, allowed to clot and centrifuged. The serum was collected for analysis.

Serum alanine aminotransferase (ALT) activity, serum iron concentration and total iron binding capacity (TIBC) were determined using commercial reagents on an automated analyser (Roche).

Transferrin saturation was calculated by the formula:

100 x (serum iron/TIBC).

Serum ferritin concentration was measured using fixed-time immuno-nephelometry (Dade Behring).

Liver biopsy

Liver biopsy was performed at diagnosis. Activity and staging were examined according the METAVIR scoring system (23). Perls' stain was performed to demonstrate the presence of iron.

HFE missense mutation

The Cys282Tyr missense mutation was searched for according to the method described by Feder et al (24). In short, after extraction of genomic DNA from peripheral blood mononuclear cells DNA was amplified by PCR. After amplification these fragments were further investigated by restriction enzyme digestion followed by agarose gel electrophoresis. The missense mutation Cys282Tyr resulted in an additional restriction site for the restriction enzyme *Rsa*I. Homozygotes, heterozygotes or normals were easily recognized. Recently, a polymorphism in intron 4 of HFE was described causing an overestimation of the Cys282Tyr homozygote prevalence in hemochromatosis. We examined all samples using a new antisense primer that excluded the site of this new polymorphism (25,26).

NTBI measurement

NTBI measurement was done according the method described by Gosriwatana et al (27). To prevent displacement of iron from nitrilotriacetic acid (NTA) to unsaturated transferrin during this procedure, samples were pretreated with triscarbonatecobalt (III). In short, serum samples were stored frozen at –20° C until time of analysis. The samples were thawed by incubation at 37° C for 10 min. To serum (450 µl) 100 µl $Na_3[Co(CO_3)_3]$. $3H_2O$ solution was added and the mixture was incubated at 37° C for 1 hour. Subsequently 800 mM nitrilotriacetic acid (NTA) was added to the serum mixture to obtain a final concentration of 80 mM and allowed to stand for 30 min at room temperature. The samples were ultrafiltered using an ultrafilter (Centricon-30) at 3000 g for 1 h.

The serum was analysed by using a colorimetric method. The ultrafiltrate was diluted 1:1 with 5 mM Mops buffer (pH 7.4) to obtain the volume of 800 µl. To form the chromogenic complex, 100 µl of 120 mM thioglycolic acid in deionised water and 100 µl of 60 mM bathophenanthroline in deionised water were added to the solution and allowed to stand for 30 min. The resulting complex was measured by spectrophotometry at 537 nm.

Despite the use of cobalt in this assay, negative NTBI levels can still be found in some samples. Probably this is caused by exchange of transferrin-bound cobalt with iron, which is present in 80 mM NTA in standardised small amounts (2µM), during the 30 minutes incubation (12).

Statistical analysis

Statistics were performed using the Medcalc program version 5.00.002 (28). Normality of the distribution of the different variables was tested using the Kolmogorov-Smirnov test. Variables with a normal distribution are presented as mean ± standard deviation. Variables with a non-Gaussian distribution are presented as median and interquartile range.

Parametric and non-parametric tests were used according the distribution of the variable. P-values of < 0.05 were considered significant.

Results

Patient characteristics (Table 1).

The group of treated patients is younger than the untreated patient group ($p<0.01$). This is also reflected by the higher number of patients infected with genotype 3 in the treated population. Not surprisingly patients on treatment have lower ALT activity than untreated patients.

Serum iron levels

Serum iron, serum iron saturation and serum ferritin are not different between treated and untreated patients. Twenty-two out of 29 patients (76 %) have serum ferritin concentrations above the upper limit of normal (untreated: 7/10 versus treated: 15/19, p=NS). A statistically significant correlation is found between ALT activity and serum ferritin concentration (r after logarithmic transformation=0.39, p=0.03). When treated and untreated patients are separately examined, this correlation remains significant in both groups (untreated: r=0.70, p=0.02; untreated: r=0.44, p=0.05).

Three males (19 %) have a serum transferrin saturation exceeding 62 % and three females (23 %) have a serum iron transferrin exceeding 52 %.

HFE missense mutation

The Cys282Tyr missense mutation is found in the heterozygote form in 4 of the 29 chronic hepatitis C patients. No patient is found to be homozygous for this missense mutation.

There is no difference in serum iron, serum iron saturation and serum ferritin levels between heterozygotes and normals.

Liver biopsy

The activity and fibrosis found on liver biopsy are demonstrated in Table 1. Only two patients had stainable iron in hepatocytes on the liver biopsy.

Treatment induced haemolysis

The mean drop in haemoglobin level in the patients receiving interferon-ribavirin treatment is 2.1 ± 1.1 g/dl .

NTBI values

NTBI values in the controls (0.29 ± 0.17 µM, range: -0.08 – 0.61) are not different from the values found in the hepatitis C patients (0.41 ± 0.44 µM, range: -0.31 – 2.05) (Figure 1). The NTBI values found in the control group are in accordance with values found by others (12).

NTBI values in the untreated group are 0.36 ± 0.44 µM. NTBI values in the treated group are 0.38 ± 0.29 µM. There is no statistical difference between the two groups (Figure 2).

In Cys282Tyr heterozygotes, NTBI values are not higher than in patients with the wild type (0.21 ± 0.12 µM versus 0.44 ± 0.47 µM).

There is no correlation between NTBI values and ALT activity, serum transferrin saturation, serum ferritin concentration and activity or fibrosis on liver biopsy.

Although the hepatitis C group has no higher NTBI values than the control group, 4 patients have NTBI values which are higher than the controls.

These patients will be briefly described.

Patient 1

Patient 1 is a 56 year old man (untreated group) of Egyptian origin. He has probably been infected during childhood during a prevention programme against Schistosomiasis. The infecting genotype is 4. Serum iron concentration is 50 µmol/l, serum iron saturation 96% and serum ferritin concentration 1920 µg/l. NTBI is present at a value of 2.05 µM. ALT activity is 199 U/l. Metavir score for the liver biopsy is: activity grade 2 and fibrosis stage 4. Perls'staining demonstrates iron deposits in several hepatocytes. The Cys282Tyr missense mutation is absent in this patient, he has no family history suggestive for hereditary hemochromatosis and there is no evidence for secondary iron overload disease.

Patient 2

Patient 2 is a 43 year old man (treated group) of Belgian origin. He has been infected with the hepatitis C virus genotype 3 through intravenous drug abuse. ALT activity at the moment of blood sampling is 31 U/L. Serum iron concentration is 32 µmol/l, serum iron saturation is 72 % and serum ferritin concentration is 350 µg/l. NTBI is present at a value of 0.89 µM. Metavir score for the liver biopsy is: activity grade 3 and fibrosis stage 3. Perls' staining demonstrates iron deposits in several hepatocytes. The Cys282Tyr missense mutation is absent, he has no family history suggestive for hereditary hemochromatosis and there is no evidence for secondary iron overload disease.

Patient 3

Patient 3 is a 39 year old man (treated group) infected with the hepatitis C virus of genotype 1b. The route of transmission is unknown. ALT activity is 90 U/L. Serum iron concentration is 28 µmol/l, serum iron saturation is 54% and serum ferritin concentration is 1120 µg/l. NTBI is present at a value of 0.81 µM.

Metavir score for the liver biopsy is: activity grade 3 and fibrose stage 3.
No iron deposits are found in the liver biopsy. The Cys282Tyr missense mutation is absent, there is no evidence for hereditary or secondary hemochromatosis.

Patient 4

Patient 4 is a 34 year old man (treated group) infected with the hepatitis C virus of genotype 3 through intravenous drug abuse. ALT activity is 23 U/L. Serum iron concentration is 28 µmol/l, serum iron saturation is 58 % and serum ferritin concentration is 348 µg/l. NTBI is present at a value of 0.93 µM. Metavir score for the liver biopsy is: activity grade 1 and fibrosis stage 1. No iron deposits are found in the liver biopsy. The Cys282Tyr missense mutation is absent, there is no evidence for hereditary nor secondary hemochromatosis.

When these four patients are compared to the other patients, higher serum iron, serum iron saturation and serum ferritin concentrations are found (Table 2). The two patients with the highest serum iron saturation, are the only two patients with stainable iron on liver biopsy.

Discussion

Patients with chronic hepatitis C often have elevated serum iron studies (1). In our series of 29 patients 22 (76 %) have elevated serum ferritin concentrations. A significant correlation is found between serum ferritin concentration and serum ALT activity. This suggests, as published by others (2,3), that the release of ferritin from damaged hepatocytes is, at least partly, responsible for the elevations in serum ferritin levels. However the correlation is more significant in untreated patients than in treated patients. This can be explained by the capacity of ribavirin to induce normal ALT activity despite ongoing hepatoinflammatory activity (29).

Although none of the patients is homozygous for the Cys282Tyr missense mutation, 19 % of the male patients and 23 % of the female patients have serum iron saturation levels in the range that is suggestive for hereditary hemochromatosis.

Several authors have demonstrated the presence of oxidative stress in patients with chronic hepatitis C (3). Considering the high serum iron concentrations and the presence of oxidative stress, it is tempting to look for NTBI. Since NTBI is likely a factor to contribute to oxidative stress (3), we have investigated the presence of NTBI in chronic hepatitis C patients.

Despite the presence of elevated serum iron indices in a substantial number of our patients, we have found that in the majority of the patients with chronic hepatitis C, NTBI values are normal. However in 4 out of 29 patients (14 %), high NTBI values are found. Similar percentages of elevated NTBI values are found in patients with end stage renal disease, in neonates and during cardiopulmonary bypass. However in these conditions reported levels are generally in the range from 1 to 10 µM (11) which is higher than those observed in our patient group.

In the 4 patients with high NTBI values, serum iron, serum iron saturation and serum ferritin concentration are higher than in the rest of the patients.

In these 4 patients hereditary and secondary hemochromatosis have been excluded. Although it is possible that other genetic mutations causing iron overload are present, this hypothesis seems less likely, considering the absence of a positive family history for iron overload.

In 2 of these 4 patients iron deposits are observed in the hepatocytes. This is not surprising, since it has been demonstrated that hepatocytes have the possibility to take up free iron (30). The presence of NTBI in chronic hepatitis C could be a hallmark of tissue iron overload. However this needs confirmation by comparing the hepatic iron concentration in the presence and absence of NTBI.

In healthy individuals heterozygous for the Cys282Tyr missense mutation, higher NTBI values are detected (12). However in our chronic hepatitis C patient group, no difference could be found between NTBI values in the four heterozygotes and normals.

Treatment of chronic hepatitis C with ribavirin causes haemolytic anaemia in a substantial portion of the patients. In ribavirin treated patients, iron deposits in the hepatocytes and increases in hepatic iron concentrations have been demonstrated (10). Hepatic iron accumulation is well known to occur in association with other chronic haemolytic conditions. Iron released by haemolysis would be expected to accumulate in the reticuloendothelial system. However increased iron staining is noted in both the cells of the reticuloendothelial system and in hepatocytes. The deposition of iron in hepatocytes suggests that the mechanism of iron loading is enhanced mucosal absorption associated with accelerated erythropoiesis (10).

We have failed to demonstrate higher NTBI values in the group of treated patients. They all had substantial haemolysis as demonstrated by the drop in haemoglobin level during the treatment. Interferon in monotherapy decreases hepatic iron concentration and iron deposits in the mesenchymal cells (9).

Combining interferon with ribavirin in the treatment of chronic hepatitis C, could counteract the ribavirin induced increases in hepatic iron concentration. However, this seems unlikely since a) ribavirin induced haemolytic anaemia remains present in the combination treatment and b) iron deposits caused by ribavirin are mainly seen in the hepatocytes and less in the macrophages (10). We have not performed control biopsies during or immediately after the treatment. Therefore we have not been able to look for the presence of excess of iron in the control biopsy.

A longitudinal study of a group of patients treated with the combination treatment should be performed to definitely exclude the occurrence of NTBI during treatment.

We conclude that in the majority of chronic hepatitis C patients, despite the presence of high serum iron indices, NTBI values are not different from controls. In the few cases where NTBI values are higher than in controls, higher serum iron concentration, serum iron saturation and serum ferritin concentration are observed.

Despite the presence of haemolytic anaemia, treatment with ribavirin does not result in higher NTBI levels.

Further studies (longitudinal follow up studies of chronic hepatitis C patients before, during and after treatment and comparison with hepatic iron concentration) are necessary to give a more detailed view of the importance of NTBI in chronic hepatitis C.

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Table 1.

Patient characteristics

	Untreated patients (n=10)	Treated patients (n=19)	Reference range
Gender (male/female)	**5/5**	**11/8**	
Age (years) *	**50 ± 7**	**38 ± 11**	
ALT activity(U/l) *	**114 ± 72**	**31 ± 30**	**7 - 31**
Serum iron (µmol/l)	**27 ± 9**	**22 ± 9**	**7 - 22**
Serum iron saturation (%)	**38 (31-51)**	**37 (23-55)**	**Female <52** **Male <62**
Serum ferritin concentration (µg/l)	**292 (121-390)**	**318 (211-430)**	**36 - 262**
Liver biopsy			
Activity scale (n): 1	**5**	**7**	
2	**5**	**6**	
3		**6**	
Fibrosis scale (n) : 0	**1**	**0**	
1	**2**	**8**	
2	**3**	**5**	
3	**1**	**5**	
4	**3**	**1**	
Genotype (n) 1a	**1**	**2**	
1b	**8**	**7**	
3		**8**	
4	**1**	**1**	
unknown		**1**	
HFE missense mutation (n)			
Cys282Tyr -/-	**8**	**17**	
Cys282Tyr +/-	**2**	**2**	

* $p<0.01$

Table 2.

Serum iron, iron saturation and ferritin concentration in chronic hepatitis C patients with higher than normal NTBI levels (left) and normal NTBI levels (right).

	'High' NTBI levels (n=4)	'Normal' NTBI levels (n=25)	Reference range	p-value
Serum iron (µmol/l)	35 ± 10	22 ± 8	7 – 22	<0.001
Serum iron saturation (%)	65 (56-84)	36 (24-41)	Female <52 Male <62	<0.001
Serum ferritin concentration (µg/l)	735 (349-1520)	284 (120-390)	36-262	0.02

Figure 1.

NTBI values in the controls (left) and in patients with chronic hepatitis C (right).

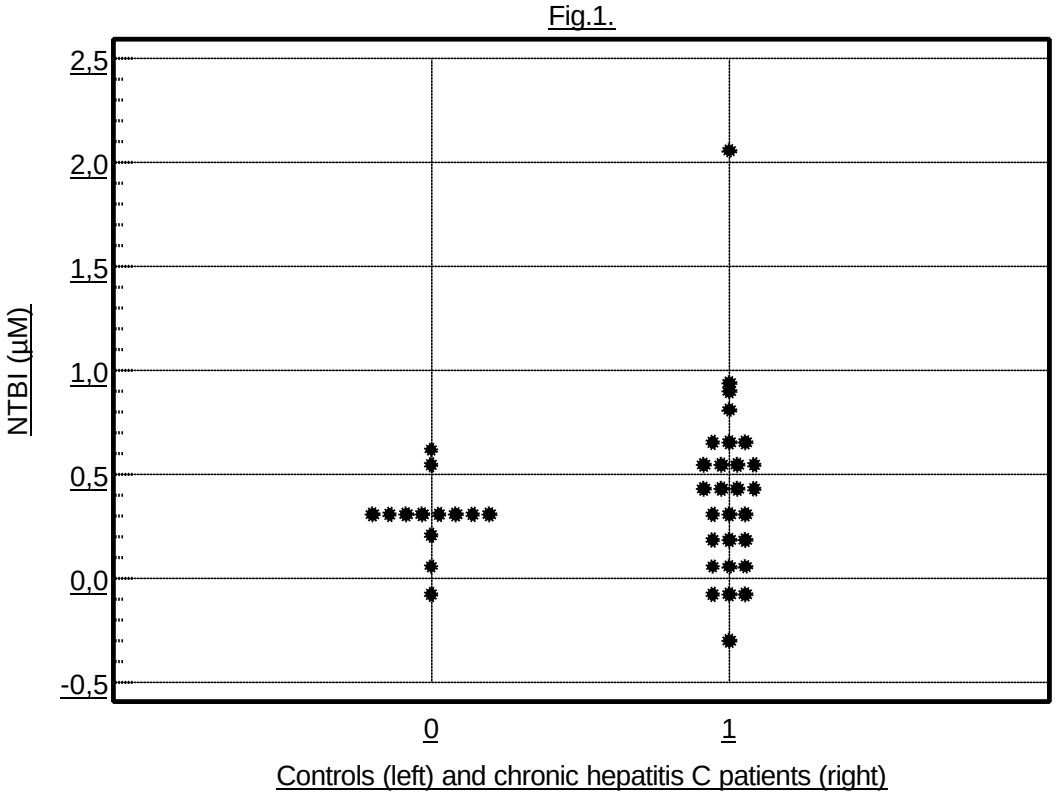
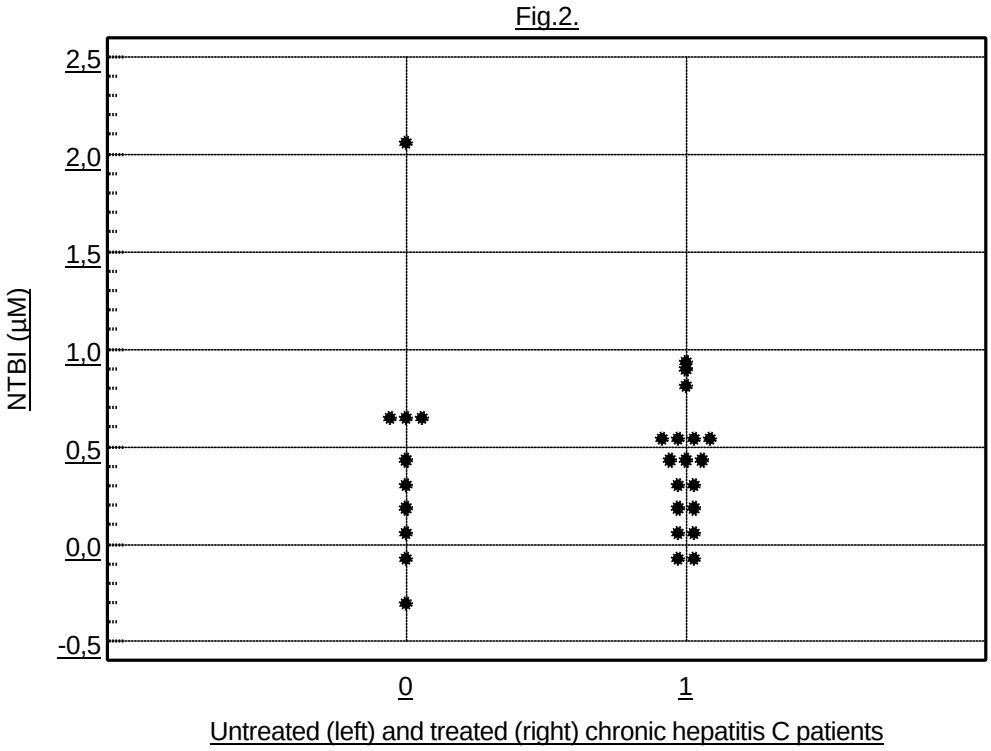


Figure 2.

NTBI values in untreated (left) and treated (right) chronic hepatitis C patients.



GENERAL DISCUSSION

The *HFE* mutation and the haptoglobin polymorphism both influence iron storage capacity (1,2) and are related with different immunological properties (3).

The *HFE* mutation is found in the majority of patients with hereditary hemochromatosis (4). Convincing data are available demonstrating that this gene is not just an innocent bystander, but really contributes to tissue iron overload (5-11). In the presence of tissue iron overload and the *HFE* mutation, different perturbations in the immune status of hereditary hemochromatosis patients have been reported (3).

Haptoglobin is a protein with haemoglobin-binding capacity and prevents iron loss and kidney damage during periods of haemolysis. The three major haptoglobin phenotypes have different effects on cellular and humoral immune responses (12). Individuals with the Hp 2-2 phenotype carry higher numbers of CD4+ T-lymphocytes and B-lymphocytes (13). Hp 2-2 also displays higher agglutination capacities against some bacteria (12).

Healthy male individuals with the Hp 2-2 phenotype have higher levels of both serum and monocyte ferritin than individuals with the Hp 2-1 or 1-1 phenotype (14).

Since both hereditary hemochromatosis and chronic hepatitis C infection are associated with higher serum and tissue iron stores and with differences in immune status, we examined the influence of the *HFE* mutation and the Hp polymorphism on both diseases.

Hereditary hemochromatosis is the most frequent genetically determined iron storage disease in Western Europe (15). Before the discovery of the hemochromatosis gene in 1996 by Feder et al (4), clinicians had to rely on phenotypic characteristics of the disease (e.g. serum iron saturation, serum ferritin level, hepatic iron index, hereditary character ...) to establish the diagnosis. Since the detection of the *HFE* mutation, diagnosis in a single patient, family screening and in a certain way, population screening have become easier.

Since more patients with hereditary hemochromatosis are diagnosed, it has become obvious that not all subjects homozygous for the Cys282Tyr missense mutation have the typical phenotypic characteristics of patients with hereditary hemochromatosis (16,17). Moreover other missense mutations (e.g. His63Asp) could play a role in iron overload disorders in patients not homozygous for the Cys282Tyr missense mutation.

In **Chapter I**, we determined the Cys282Tyr and the His63Asp missense mutation in a cohort of patients displaying the phenotype of hereditary hemochromatosis and in group of healthy controls. The patients were followed in the out-patient clinic between 1985 and 1997 (18). In the healthy control group 10.5 % of the 96 studied subjects were heterozygous for the Cys282Tyr missense mutation and 2 % were homozygous for this missense mutation. Of the 49 patients, 46 (94 %) were homozygous for the Cys282Tyr missense mutation; 2 were compound heterozygous (Cys282Tyr +/-, His63Asp +/-) and one patient was only heterozygous for the His63Asp missense mutation. These results are in accordance with the reported prevalences in other countries and confirm that the Cys282Tyr missense mutation in the *HFE* gene is present in a majority of patients with hereditary hemochromatosis and contributes to the phenotypic expression of the disease (4).

The significance of the His63Asp mutation is less clear. It is suggested that it is only of importance in the compound heterozygote setting. In our patient group, the 2 patients who are compound heterozygotes have the clinical picture of hereditary hemochromatosis. The identification of one patient with hemochromatosis which is only heterozygous for the His63Asp mutation suggests the existence of other genetic (or non-genetic) causes.

Since in healthy subjects the Hp 2-2 phenotype is associated with higher serum ferritin and monocyte ferritin content (14), we have investigated in **Chapter II**, the influence of the Hp polymorphism on the phenotypic presentation of 167 patients with hereditary hemochromatosis. A low Hp1 allele frequency is found and is attributable to an overrepresentation of the Hp 2-2 phenotype ($p<0.01$). Hp 2-2 is associated with a relative risk of 1.73 (95 % CI 1.14-2.62) for hemochromatosis. The underrepresentation of the Hp1 allele is only observed in the male patients ($p<0.01$) but is not significant in the female patients ($p=0.3$). In the male population serum iron levels, serum ferritin levels and the amount of iron removed by phlebotomy to achieve iron depletion, are higher in hemochromatosis patients carrying the Hp 2-2 phenotype. The absence of similar findings in females is possibly related to the lower number of females included, although other factors (e.g. menstruation, pregnancy) could have a greater influence on the phenotypic expression of hereditary hemochromatosis in female patients (19).

The higher levels of serum ferritin both in Hp 2-2 healthy men and in Hp 2-2 male hemochromatosis patients could be explained by the Hp 2-2 specific transfer of Hb-Hp complexes into monocytes-macrophages. The Hb-Hp related iron uptake in monocytes-macrophages has recently been demonstrated to occur via the Hb scavenger receptor CD163.

This receptor has a higher affinity for the multimeric Hb-Hp 2-2 complex than for the monomeric Hb-Hp 1-1 complex (20). Since the CD163 receptor could play a role in the iron status of healthy subjects and patients with hereditary hemochromatosis, we examined the percentage of CD163 positive monocytes-macrophages in both populations (**Chapter III**). In the control group of 30 male healthy volunteers there is neither difference in the percentage of CD163 positive monocytes-macrophages between the different haptoglobin phenotypes nor any correlation between the CD163 receptors and serum iron indices.

In patients with hereditary hemochromatosis, we found no different percentage of CD163 positive monocytes-macrophages than in patients with secondary hemochromatosis or in controls. It seems unlikely that the low macrophage iron content seen in patients with hereditary hemochromatosis is influenced by a different percentage of CD163+ monocytes-macrophages.

The prevalence of chronic hepatitis C infection in the Belgian population is 0.9 % (21). Chronic hepatitis C virus infection is associated with iron overload and increased oxidative stress (22). Both viral factors (genotype, viral load) and/or host factors (immune response) seem to influence the outcome of a hepatitis C virus infection. In **Chapter IV** we have demonstrated that both the Cys282Tyr missense mutation and the Hp 1-1 phenotype are overrepresented in a group of 142 chronic hepatitis C patients (23). Remarkably, the presence or absence of one gene seems to determine the impact of the other gene on the risk to develop chronic hepatitis C virus infection. More precisely, in the absence of the Cys282Tyr missense mutation, the Hp phenotype determines the risk to develop a chronic hepatitis C infection. Only in this group of patients, a significant overrepresentation of the Hp 1-1 phenotype is found.

In addition we see a significant higher Cys282Tyr allele frequency in the group of patients with the Hp 2-1 and 2-2 phenotype. We assume that the higher oxidative stress that could be present in individuals with both the Cys282Tyr allele and the Hp2 allele results in a higher risk in this subgroup to develop a chronic hepatitis C infection.

Since we have not followed our patient group for a time period and our study is only a single time point measurement, it is difficult to comment on progression of the disease. However, since we have found an overrepresentation of the Hp 1-1 phenotype in our group of patients with chronic hepatitis C, we make the assumption that the presence of this phenotype makes the infected patient more susceptible to develop a chronic infection. To strengthen this assumption, it should be interesting to look for a patient group with acute hepatitis C. In this group of patients, a difference in haptoglobin phenotype distribution should be looked for between the group of patients with a self-resolving hepatitis C and the group of patients that progresses to chronicity. However, for practical reasons, this is very difficult since acute hepatitis C is mostly asymptomatic, the number of patients with acute hepatitis C is very low (approximately 30 registered acute hepatitis C patients annually in Belgium) and since a recent study by Jaeckel et al (24), it is not ethical any more to deny patients with an acute hepatitis C a treatment with interferon alfa.

Together with interferon alfa, ribavirin has become the standard treatment of chronic hepatitis C infection (25,26). Ribavirin treatment induces higher serum and liver iron levels, probably due to the haemolytic anaemia it causes (27,28). Because little is known about the host, viral and treatment factors that may influence ribavirin-induced haemolysis, we have examined in **Chapter V** several factors that could influence the drop in haemoglobin level during treatment with interferon and ribavirin (29).

We have demonstrated that the degree of haemoglobin loss is higher in patients with a higher pre-treatment haemoglobin level, with a low number of platelets, during daily administration of interferon-alfa, and in the presence of the Hp 1-1 phenotype.

The correlation between the pre-treatment haemoglobin level and the drop in haemoglobin can be explained by the fact that ribavirin-induced haemolysis breaks down a certain proportion of the available erythrocytes pool. Higher absolute drops in the haemoglobin level will therefore be observed in subjects with the higher pre-treatment haemoglobin levels.

Patients with lower platelet counts (<110,000/µl) have a significantly higher drop in haemoglobin level than those with higher counts. The low pre-treatment platelet count can be considered as a hallmark of the presence of liver cirrhosis with hypersplenism.

Patients receiving a higher dose of interferon-alfa experience a higher drop in haemoglobin level than those patients receiving a standard dose. Interferon-alfa is known to be myelosuppressive and by itself a cause of anaemia, especially when given in higher doses.

Patients with the Hp 1-1 phenotype experience a higher drop in haemoglobin level during interferon-ribavirin treatment. Complexes of haemoglobin and dimeric haptoglobin (Hp 1-1) exhibit lower functional affinity for the CD163 receptor than do complexes of haemoglobin and the multimeric Hp 2-2 haptoglobin (20). In situations of haemolysis, this could result in less adequate re-utilisation of haem-iron and a higher degree of drop in haemoglobin.

Several recent studies have demonstrated that serum iron indices and liver iron content are frequently elevated in patients with chronic hepatitis C (30). Also, a pattern of free radical production and lipid peroxidation is observed (22).

This could be the consequence of a local and systemic derangement of iron metabolism. Since NTBI is likely to be one of the several factors responsible for oxidative stress in situations with iron overload, we investigated in **Chapter VI** if NTBI is present in patients with chronic hepatitis C. In the majority of the patients (86 %) we cannot demonstrate the presence of NTBI. Even during a treatment with interferon and ribavirin where haemolysis contributes to higher serum iron indices and liver iron content, NTBI values cannot be detected. In the few patients where NTBI is high, it could be a hallmark of real iron overload.

GENERAL CONCLUSION

We could demonstrate that the Hp polymorphism affects the iron metabolism in patients with hereditary hemochromatosis and influences the evolution to chronicity in patients with hepatitis C virus infection. The Hp polymorphism also influences the degree of haemolysis during a treatment with interferon-alfa and ribavirin. This results in the clinical implication that in patients with chronic hepatitis C and low platelets, a high dose interferon treatment results in a higher degree of haemolysis in patients with the Hp 1-1 phenotype. Therefore high dose interferon treatment should be avoided in this subgroup of patients.

The percentage of CD163 positive monocytes-macrophages is not influenced by the Hp phenotype. It seems unlikely that the low macrophage iron content seen in patients with hereditary hemochromatosis is influenced by a different percentage of CD163+ monocytes-macrophages.

Despite the fact that serum and liver iron content are often higher than normal in patients with chronic hepatitis C, the majority of the investigated patients have no detectable NTBI. However in a minority of these patients elevated NTBI levels are found. Further research will focus on the correlation between the presence or absence of NTBI, haptoglobin phenotype polymorphism and HII, the influence of the presence of NTBI on response after antiviral treatment and the possible influence of phlebotomy in patients with high NTBI on response after antiviral treatment.

SAMENVATTING

Zowel de HFE mutatie als het haptoglobine polymorfisme beïnvloeden het ijzermetabolisme (1,2) en houden verband met verschillende immunologische eigenschappen (3).

De *HFE* mutatie wordt teruggevonden bij de meerderheid van de patiënten met erfelijke hemochromatose (4). Er zijn overtuigende gegevens voorhanden dat deze mutatie werkelijk bijdraagt tot de overmaat aan ijzeropstapeling, eigen aan deze ziekte (5-11). In situaties waar er een teveel aan weefselijzer aanwezig is, ziet men in de aanwezigheid van de *HFE* mutatie diverse wijzigingen in de immuunstatus van patiënten met erfelijke hemochromatose ontstaan (3).

Haptoglobine is een eiwit dat hemoglobine bindt en daardoor ijzerverlies en nierbeschadiging voorkomt tijdens episodes van hemolyse. De drie belangrijkste haptoglobine fenotypes hebben elk een verschillend effect op de cellulaire en humorale immuunrespons (12). Bij individuen met het Hp 2-2 fenotype vindt men een hoger aantal CD4+ T-lymfocyten en B-lymfocyten (13). Het Hp 2-2 eiwit vertoont een hogere agglutinatiecapaciteit tegen bepaalde bacteriën dan de andere fenotypes (12). Bij gezonde mannen met het Hp 2-2 fenotype vindt men zowel een hogere serum ferritine concentratie als een hoger ferritine gehalte in monocyten terug dan bij mannen met het Hp 2-1 of Hp 1-1 fenotype (14).

Gezien erfelijke hemochromatose en chronische hepatitis C infectie geassocieerd zijn met zowel hoog serum en weefselijzer als met verschillen in immuun status, onderzochten we de invloed van de *HFE* mutatie en het haptoglobine polymorfisme op deze twee aandoeningen.

In West-Europa is erfelijke hemochromatose de meeste frequente erfelijke aandoening met invloed op het ijzermetabolisme (15).

Vooraleer de groep van Feder (4) in 1996 de hemochromatosemutatie ontdekte, waren de fenotypische karakteristieken van de aandoening (vb. serum ijzersaturatie, serum ferritine concentratie, hepatische ijzerindex, erfelijk karakter …) de belangrijkste aanknopingspunten voor de clinicus om de diagnose te stellen. Actueel worden, dankzij de ontdekking van de *HFE* mutatie, diagnosestelling, familiescreening en populatiescreening eenvoudig.

Het werd dan ook duidelijk dat niet alle individuen die homozygoot zijn voor de Cys282Tyr missense mutatie, de typische fenotypische karakteristieken hebben van erfelijke hemochromatose (16,17). In de afwezigheid van Cys282Tyr homozygotisme zouden ook andere mutaties (vb. His63Asp) een rol kunnen spelen in de etiologie van ijzeropstapelingsziekten. Bij een controlegroep en bij een groep van patiënten met de fenotypische karaktertrekken van erfelijke hemochromatose bepaalden we de aan- of afwezigheid van de Cys282Tyr en His63Asp missense mutatie (**Hoofdstuk I**). De patiënten werden ambulant gevolgd in de periode 1985-1997 (18). In de gezonde controlegroep waren 10,5 % van de 96 individuen heterozygoot voor de Cys282Tyr missense mutatie. Twee procent van hen was homozygoot voor deze mutatie. In de patiëntengroep waren er 46 van de 49 patiënten (94 %) homozygoot voor de Cys282Tyr missense mutatie; 2 waren compound heterozygoot (Cys282Tyr +/-, His63Asp +/-) en 1 individu was enkel heterozygoot voor de His63Asp missense mutatie. Deze resultaten zijn perfect te vergelijken met de prevalenties teruggevonden in andere studies en landen. Ze bevestigen ook dat de Cys282Tyr missense mutatie teruggevonden wordt bij de meerderheid van patiënten met erfelijke hemochromatose en dat deze mutatie zou kunnen bijdragen tot de fenotypische expressie van deze ziekte (4). Of de His63Asp missense mutatie ook bijdraagt tot deze fenotypische expressie is ver van duidelijk.

Deze mutatie lijkt enkel belangrijk te zijn wanneer ze samen voorkomt met de Cys282Tyr missense mutatie (compound heterozygotisme). We vonden deze toestand terug bij twee patiënten van onze groep; beiden hadden de fenotypische presentatie passend bij erfelijke hemochromatose. De identificatie van 1 patiënt met fenotypische hemochromatose maar enkel heterozygoot voor de His63Asp missense mutatie, suggereert het bestaan van andere genetische of niet genetische oorzaken.

Bij gezonde mannelijke vrijwilligers met het Hp 2-2 fenotype vond men hogere serum ferritineconcentraties en een hogere ferritine inhoud in monocyten terug (14). We onderzochten daarom in **Hoofdstuk II**, de invloed van het Hp polymorfisme op de fenotypische presentatie van 167 patiënten met erfelijke hemochromatose. Een significante lage Hp1 allel frequentie tengevolge van een Hp 2-2 fenotype overrepresentatie ($p<0,01$) werd teruggevonden. De aanwezigheid van het Hp 2-2 fenotype induceert een relatief risico van 1,73 (95 % CI 1,14-2.62) tot het ontwikkelen van erfelijke hemochromatose. De lage Hp1 allel frequentie vond men enkel terug bij mannen ($p<0,01$), maar niet bij vrouwen ($p=0,3$). Bij Hp 2-2 mannen werd een hogere serum ijzerconcentratie, serum ferritineconcentratie en aantal aderlatingen tot ijzerdepletie teruggevonden. Het laag aantal vrouwelijke patiënten geïncludeerd in deze studie zou het ontbreken van significantie in deze subgroep kunnen verklaren. Evenmin is het uitgesloten dat andere factoren (menstruatie, zwangerschap) een grotere invloed hebben op de fenotypische expressie van erfelijke hemochromatose (19).
De hogere serum ferritine concentraties, zowel bij gezonde mannen als bij mannelijke hemochromatosepatiënten zou kunnen verklaard worden door het Hp 2-2 specifiek transfer van hemoglobine-haptoglobine complexen in monocyten-macrofagen.

Recent werd aangetoond dat het hemoglobine-haptoglobine complex, en het daarmee gerelateerde ijzer, opgenomen wordt in monocyten-macrofagen via de CD163 receptor op deze cellen. Deze receptor heeft een hogere affiniteit voor Hb-Hp 2-2 complexen dan voor Hb-Hp 1-1 complexen (20). Zowel bij gezonden als bij patiënten met erfelijke hemochromatose zou deze CD163 receptor een rol kunnen spelen in de ijzerstatus van het individu. We bepaalden daarom het percentage CD163 positieve monocyten-macrofagen bij gezonden en bij patiënten met erfelijke hemochromatose (**Hoofdstuk III**). Bij de gezonde controlegroep vonden we geen haptoglobine-gerelateerd verschil in het percentage CD163 positieve monocyten-macrofagen. Evenmin was er een correlatie tussen het percentage CD163 positieve monocyten-macrofagen en serum ijzerparameters.
Er werd geen verschil opgemerkt tussen het percentage CD163+ monocyten-macrofagen tussen patiënten met erfelijke hemochromatose en patiënten met secundaire hemochromatose of individuen uit de controlepopulatie. Het lijkt daarom zeer onwaarschijnlijk dat de lage ijzerinhoud van macrofagen bij patiënten met erfelijke hemochromatose beïnvloed wordt door een verschil in percentage aan CD163+ monocyten-macrofagen.

In België bedraagt de prevalentie van chronische hepatitis C infectie 0,9 % (21). Bij een chronische hepatitis C infectie vindt men vaak een overmaat aan ijzer en verhoogde oxidatieve stress (22). Zowel virale (genotype, hoeveelheid virus) als gastheerfactoren (immuunantwoord) beïnvloeden het verloop van de hepatitis C infectie.

In **Hoofdstuk IV** toonden we aan dat bij een groep van 142 chronische hepatitis C patiënten, zowel de Cys282Tyr missense mutatie als het Hp 1-1 fenotype in een statistisch hoger frequentie aanwezig zijn dan bij een controlepopulatie (23). Heel opvallend blijkt de aan- of afwezigheid van het ene gen, de impact van het andere gen te beïnvloeden.

Zo blijkt enerzijds, dat bij de afwezigheid van de Cys282Tyr missense mutatie, het Hp fenotype bepalend is voor het risico tot het ontwikkelen van een chronische hepatitis C infectie. Enkel in deze subgroep van patiënten zien we een hogere frequentie van het Hp 1-1 fenotype. Anderzijds ziet men een hogere frequentie van de Cys282Tyr missense mutatie in de groep van patiënten met het Hp 2-1 en Hp 2-2 fenotype. We veronderstellen dat de verhoogde oxidatieve stress aanwezig in de patiënten met zowel het Cys282Tyr allel als het Hp2 allel, resulteert in een hogere risico in deze subgroep om een chronische hepatitis C infectie te ontwikkelen.

Omdat we patiënten niet over een tijdspanne gevolgd hebben, maar dat deze observatie enkel een momentopname betreft, is het moeilijk commentaar te geven over de ziekteprogressie. Echter omdat we een significante overrepresentatie vinden van het Hp 1-1 fenotype bij deze groep van patiënten met een chronische hepatitis C infectie, maken we de veronderstelling dat de aanwezigheid van dit fenotype de patiënt gevoeliger maakt voor het ontwikkelen van een chronische hepatitis C infectie. Om dit te kunnen staven, is er interesse in een studie bij patiënten met een acute hepatitis C infectie, waarbij gepoogd wordt een verschil in haptoglobine fenotypedistributie aan te tonen tussen de groep met een zelflimiterende infectie en de groep welke evolueert naar een chronische hepatitis C. Dit is praktisch echter niet haalbaar omdat de meerderheid van de patiënten met een acute hepatitis C infectie asymptomatisch zijn, het aantal gerapporteerde acute hepatitis C infecties laag is (ongeveer 30 geregistreerde acute hepatitis C gevallen in België per jaar) en het sinds de studie van Jaeckel et al (24) niet ethisch meer is een patiënt met een acute hepatitis C infectie een behandeling met interferon alfa te ontzeggen.

Ribavirine, in combinatie met interferon alfa, is de standaardbehandeling van patiënten met chronische hepatitis C infectie (25,26).

Ribavirine veroorzaakt hemolyse, waardoor een stijging in serum ijzer en ijzerinhoud van de lever wordt waargenomen (27,28). Er is weinig gekend over factoren die deze hemolyse kunnen beïnvloeden. Wij onderzochten daarom in **Hoofdstuk V**, welke factoren de hemoglobinedaling kunnen beïnvloeden gedurende een behandeling met interferon en ribavirine (29).

We konden aantonen dat een ernstiger hemoglobinedaling werd geobserveerd bij patiënten met een hoger hemoglobinegehalte bij het opstarten van de behandeling, een laag aantal trombocyten, dagelijkse toediening van interferon alfa en de aanwezigheid van het Hp 1-1 fenotype.

Omdat ribavirine een zekere, relatieve constante, proportie van de aanwezige rode bloedcellen afbreekt ziet men een correlatie tussen het hemoglobinegehalte bij het opstarten en de hemoglobinedaling gedurende de behandeling. Daardoor resulteren hogere startwaarden van hemoglobine in een hogere absolute daling van het hemoglobinegehalte.

Bij patiënten met lagere trombocyten (<110.000/µl) ziet men een belangrijkere hemoglobinedaling dan bij patiënten met trombocytenaantallen die hoger zijn. Het laag aantal trombocyten wordt beschouwd als een handtekening van de aanwezigheid van levercirrose met hypersplenisme.

Patiënten die een hogere dosis interferon alfa toegediend krijgen (onder de vorm van een dagelijkse toediening), ondervinden ook een hogere hemoglobinedaling dan de patiënten die een standaard dosis interferon krijgen (drie keer per week). Het is immers gekend dat interferon alfa myelosuppressief werkt en, vooral wanneer toegediend in een hoge dosis, anemie kan veroorzaken.

Patiënten met het Hp 1-1 fenotype ondervinden eveneens een hogere hemoglobinedaling dan patiënten met de andere fenotypes. Hb-Hp 1-1 complexen hebben een lagere affiniteit voor de CD163 receptor dan Hb-Hp 2-2 complexen (20). In geval van hemolyse kan dit leiden naar een minder adequaat hergebruik van heemijzer en een belangrijker daling in het hemoglobinegehalte.

Bij patiënten met chronische hepatitis C infectie vindt men frequent verhoogde serumijzer-waarden en een hogere ijzerinhoud in de lever terug (30). Daarnaast observeert men eveneens situaties die gepaard gaan met vrijstelling van vrije radicalen en vetperoxidatie (22). Dit zou kunnen het gevolg zijn van een lokaal of systemische stoornis in het ijzermetabolisme. Omdat NTBI één van de factoren is die verantwoordelijk kan gesteld worden voor oxidatieve stress in situaties met overmaat aan ijzer, onderzochten we in **Hoofdstuk VI,** de aanwezigheid van NTBI bij patiënten met chronische hepatitis C infectie. In de meerderheid van hen (86 %) werd geen NTBI aangetroffen. Evenmin werd gedurende een behandeling met interferon en ribavirine NTBI aangetroffen. Bij de zeldzame patiënt waar NTBI wel aangetroffen wordt, kan dit een handtekening zijn van echte overmaat in weefselijzer.

ALGEMEEN BESLUIT

We konden aantonen dat het Hp fenotype het ijzermetabolisme van patiënten met erfelijke hemochromatose en de evolutie naar chroniciteit bij patiënten met een chronische hepatitis C infectie beïnvloedt. Het Hp fenotype beïnvloedt ook de graad van hemolyse gedurende een behandeling met interferon alfa en ribavirine. Klinisch betekent dit dat een behandeling met hoog gedoseerde interferon bij een patiënt met lage plaatjes en het Hp 1-1 fenotype resulteert in een belangrijker graad van anemie. Hoog gedoseerde interferonbehandeling moet dan ook vermeden worden in deze subgroep van patiënten.

Bij een gezonde mannelijke controlepopulatie wordt het percentage CD163 positieve monocyten-macrofagen niet beïnvloed door het Hp fenotype. Het lijkt onwaarschijnlijk dat de lage ijzerinhoud van macrofagen bij patiënten met erfelijke hemochromatose beinvloed wordt door een verschil in percentage aan CD163+ monocyten-macrofagen.

Ondanks het feit dat het serumijzer en de inhoud aan leverijzer vaak verhoogd zijn bij patiënten met chronische hepatitis C, vindt men in de meerderheid van deze patiënten geen NTBI in het serum terug. Nochtans heeft een minderheid van deze patiënten een verhoogd NTBI gehalte. Verder onderzoek zal zich toespitsen op het verband tussen NTBI, het haptoglobine fenotype polymorfisme en HII, de invloed van het NTBI gehalte op het antwoord na een antivirale behandeling. Tenslotte zal bestudeerd worden of de invloed van aderlatingen bij patiënten met een hoog NTBI gehalte het antwoord na een antivirale behandeling kunnen beïnvloeden.

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LIST OF ABBREVIATIONS

ALT :	Alanine aminotransferase
AST:	Aspartate aminotransferase
CTL :	Cytotoxic T Lymphocyte
DCT1 :	Divalent cation transporter 1
DMT1 :	Divalent metal transporter 1
E1 :	Envelope protein 1
E2 :	Envelope protein 2
EASL :	European Association for the Study of the Liver
EDTA :	Ethylene diaminetetraacetic acid
Hb :	Haemoglobin
HCV :	Hepatitis C virus
HFE :	Hemochromatosis gene
HFE :	Hemochromatosis protein
HH :	Hereditary hemochromatosis
HIC:	Hepatic iron content
HII:	Hepatic iron index
HLA :	Human major histocompatibility complex
Hp:	Haptoglobin gene
Hp:	Haptoglobin protein
HPLC:	High performance liquid chromatography
Ireg1:	Iron regulating protein 1
MHC:	Major histocompatibility complex
MU:	Million units
Nramp2:	Natural resistance associated macrophage protein 2
NTBI:	Non-transferrin-bound iron
PCR:	Polymerase chain reaction
Th1:	T helper 1 lymphocyte
Th2:	T helper 2 lymphocyte

Phenotype (in this work): biochemical characteristics of the different Hp proteins found in serum, reflecting differences in the genetics of the Hp protein.