

HEREDITARY HEMOCHROMATOSIS

With a special emphasis on HFE genotyping

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Abstract

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder estimated to affect one out of every 250–400 Caucasian individuals. It is a disorder of iron metabolism, in which excessive iron accumulation in the body may induce serious clinical manifestations (e.g. liver cirrhosis, hepatocellular carcinoma, diabetes, and cardiomyopathy). HH is caused by mutations in the *HFE* gene, and *HFE* genotyping thus enables early diagnosis of the disease and detection of the individuals at risk for HH. *HFE* mutations have also been proposed to predispose to certain other diseases, such as various hematological malignancies and cardiomyopathy.

The present evaluation of the clinical utility and outcome of *HFE* genotyping in search for HH was based on data obtained from 137 subjects referred for *HFE* mutation analysis during the years 1999–2001. The C282Y and H63D mutations were determined for each subject. *HFE* genotyping was also used to examine the association between *HFE* mutations with various hematological disorders and idiopathic dilated cardiomyopathy (IDCM). The C282Y and H63D mutations were determined from 232 patients with various hematological disorders and the C282Y, H63D, and S65C mutations from 91 patients with IDCM and 102 control subjects.

High frequencies of C282Y homozygotes (16.8%) and C282Y/H63D compound heterozygotes (5.1%) were found among the subjects referred for *HFE* genotyping, and the rate of positive findings for HH increased steadily over the years 1999–2001. The frequencies of *HFE* mutations did not differ significantly in patients with various hematological disorders and IDCM compared to controls. At the end of the follow-up period, left ventricular end-diastolic diameter (LVEDD) was significantly higher in IDCM patients carrying the C282Y mutation than in those without this mutation ($p = 0.037$).

The present study supports active testing for the *HFE* gene mutations C282Y and H63D in public health care. Serum transferrin saturation is considered the most useful test for selecting subjects for such analysis. Although increasing numbers of HH cases are recognized by physicians, it may still be an underdiagnosed disease. *HFE* mutations do not seem to significantly increase the risk for various hematological disorders or IDCM. The C282Y mutation may, nevertheless, mediate the progression of IDCM by modifying LV dilation and remodeling.

Keywords: hematological disorders, idiopathic dilated cardiomyopathy, iron

To Katja

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Oulu, October 2004

Jokke Hannuksela

Abbreviations

95% CI	95% confidence interval
AD	Alzheimer's disease
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APOE	apolipoprotein E
BSA	bovine serum albumin
CaCo-2 cells	a human intestinal cell line
CHD	coronary heart disease
CHO	Chinese hamster ovary
CML	chronic myeloid leukemia
DcytB	duodenal cytochrome b
DMT1	duodenal metal transporter
ET	essential thrombocythemia
FAB	French-American-British
Fe ²⁺	ferrous iron
Fe ³⁺	ferric iron
FITC	fluorescein isothiocyanate
FVL	factor V Leiden
HCC	hepatocellular carcinoma
HEK	human embryonic kidney
HeLa cells	human cervix adenocarcinoma cells
HH	hereditary hemochromatosis
HIC	hepatic iron concentration
HII	hepatic iron index
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IDCM	idiopathic dilated cardiomyopathy
IRE	iron responsive element
IRPs	iron regulatory proteins
LVEDD	left ventricular end diastolic diameter
LVEF	left ventricular ejection fraction

MCI	mild cognitive impairment
MDS	myelodysplastic syndromes
MHC	major histocompatibility complex
MI	myocardial infraction
MM	multiple myeloma
MRI	magnetic resonance imaging
NASH	nonalcoholic steatohepatitis
NTBI	non-transferrin-bound iron
NYHA	New York Heart Association
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCT	porphyria cutanea tarda
PD	Parkinson's disease
PV	polycythemia vera
RBC	red blood cell
RES	reticuloendothelial system
SD	standard deviation
SDS	sodium dodecyl sulphate
s-ALP	serum alkaline phosphatase
s-ALT	serum alanine aminotransferase
s-AST	serum aspartate aminotransferase
s-Fe	serum iron concentration
s-ferrit	serum ferritin concentration
s-TS	serum transferrin saturation
TBST	Tris-buffered saline with Tween-20
TfR	transferrin receptor
TIBC	total iron-binding capacity
UIBC	unsaturated iron-binding capacity
URO-D	uroporphyrinogen decarboxylase
UTR	untranslated region
β_2 M	β_2 -microglobulin

List of original publications

This thesis is based on the following original articles, which are referred to in the text by Roman numerals I-IV:

- I Hannuksela J, Niemelä O, Leppilampi M, Parkkila AK, Koistinen P, Nieminen P & Parkkila S (2003) Clinical utility and outcome of HFE-genotyping in the search for hereditary hemochromatosis. *Clin Chim Acta* 331:61-67.
- II Hannuksela J, Parkkila S, Waheed A, Britton RS, Fleming RE, Bacon BR & Sly WS (2003) Human platelets express hemochromatosis protein (HFE) and transferrin receptor 2. *Eur J Haematol* 70:201-206.
- III Hannuksela J, Savolainen E-R, Koistinen P & Parkkila S (2002) Prevalence of HFE genotypes, C282Y and H63D, in patients with hematologic disorders. *Haematologica* 87:131-135.
- IV Hannuksela J, Leppilampi M, Peuhkurinen K, Kärkkäinen S, Saastamoinen E, Heliö T, Nieminen MS, Nieminen P & Parkkila S. Hereditary hemochromatosis gene (HFE) mutations C282Y, H63D and S65C in patients with idiopathic dilated cardiomyopathy. *Eur J Heart Fail*, in press.

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1 Introduction

Iron is a crucial metal for living organisms due to its participation in multiple biochemical reactions. Excessive iron, however, may induce oxidative stress leading to cell damage. (Emerit *et al.* 2001, Fraga & Oteiza 2002.) Indeed, iron homeostasis plays a significant role in health and disease. Because humans do not possess any regulated pathways for iron excretion, iron homeostasis in the body is determined by the level of iron absorption from the diet.

Hereditary hemochromatosis (HH) is a common disorder of iron homeostasis characterized by increased intestinal iron absorption that leads to a progressive iron overload. Most HH patients are homozygous for the same missense mutation (C282Y) in the *HFE* gene, while a few are compound heterozygotes for the C282Y and H63D mutations. (Feder *et al.* 1996.) The estimated carrier frequencies of the C282Y and H63D mutations in the European population are 9.2% and 22%, respectively (Hanson *et al.* 2001). HH may be an underdiagnosed disease that can cause early death if untreated. The most important clinical manifestations of the disease include e.g. liver cirrhosis, hepatocellular carcinoma, diabetes mellitus, and cardiomyopathy. (Parkkila *et al.* 2001a.) These irreversible conditions can be prevented by early detection and proper treatment of HH patients. Recurrent phlebotomies are an effective, inexpensive and safe treatment method for HH (Adams *et al.* 2000a.), and the patients treated before the development of diabetes or cirrhosis of the liver have normal life expectancy (Niederau *et al.* 1985, Niederau *et al.* 1996, Barton *et al.* 1998, McCullen *et al.* 2002).

HFE genotyping is a novel tool in the diagnostics of HH that enables early diagnosis of the disease. It is a safe and convenient method, and the results of DNA mutation analyses remain unchanged for the rest of the individual's life. HH patients can be identified even before the onset of clinical symptoms with the help of serum iron parameters. The diagnosis of HH can usually be confirmed by *HFE* genotyping in iron-overloaded patients, while liver biopsy is no longer necessary for basic diagnostics. *HFE* genotyping of first-degree family members of affected subjects is also recommended for the detection of HH. (Brandhagen *et al.* 2002, McCullen *et al.* 2002, Powell 2002).

HFE mutations are known to induce HH, but it has been suggested that these mutations may also increase the risk for other diseases, such as various hematological malignancies and cardiomyopathy. *HFE* mutations have been found to increase the body

iron stores (de Valk *et al.* 2000a, Rossi *et al.* 2000a, Raddatz *et al.* 2003, Sánchez *et al.* 2003). This may subsequently be involved in carcinogenesis (Toyokuni 1996, Weiss 2002, Huang 2003), and it has been reported that heterozygosity for HH is associated with an increased risk of hematological malignancy (Nelson *et al.* 1995). Cardiomyopathy is known as one of the most severe complications of HH. However, previous studies elucidating the association between *HFE* gene mutations and cardiomyopathy have yielded conflicting results (Mahon *et al.* 2000, Hetet *et al.* 2001a, Pereira *et al.* 2001). The present study set out to evaluate the clinical utility and outcome of *HFE* genotyping in the search for HH and to investigate the association of *HFE* mutations with various hematological disorders and cardiomyopathy. This thesis also elucidates the expression of HFE, TfR1, and TfR2 in human platelets.

2 Review of the literature

2.1 Iron homeostasis

2.1.1 General

Most living organisms need iron for their normal growth and development. Iron is indispensable in many biochemical reactions, being an element of cytochromes, oxygen-binding molecules, and multiple enzymes. Iron can exist in two redox states, and it can readily accept and donate electrons. Despite the crucial role of this capacity in physiological events, it also makes excess iron toxic, and iron can damage tissues by inducing free radical formation. (Emerit *et al.* 2001, Fraga & Oteiza 2002.)

An adult male body contains approximately 4 g of iron and is incapable of excreting substantial amounts of it. The daily loss of iron in men is 1-2 mg and it is mainly caused by desquamation of skin and enterocytes. (Conrad & Umbreit 2002.) Additionally, premenopausal women lose iron during menstruations (3-60 mg/month) (Hallberg & Rossander-Hultén 1991) and pregnancy (~580 mg) (Bothwell 2000), which explains their lower iron stores (~3 g). Most of the body iron is bound to hemoglobin (65%), while 10% is bound to myoglobin, cytochromes, and respiratory enzymes, and 20-30% is stored in cells as ferritin and hemosiderin (Fraga & Oteiza 2002). (Figure 1)

Iron deficiency and iron overload are common disorders affecting widely human health. Pathological conditions of iron deficiency includes e.g. anemia and growth arrest (Prasad & Prasad 1991), while excessive iron may lead to other severe manifestations, such as fibrosis and cirrhosis of the liver, cancer, cardiac diseases, endocrine abnormalities, including diabetes mellitus, immune system dysfunctions, and/or neurodegenerative disorders (Bacon & Britton 1989, Yaouanq 1995, Sipe *et al.* 2002, Weiss 2002).

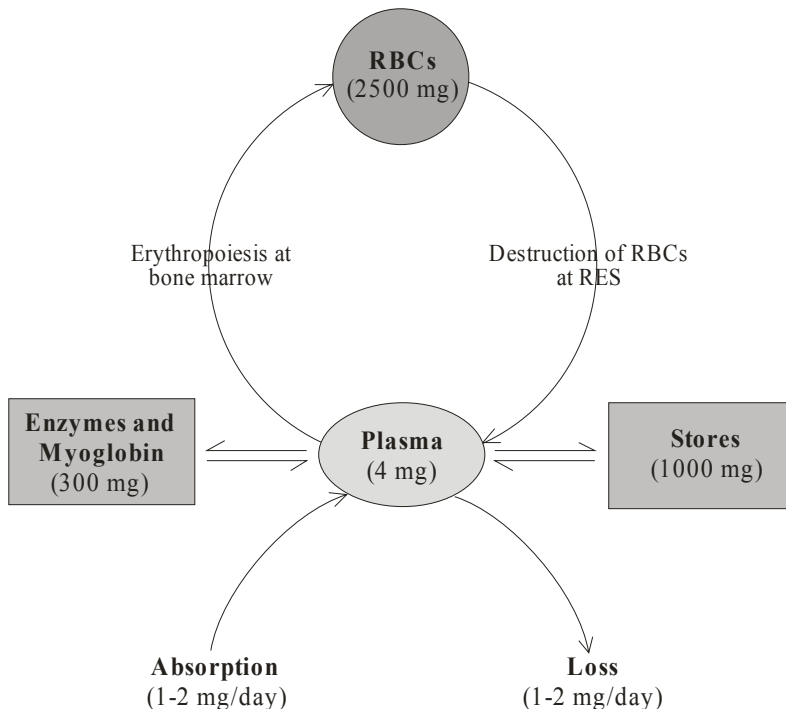


Fig. 1. Normal iron homeostasis according to Conrad and Umbreit (2002). Adult men have approximately 4 g and women 3g of iron in the body. RBCs, red blood cells; RES, reticuloendothelial system.

2.1.2 Iron absorption

Body iron status is directly proportional to the level of absorption from the diet, since humans lack a regulatory pathway for iron excretion. Approximately 1-2 mg of iron is absorbed from the small intestine each day. Iron uptake into the body takes place in the duodenum and upper jejunum exclusively through mature enterocytes. Dietary iron has to traverse two interfaces of enterocytes before access into the circulation. The apical cell membrane of a mature enterocyte faces the intestinal lumen and is specialized to transport heme and inorganic iron into the cell. Iron efflux from the enterocyte into the bloodstream takes place across the basolateral cell membrane. (Parkkila *et al.* 2001a, Conrad & Umbreit 2002, Trinder *et al.* 2002a.)

Iron can be delivered across the apical membrane of an intestinal enterocyte via the divalent metal transporter (DMT1), the integrin-mobilferrin pathway, and the heme iron uptake pathway. DMT1 transports ferrous iron (Fe^{2+}) and other divalent metals from the intestinal lumen into the enterocyte (Gunshin *et al.* 1997, Andrews 1999, Garrick *et al.*

2003). Inorganic dietary iron is predominantly in the ferric form (Fe^{3+}) (Conrad & Umbreit 2002), and before it can be absorbed via DMT1, it is reduced into the ferrous form by duodenal cytochrome b (DcytB) (McKie *et al.* 2001). Mobilferrin and β_3 -integrin may provide a pathway specific for ferric iron into the absorptive cell. Once ferric iron is within the enterocyte, a large protein complex is formed (paraferritin), which reduces it into the ferrous form. (Conrad & Umbreit 2002.) The mechanism by which heme iron enters the enterocyte from the diet is poorly recognized. The proposal that it involves a specific heme receptor (Mills & Payne 1995) was supported by a recent study, which introduced a candidate transporter for heme (Brissot *et al.* 2004). After internalization, inorganic iron is released from heme by heme oxygenase and further reduced in a complex comprised of mobilferrin and paraferritin (Uzel & Conrad 1998). Thus, the iron delivered into the enterocyte via these separate pathways is in the ferrous form and can be stored as ferritin within the cell or delivered into the portal circulation. The basolateral enterocyte iron transporter ferroportin1 (also called Ireg1 or MTP1) (Abboud & Haile 2000, Donovan *et al.* 2000, McKie *et al.* 2000) transports ferrous iron across the basolateral membrane. Ferroportin1 requires an accessory protein (hephaestin), which may function as a ferroxidase, oxidizing ferrous iron into the ferric form that binds avidly to circulating transferrin (Vulpe *et al.* 1999). (Figure 2)

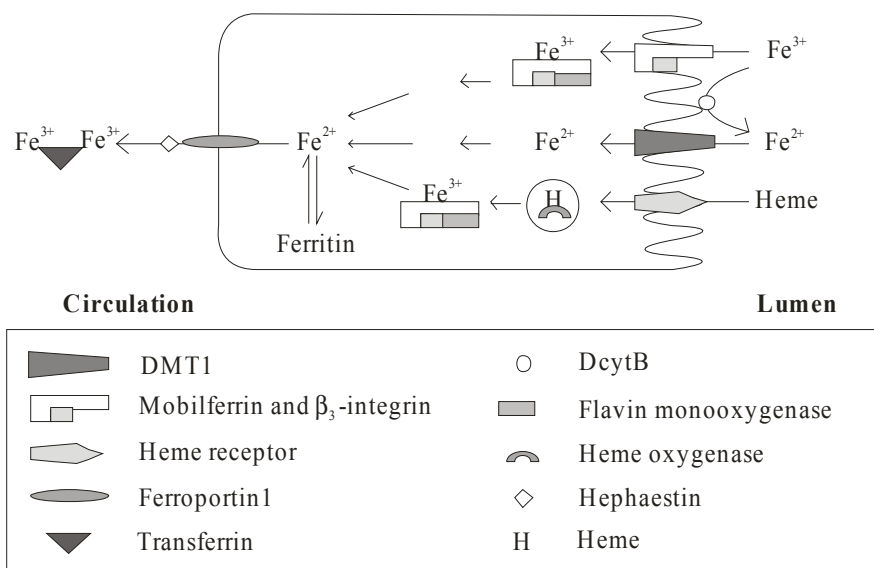


Fig. 2. Three separate pathways have been presented for the uptake of dietary iron. The best characterized pathway is via the divalent metal transporter (DMT1) for ferrous iron (Fe^{2+}) (Gunshin *et al.* 1997, Andrews 1999, Garrick *et al.* 2003). The integrin-mobilferrin pathway has been postulated to transport ferric iron (Fe^{3+}) (Conrad & Umbreit 2002). Heme iron is thought to enter the enterocyte via a specific heme receptor (Mills & Payne 1995, Brissot *et al.* 2004). Ferrous iron is stored as ferritin or transported into the circulation via Ferroportin1 (Abboud & Haile 2000, Donovan *et al.* 2000, McKie *et al.* 2000).

2.1.3 Regulation of iron absorption

Iron absorption is a strictly regulated process that depends on the body iron status and is normally equivalent to the body iron needs. An average of only 10% of ingested iron is usually absorbed from the small intestine, and the uptake of dietary iron is increased in the presence of iron deficiency and decreased in iron excess. (Conrad & Umbeirt 2002.)

The regulatory mechanisms that control the level of iron absorption influence the synthesis of iron transport proteins in intestinal cells. This has been considered to mediate through changes in cellular free iron and iron regulatory proteins (IRPs). The expression levels of iron transport proteins in enterocytes ultimately determine the rate of iron absorption from the intestinal lumen. IRPs exert their effects on the synthesis of the key proteins involved in iron metabolism in response to fluctuations in the cellular free iron pool. The molecular target of IRPs is the iron-responsive element (IRE) present in the mRNAs of certain proteins. (Cairo *et al.* 2002, Pietrangelo 2002.) It has been detected that the apical iron transport protein DMT1 possesses an IRE in the 3' untranslated region (UTR) of the mRNA sequence (Gruenheid *et al.* 1995, Pinner *et al.* 1997). Thus, the low levels of cellular iron stabilize DMT1 mRNA, which increases the expression of DMT1 in the apical surface of mature enterocytes (Pietrangelo 2002). The specific mechanisms that alter the gene expression of the intestinal iron transport molecules other than DMT1 have remained unclear. Another iron transport protein, ferroportin1, seems to have an IRE in the 5' UTR (Abboud & Haile 2000), but it was recently found that enterocyte iron levels had no effect on the expression of ferroportin1 (Frazer *et al.* 2003). Regulation through IRPs is also unlikely in the case of Dcytb and hephaestin (Vulpe *et al.* 1999, Frazer *et al.* 2001, McKie *et al.* 2001, Frazer *et al.* 2003).

The level of iron absorption is regulated by mechanisms that supply information about the body iron requirements to the intestinal enterocytes. The “stores regulator” is considered an important regulatory mechanism that adjusts slow non-heme dietary iron uptake, but does not have a substantial effect on heme iron accumulation. This mechanism increases iron absorption when the body iron stores are low. It also plays a crucial role in preventing excess iron accumulation and an iron overload after the body iron requirements have been met. Soluble components of plasma, such as transferrin-bound iron, serum ferritin, serum transferrin receptors (TfR1, TfR2), and hepcidin are considered to potentially communicate between the iron stores (e.g. liver, skeletal muscle, blood) and the intestine. Failure in the stores regulator function may induce iron accumulation and an iron overload in the body and lead to hemochromatosis. (Roy & Enns 2000, Pietrangelo 2002.)

The second major regulatory mechanism of iron absorption mediates the signal between the hematopoietic bone marrow and the intestine. This “erythropoietic regulator” is probably also a soluble component of plasma, and it can regulate iron uptake according to the demands of erythropoiesis. An increase in erythropoiesis alone does not increase iron absorption. It rather seems that the ratio between the bone marrow iron supply and erythropoiesis is critical for the regulation of iron absorption. The erythropoietic regulator can induce iron absorption in anemic individuals much more than the stores regulator, suggesting that the erythropoietic regulator is distinct from the stores regulator. Hypoxia has also been introduced as a potential regulatory factor that induces the level of iron

absorption. However, it is not clear whether the effect of hypoxia is independent of the erythropoietic regulator. (Roy & Enns 2000, Pietrangelo 2002.)

Hepcidin is a circulating antimicrobial peptide that is mainly synthesized in the liver (Krause *et al.* 2000, Park *et al.* 2001, Pigeon *et al.* 2001). It is likely to act as an important component of the stores and/or erythroid regulator, and a number of reports have suggested that hepcidin mediate the body signals of iron requirements to intestinal enterocytes (Nicolas *et al.* 2001, Pigeon *et al.* 2001, Nicolas *et al.* 2002a, Frazer & Anderson 2003, Miret *et al.* 2003). Observations of iron-overloaded mice have shown that excess iron leads to overexpression of hepcidin, while iron depletion leads to a decrease of its expression (Pigeon *et al.* 2001). Transgenic mice have provided functional implications for hepcidin in the regulation of iron absorption. It has been found that mice lacking hepcidin develop a severe iron overload (Nicolas *et al.* 2001), while transgenic mice overexpressing this protein die shortly after birth from severe iron deficiency anemia (Nicolas *et al.* 2002b). These data suggest that hepcidin is a negative regulator of iron absorption. Additionally, anemia and hypoxia have been demonstrated to reduce the expression of hepcidin (Nicolas *et al.* 2002a). Reduced levels of hepcidin thus appear to induce intestinal iron absorption in these conditions. Hepcidin also seems to be a mediator of anemia of inflammation (Ganz 2003). This is a common anemia in patients with infections, cancer, trauma, and autoimmune diseases. It was recently shown that, in inflammation, hepcidin expression is induced by interleukin 6 (Nemeth *et al.* 2003, Nemeth *et al.* 2004). Increased hepcidin expression then leads to the low serum iron levels observed in anemia of inflammation by inhibiting macrophage iron release and iron absorption from the intestine (Andrews 2004).

It is widely supported that iron uptake from plasma across the basolateral membrane of a cryptal enterocyte plays a significant role in regulating the level of dietary iron absorption in mature enterocytes (Anderson 1996, Roy & Enns 2000, Parkkila *et al.* 2001a, Trinder *et al.* 2002a). Changes in iron absorption do not commonly occur until 2 to 3 days after the iron absorption stimulus. This time lag is identical to the time duodenal crypt cells need to migrate up to the villus and to differentiate into mature iron-absorbing enterocytes. (Lombard *et al.* 1997.) Iron from circulation enters the cryptal enterocyte across the basolateral membrane via a TfR1-mediated pathway. TfR1 is physically associated with the hereditary hemochromatosis protein HFE in duodenal crypt cells (Waheed *et al.* 1999), and the interaction between these two proteins is likely to be critical for the acquisition of iron in these cells. This enables cryptal enterocytes to be programmed in response to the body iron stores and to absorb the required quantity of iron from the intestinal lumen once they have differentiated into mature enterocytes (Parkkila *et al.* 2001a). This model, however, does not account a role for hepcidin, although there is a possibility that hepcidin interacts with cryptal enterocytes and is subsequently involved in the programming of these cells (Nicolas *et al.* 2001). (Figure 3)

A large oral dose of iron is known to reduce the absorption of a smaller dose administered several hours later (Hahn *et al.* 1943). This rapid change of iron absorption cannot result from the series of events that involves the maturation of crypt cells, and it further led to the proposal of a mucosal block in iron absorption. The molecular mechanisms of the hypothesized mucosal block, nevertheless, remained unknown for several decades. It was only recently discovered that, after an oral dose of iron, the expression of DMT1 and Dcytb is rapidly reduced, leading to decreased iron absorption

at the luminal surface of intestinal enterocytes in rats (Oates *et al.* 2000, Yeh *et al.* 2000, Frazer *et al.* 2003). These findings support the existence of a mucosal block and the local regulation of iron absorption by mature enterocytes. Other situations where iron absorption is regulated more rapidly than can be explained by the mechanism involving differentiation of cryptal enterocytes have also been described. Iron absorption is rapidly decreased after an acute phase response and increased following RBC transfusions (Cortell & Conrad 1967, Finch *et al.* 1982). The decreased iron absorption observed in the acute phase response is also associated with increased expression of hepcidin, but the effect of inflammation on iron absorption is suppressed in hepcidin-deficient mice (Nicolas *et al.* 2002a).

Interestingly, Frazer and Anderson (2003) recently proposed that mature enterocytes receive signals to alter iron absorption directly rather than via differentiation of cryptal enterocytes. They suggested that the lag period of several days usually seen in response to an iron absorption stimulus may not represent the time required for crypt cells to differentiate into mature enterocytes. This lag in response may be explained by the time needed by the body to recognize its iron requirements. In this scheme, the liver regulates the expression of hepcidin in response to alterations in transferrin saturation. Hepcidin levels would directly influence the mature enterocytes contributing to changes in iron absorption from the intestinal lumen equivalent to the body iron needs. The expression of hepcidin has, indeed, been demonstrated to closely correlate with the expression of the duodenal iron transport proteins Dcytb, DMT1, and ferroportin1 (Frazer *et al.* 2002). (Figure 4)

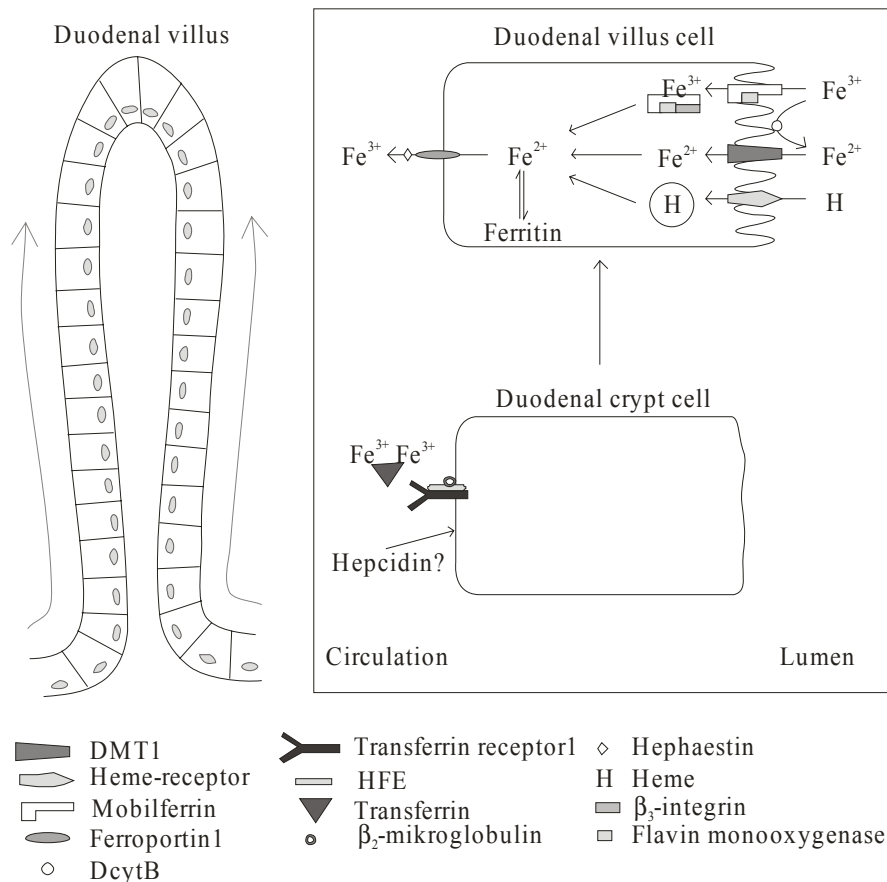


Fig. 3. A widely supported model for the regulation of iron absorption. Duodenal crypt cells are thought to sense the body iron stores by taking up transferrin-bound iron from the circulation via a TfR1-dependent pathway. HFE protein is considered to facilitate the transport of transferrin-bound iron via TfR1. The intracellular iron pool of cryptal enterocytes is thus directly proportional to serum transferrin saturation, which, in turn, reflects the body iron stores. The intracellular iron pool is considered to influence the synthesis of the iron transport proteins of mature enterocytes. In this way, enterocytes may be programmed to absorb the required amount of dietary iron once they have migrated up to the villus. (Parkkila *et al.* 2001a.) Heparidin may theoretically be involved in the programming of cryptal enterocytes (Nicolas *et al.* 2001), but its exact role has not been established.

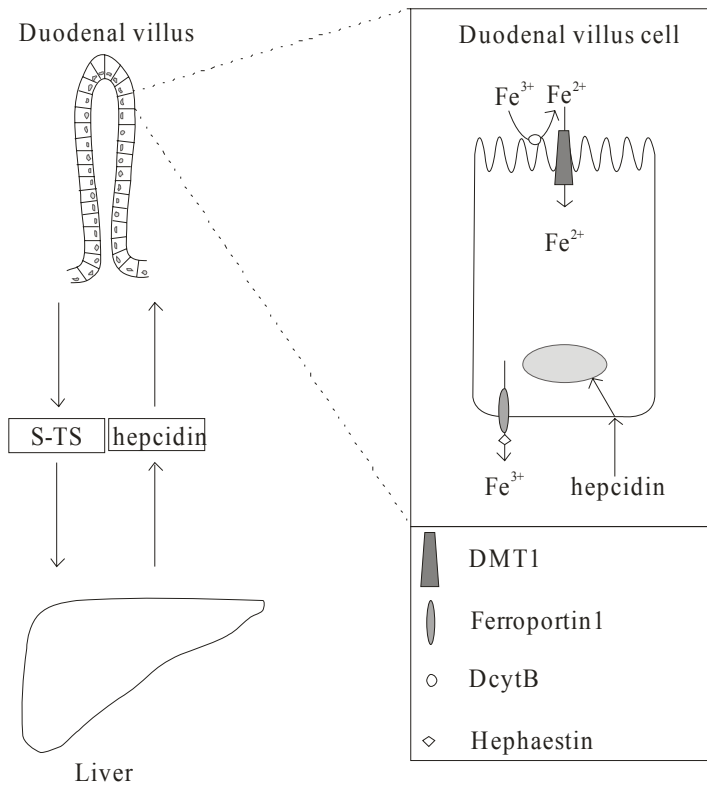


Fig. 4. A recently described model for the regulation of iron absorption (Frazer & Anderson 2003). The liver regulates the expression of hepcidin in response to alterations in serum transferrin saturation. An increase in serum transferrin saturation normally results in increased hepcidin expression in the liver. The specific mechanism by which serum transferrin saturation is sensed by the liver must be further confirmed, but it is likely to involve Tfr1, Tfr2, and HFE. Hepcidin interacts directly with mature villus cells. An increase of serum hepcidin level suppresses the expression of iron transport proteins, while a decrease of hepcidin level increases the expression of these proteins. In this way, mature enterocytes are programmed to absorb iron according to the body iron requirements.

2.2 Hereditary hemochromatosis

2.2.1 General

Hereditary hemochromatosis (HH) is the most common autosomal recessive disorder in Caucasians, affecting approximately 1/300 in the Western and Northern European populations. It results from increased intestinal iron absorption and leads to excessive iron deposition into the parenchymal cells of the body. An iron overload within these cells, in turn, may induce cell injury and pathological involvement of internal organs, such as the liver, pancreas, and heart. (Parkkila *et al.* 2001a.)

HH may be an underdiagnosed disease and it can cause early death if left untreated. Phlebotomy is an effective, inexpensive, and safe treatment method available for iron overload, especially when combined with early detection of affected subjects. Patients whose diagnosis is established and proper treatment given at an early stage of the disease progression have normal life expectancy. (Adams *et al.* 2000a.)

Various forms of hereditary iron overload disorders are shown in Table 1 (Bomford 2002, Pietrangelo 2003). The gene mutated in most patients with hereditary iron overload disorder is designated as *HFE*. 80-90% of the patients with primary iron overload are homozygous for the same missense mutation 845G→A (C282Y) resulting in a cysteine to tyrosine substitution at amino acid 282 in the HFE protein, while a few are compound heterozygous for the C282Y and 187C→G (H63D) mutations (Feder *et al.* 1996). The H63D mutation causes a substitution of histidine to aspartate at amino acid position 63. Furthermore, an A-to-T transition at nucleotide 193 that results in a serine to cysteine substitution at position 65 (S65C) in the HFE protein has recently been associated with a mild form of HH (Mura *et al.* 1999, Holmström *et al.* 2002, Wallace *et al.* 2002).

Table 1. Classification of various forms of hereditary iron overload disorders.

Hemochromatosis subgroup	Inheritance	Gene mutated	Dysfunctional protein product	References
type 1 or HFE-related or hereditary hemochromatosis	autosomal recessive	<i>HFE</i>	HFE	Feder <i>et al.</i> 1996
type 2 or juvenile hemochromatosis	autosomal recessive	1) <i>HJV</i> 2) <i>HAMP</i>	1) hemojuvelin 2) hepcidin	Roetto <i>et al.</i> 2003, Lee <i>et al.</i> 2004, Papanikolaou <i>et al.</i> 2004
type 3 hemochromatosis	autosomal recessive	<i>Tfr2</i>	Tfr2	Camashella <i>et al.</i> 2000, Roetto <i>et al.</i> 2001
type 4 hemochromatosis or ferroportin disease	autosomal dominant	<i>SLC40A1</i> (previously called <i>SLC11A3</i>)	ferroportin1 (also called MTP1 or IREG1)	Montosi <i>et al.</i> 2001, Njajou <i>et al.</i> 2001, Pietrangelo 2004
type 5 hemochromatosis	autosomal dominant	<i>H-ferritin</i>	ferritin (H-subunit)	Kato <i>et al.</i> 2001

2.2.2 *HFE* gene and its transcript

Genes related to the MHC class I family are conventionally linked with immune functions. The first evidence supporting the linkage between the HLA locus and hemochromatosis was reported in 1976 (Simon *et al.* 1976). Twenty years later, Feder *et al.* (1996) finally succeeded in identifying a novel MHC class I-like gene involved in iron homeostasis, which they called *HLA-H*. This gene was subsequently designated as *HFE* (Bodmer *et al.* 1997, Mercier *et al.* 1997). It has been localized on the short arm of chromosome 6 at 6p21.3, covering 10 kb approximately 4 Mb telomeric to *HLA-A* (Rhodes & Trowsdale 1999).

The genomic structure of the *HFE* gene resembles closely MHC class I molecules, and each of the first six exons encodes a distinct domain of the HFE protein (Britton *et al.* 2002, Fleming & Sly 2002). *HFE* mRNA is expressed at low levels in multiple cell lines and most human tissues (Feder *et al.* 1996). The major transcript of the *HFE* gene is approximately 4.2 kb in size. Additionally, both longer and shorter minor transcripts have been detected, probably due to alternative splicing events and differential use of polyadenylation signals in exon 7. The expression levels and physiological implications of the proteins translated from these minor transcripts await further investigation. (Fleming & Sly 2002.)

Regulation of the expression level of HFE seems to differ from the other MHC molecules. Although the great majority of MHC molecules are up-regulated by interferon, various cytokines do not induce HFE expression in human embryonic kidney

(HEK 293) or human cervix adenocarcinoma (HeLa) cells (Salter-Cid *et al.* 1999). The definite factors that regulate the transcription of the *HFE* gene have remained unknown. Iron has naturally been considered as a potential factor influencing HFE expression, but neither sequences conferring transcriptional regulation by metal ions nor IREs have been found in the *HFE* gene or *HFE* mRNA, respectively (Sánchez *et al.* 1998). Han *et al.* (1999) observed that an increased cellular iron pool results in increased *HFE* mRNA and HFE protein levels in cultured human intestinal (CaCo-2) cells. Contrary to these results, another research group did not observe any alterations in *HFE* mRNA levels attributable to iron chelation or replacement in these cells (Fleming & Sly 2002). Dupic *et al.* (2002) demonstrated that iron does not influence duodenal *HFE* mRNA expression in four different mouse strains.

2.2.3 *HFE* protein

The *HFE* gene encodes a 343-amino acid glycoprotein, which has similarities in sequence and three-dimensional structure to MHC class I molecules. The HFE protein contains six different domains: a 22-residue signal peptide, three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$ loop), a transmembrane region, and a short intracellular C-terminal tail. The tertiary structure of the HFE protein is stabilized by disulfide bridges, and HFE is physiologically associated with β_2 -microglobulin (β_2M) analogous to MHC I class proteins. (Feder *et al.* 1996, Lebrón *et al.* 1998.) (Figure 5.)

Immunohistochemical studies have demonstrated the localization of HFE protein in several human tissues. HFE is expressed in the epithelium throughout the alimentary tract, where it shows the most intensive staining in the cryptal enterocytes of the duodenum but is not detectable in villus cells (Parkkila *et al.* 1997a). HFE has also been found in sinusoidal lining cells, bile duct epithelial cells and Kuppfer cells of the liver, placental syncytiotrophoblasts, and capillary endothelial cells of brain (Parkkila *et al.* 1997b, Bastin *et al.* 1998). Furthermore, HFE is expressed in tissue macrophages and in circulating granulocytes and monocytes (Parkkila *et al.* 2000).

It is notable that the HFE protein has three distinctly different localization patterns (Parkkila *et al.* 2001a): (1) staining of the entire plasma membrane in nonpolarized epithelial cells (in esophagus) and leukocytes, (2) staining restricted to the basolateral membranes in most polarized epithelial cells (in stomach, large intestine, and biliary tract), and (3) a unique pattern of intracellular, perinuclear staining in some polarized cells (in crypt enterocytes of small intestine).

The finding that HFE binds to TfR1 has pointed out a regulatory role for HFE in iron metabolism. The $\alpha 1$ and $\alpha 2$ domains of the HFE protein together form a groove that resembles the peptide-binding groove in MHC class I antigen-presenting proteins. The groove is narrower in HFE than in MHC class I molecules, and HFE is thus conceivably unable to bind short peptides and participate in antigen presentation. This characteristic feature of HFE protein, however, provides a binding site for the interaction between HFE and TfR1. (Feder *et al.* 1996, Lebrón *et al.* 1998.) It has been shown that HFE is

physically associated with TfR1 not only in duodenal crypt cells but also in placental cytotrophoblasts (Parkkila *et al.* 1997b, Waheed *et al.* 1999).

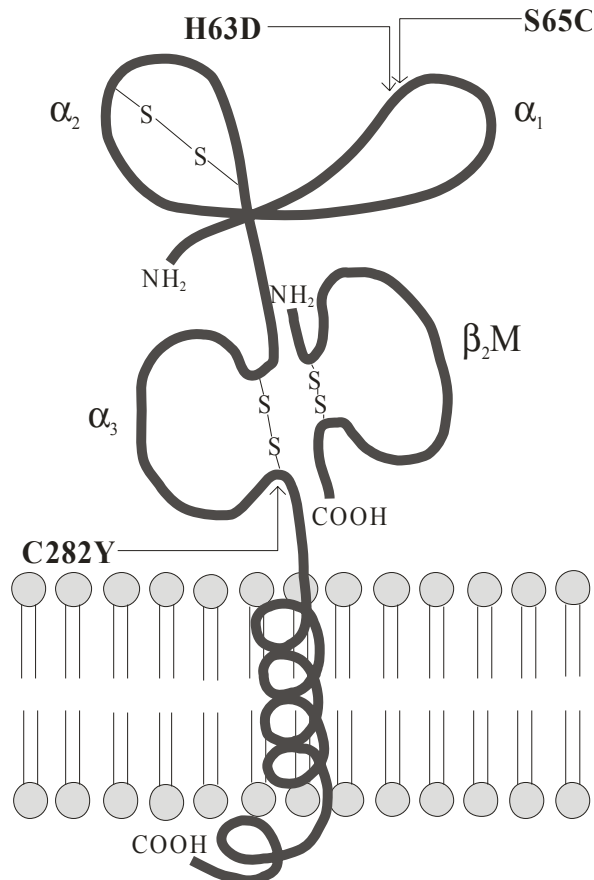


Fig. 5. Schematic model of HFE protein. HFE is physically associated with β_2 -microglobulin (β_2M). The positions of C282Y, H63D, and S65C mutations are presented. Modified from Feder *et al.* (1996).

2.2.4 Proof for causality between HFE and HH

Studies in mice with *HFE* gene disruption have offered definitive proof for a causal link between the *HFE* gene and HH (Parkkila *et al.* 2001a). The manifestations in these mice mimic the situation observed in human HH patients: 1) The mice have increased iron absorption that leads to elevated transferrin saturation and hepatic iron content. 2) They also show relative sparing of iron loading in reticuloendothelial cells and 3) do not demonstrate any immunological manifestations. These studies showed that iron

accumulation was less abundant in C282Y homozygous mice than in *HFE* knockout mice, which indicates that C282Y is not a null allele. (Zhou *et al.* 1998, Bahram *et al.* 1999, Levy *et al.* 1999.) Compound mutant mice lacking *HFE* and *TfR1* showed more severe iron loading than *HFE* knockout mice, which supported the hypothesis that the formation of the *HFE*-*TfR1*-complex is important for the regulation of iron homeostasis (Levy *et al.* 2000).

Similar iron loading as seen in HH was observed in β_2 M knockout mice, suggesting that an association between *HFE* and β_2 M is also necessary for the normal control of iron homeostasis (de Sousa *et al.* 1994, Santos *et al.* 1996). It has been demonstrated that the C282Y mutation abrogates the interaction between *HFE* and β_2 M and, consequently, leads to decreased intracellular transport, accelerated degradation and decreased presentation of C282Y mutant protein on the cell surface (Feder *et al.* 1997, Waheed *et al.* 1997). Reduced levels of *HFE* protein expression have been detected in duodenal crypt cells, Kupffer cells, gastric epithelial cells, tissue macrophages, and circulating monocytes in C282Y homozygous HH patients (Bastin *et al.* 1998, Byrnes *et al.* 2000, Parkkila *et al.* 2000). Zuccon *et al.* (2000) reported that duodenal crypt and villus enterocytes show granular staining in most C282Y homozygotes, but no such staining is seen in controls. They proposed that aggregated C282Y mutant protein may not be degraded and remains constant in villus enterocytes. Furthermore, C282Y mutation appears to block much of the interaction between *HFE* and *TfR1* in cultured cells, and interaction between *HFE* and β_2 M is probably required to enable the binding of *HFE* and *TfR1* (Feder *et al.* 1998, Salter-Cid *et al.* 2000).

While the C282Y mutant protein fails to associate with β_2 M and largely with *TfR1*, this is not the case with the H63D mutant protein. The H63D mutation does not affect the binding of *HFE* and β_2 M (Feder *et al.* 1997, Waheed *et al.* 1997). Although amino acid H63 seems to form a salt bridge with a residue in the α_2 loop of *HFE* that binds to *TfR1* (Lebrón *et al.* 1998, Fleming & Sly 2002), the H63D mutant protein appears to be able to associate with *TfR1* (Feder *et al.* 1998). Hence, the mechanism by which this mutation interrupts iron metabolism has remained unclear.

2.2.5 Pathogenesis

Iron absorption in HH is inappropriately regulated relative to the body iron stores. This regulatory defect leads to increased iron absorption and a positive iron balance (Pitrangelo 2002).

Several *in vitro* studies have found that overexpression of *HFE* protein decreases *TfR1*-mediated iron uptake (Gross *et al.* 1998, Corsi *et al.* 1999, Riedel *et al.* 1999, Roy *et al.* 1999, Salter-Cid *et al.* 1999, Arredondo *et al.* 2001, Roy *et al.* 2002). Hence, it was thought that *HFE* would also inhibit *TfR1*-mediated iron uptake *in vivo*, and that iron uptake in cells expressing mutant *HFE* protein would be increased. This hypothesis, however, is in contrast to the HH phenotype. *HFE* protein is probably not highly expressed in human hepatocytes, which are the main target of iron accumulation in HH, while *HFE* protein is highly expressed in macrophages, Kupffer cells, and duodenal crypt

cells, which are iron-deficient in HH (Parkkila *et al.* 1997a, Bastin *et al.* 1998, Parkkila *et al.* 2000, Parkkila *et al.* 2001a). This is consistent with the finding of elevated IRP activity in HH macrophages indicating a low intracellular iron concentration in these cells (Cairo *et al.* 1997). It has also been shown that macrophages from HH patients accumulate significantly less transferrin-delivered ^{55}Fe compared with controls expressing normal HFE protein. The 40-60% recovery of iron uptake in HH macrophages after transfection of wild type HFE protein further indicated that the decreased iron accumulation in HH macrophages was due to the mutant HFE protein. (Montosi *et al.* 2000.) Because binding between HFE and $\beta_2\text{M}$ is critical for normal functioning of the HFE protein (Feder *et al.* 1998), the discrepancy between these studies is likely to be explained by the fact that the HFE overexpression studies were carried out without parallel overexpression of $\beta_2\text{M}$. Waheed *et al.* (2002) recently showed in Chinese hamster ovary (CHO) cells that overexpression of HFE protein alone decreases the uptake of transferrin-bound iron, but that overexpression of both HFE and $\beta_2\text{M}$ actually increases transferrin-mediated iron uptake. Thus, it can be concluded that coexpression of HFE and $\beta_2\text{M}$ facilitates TfR1-mediated cellular iron uptake.

It has been suggested that the intracellular iron pool in duodenal crypt cells normally reflects the level of transferrin-bound iron in the circulation (Anderson 1996). These cells, however, are probably unable to sense high plasma iron levels in HH due to mutations in the *HFE* gene (Bacon *et al.* 1999a, Rolfs & Hedinger 1999, Waheed *et al.* 1999, Fleming & Sly 2002, Pietrangelo 2002, Trinder *et al.* 2002a). The normal HFE protein facilitates TfR1-mediated iron uptake from plasma in duodenal crypt cells, while the mutant HFE protein may lack this ability. Uptake of diferric transferrin and the labile iron pool of crypt cells would therefore be decreased in patients with HH. This, in turn, would ultimately lead to increased expression of DMT1 and ferroportin1 in these cells as they develop to mature enterocytes. The increased expressions of DMT1 and ferroportin1 enhance iron absorption from the intestinal lumen into enterocytes and facilitate iron transport across the basolateral cell membrane into the circulation, respectively. (Parkkila *et al.* 2001a.) The proposed model would result in increased iron absorption from the diet regardless of the plasma iron content.

Another plausible model of the pathophysiology of HH was recently introduced. This includes the assumptions that normal regulation of hepcidin synthesis in the liver is abrogated in HH patients, and that hepcidin directly interacts with mature enterocytes. According to this model, dysfunctional HFE protein fails to mediate the signal to produce hepcidin in hepatocytes, which would normally occur in response to alterations in serum transferrin saturation level. This would lead to a decreased level of hepcidin in the circulation that would, in turn, induce the expression of ferroportin 1 in mature enterocytes. Due to the increased expression of ferroportin 1, intestinal iron transport into circulation would be increased, which decreases the cellular iron pool. This subsequently increases the expression of DMT1 and Dcytb, which causes an increased level of iron absorption from the intestinal lumen. (Frazer & Anderson 2003.)

These two different hypotheses representing excessive iron absorption in HH are illustrated in the Figures 6 and 7. Both hypotheses are consistent with the findings of increased duodenal reductase activity and expression of DMT1 and ferroportin 1 in patients with HH (Zoller *et al.* 1999, Zoller *et al.* 2001, Rolfs *et al.* 2002, Bridle *et al.* 2003, Zoller *et al.* 2003). The findings that DMT1 mRNA is predominantly expressed

toward crypts and ferroportin1 mRNA predominantly at the tip of the villus are in favor of the first model (Gunshin *et al.* 1997, Abboud & Haile 2000), as is the observation of decreased duodenal iron uptake from plasma transferrin in *HFE* knockout mice (Trinder *et al.* 2002b). On the other hand, this model is not in line with the observation that enterocyte iron levels do not seem to regulate ferroportin1 expression in rats (Frazer *et al.* 2003). Heparin has recently been associated with the pathogenesis of HH. Heparin is considered to act as a negative regulator of iron absorption, and its expression should hence be normally increased in the presence of an iron overload (Frazer & Anderson 2003). Heparin expression, however, is not induced in HH patients or mouse models of HH, both of which show decreased levels of heparin (Ahmad *et al.* 2002, Bridle *et al.* 2003, Gehrke *et al.* 2003, Muckenthaler *et al.* 2003). It has also been observed that constitutive expression of heparin prevents iron loading in *HFE* knockout mice (Nicolas *et al.* 2003), and mutations in the gene for heparin (*HAMP*) appear to modify the phenotype of HH (Merryweather-Clarke *et al.* 2003, Jacolot *et al.* 2004, Nicolas *et al.* 2004). Although heparin could be theoretically involved in the programming of enterocytes also in the first model, this model does not provide a transparent role for heparin. Based on this model, it is difficult to explain the effect of constitutive heparin expression on iron absorption in *HFE* knockout mice. The mechanism by which heparin expression is depressed in the liver in HH has not been explained to date, but a speculative mechanism has been proposed in the second model. High expression levels of *HFE* mRNA and protein have been found in rat hepatocytes in agreement with the presented model (Frazer & Anderson 2003), but it has been shown that *HFE* may not be highly expressed in human hepatocytes (Parkkila *et al.* 1997a). Knowledge of the pathogenesis of HH has considerably increased in the past few years, but the ultimate mechanisms still need to be clarified (Brissot *et al.* 2004). Further studies may also reveal additional genetic factors that could be involved in the pathogenesis of HH (Levy *et al.* 2000).

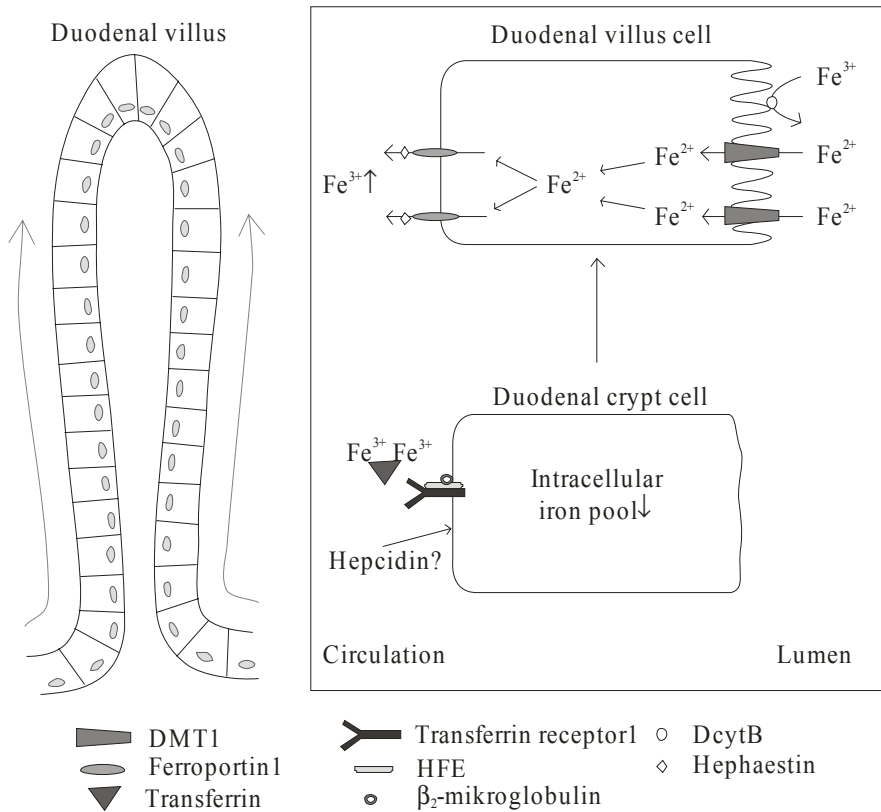


Fig. 6. A widely supported model of the pathogenic mechanisms of HH. Dysfunctional HFE protein results in decreased TfR1-mediated iron uptake from the circulation in duodenal crypt cells. This leads to a decreased intracellular iron pool in cryptal enterocytes regardless of serum transferrin saturation and, subsequently, to an increased “set point” of dietary iron absorption in mature enterocytes. A decreased intracellular iron pool is thought to induce the synthesis of DMT1 in mature enterocytes, which increases the transport of iron from the intestinal lumen into the cell. The increase in the intracellular iron pool in mature enterocytes may then increase the synthesis of ferroportin1, which facilitates iron transport across the basolateral plasma membrane. (Parkkila *et al.* 2001a.) The role of hepcidin in this model is unclear.

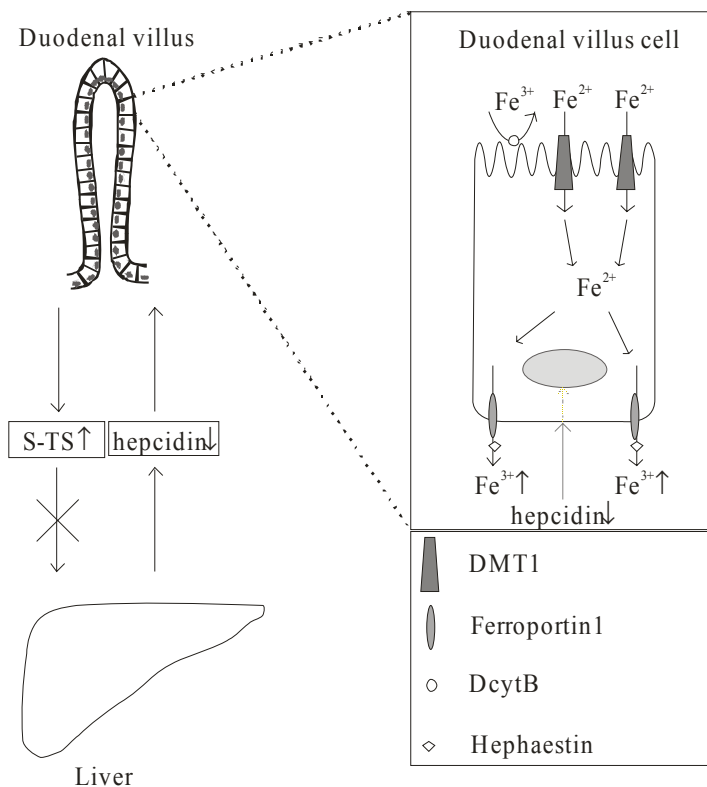


Fig. 7. A recently described model of the pathogenic mechanisms of HH (Frazer & Anderson 2003). Regardless of serum transferrin saturation, the synthesis of hepcidin is decreased in hepatocytes due to dysfunctional HFE protein. This leads to a lowered serum hepcidin level and, subsequently, to an increased “set point” of dietary iron absorption in mature enterocytes. Hepcidin may interact with mature enterocytes, whereby a decreased hepcidin level increases the expression of ferroportin1 and the iron efflux from the mature enterocyte to the bloodstream. This decreases the intracellular iron pool of mature enterocytes, which in turn, increases the expression of DMT1, leading to increased iron absorption from the intestinal lumen.

2.2.6 HFE mutations

The high frequency of C282Y mutation may be due to the potential selective advantages of this mutation. Both heterozygosity and homozygosity for C282Y mutation lead to increased intestinal iron absorption (de Valk *et al.* 2000a, Rossi *et al.* 2000a, Raddatz *et al.* 2003, Sánchez *et al.* 2003), and the C282Y mutation may thus have prevented iron deficiency in populations with limited nutritional iron availability. This would have been

and may still be beneficial, especially for premenopausal women, who lose blood through menstruations and pregnancies (Datz *et al.* 1998, Beutler *et al.* 2000).

It was initially postulated that genetic hemochromatosis originated among Celts (Simon *et al.* 1980). After the description of the C282Y mutation, the highest prevalences of this major hemochromatosis mutation have been reported in populations of Northern Europe. Although some studies support the Celtic origin of the C282Y mutation, the recent data is in favor of the "Vikings theory". This theory hypothesizes that the C282Y mutation originated among the German Iron Age population in Southern Scandinavia and spread with the Vikings. The "Vikings theory" seems to be more consistent with the estimations of the age of the C282Y mutation (60-70 generations) and with the distribution of this mutation in the European populations. (Lucotte 1998, Lucotte 2001, Milman & Pedersen 2003.)

While the C282Y mutation has emerged relatively recently, the H63D mutation is considered to be older. The highest frequencies of H63D mutation have been found in the Mediterranean area, and it has been postulated that this mutation originated in that region. It is also possible that the H63D mutation has appeared more than once, occurring separately in the Mediterranean and Asia. (Rochette *et al.* 1999.)

The C282Y and H63D mutation frequencies are high among Caucasians. The H63D mutation is also prevalent in other racial/ethnic groups, but the frequency of C282Y mutation is lower in other ancestries (Waalén *et al.* 2002a). The assessed carrier frequency of the C282Y mutation in Europe and North America is on average 9.2% and 9.0%, while that of the H63D mutation 22% and 23%, respectively (Hanson *et al.* 2001). Parkkila *et al.* (2001b) have reported carrier frequencies of 10.2% for the C282Y and 20.3% for the H63D mutation in the normal Finnish population. The prevalence estimated for C282Y homozygotes is 0.4% in the European and 0.5% in the North American population, and the corresponding frequencies for C282Y/H63D compound heterozygotes are 2% and 2.5% (Hanson *et al.* 2001). The recently observed carrier frequencies for the S65C mutation in Caucasian populations appear to range between 1% and 4% (Barton *et al.* 1999, Mura *et al.* 1999, Beutler *et al.* 2000, Holmström *et al.* 2002, Wallace *et al.* 2002, Salvioni *et al.* 2003).

Most HH patients are found to be homozygous for the C282Y mutation, and a few are compound heterozygous for C282Y and H63D mutations (Feder *et al.* 1996). H63D homozygosity slightly increases iron loading into the body, leading to a mild iron overload, which is not considered to be clinically significant, however (Gochee *et al.* 2002). The observed frequencies of C282Y homozygous subjects among patients with hereditary iron overload disorder range from 52 to 100 percent (Hanson *et al.* 2001). Compound heterozygosity for the C282Y and H63D mutations accounts for approximately 5% and H63D homozygosity for 1.5% of the cases with hereditary iron overload disorder. In addition, there is an average of 3.6% C282Y heterozygotes and 5.2% H63D heterozygotes among these patients, while 6.9% do not carry these mutations. (Hanson *et al.* 2001.) The S65C mutation is also associated with a slight increase of body iron stores. Mura *et al.* (1999) detected the S65C mutation in 7.8% of patients with primary iron overload without C282Y or H63D mutations, and compound heterozygosity for C282Y and S65C has been found to lead to HH in some individuals (Asberg *et al.* 2002, Wallace *et al.* 2002). Nevertheless, Holmström *et al.* (2002) did not observe any clinical manifestations of HH in subjects with S65C mutation.

Several other mutations of the *HFE* gene have also been discovered in patients with iron overload (e.g. G93R, I105T (Barton *et al.* 1999), Q127H (de Villiers *et al.* 1999), R74X (Beutler *et al.* 2002a), E168X, W169X (Piperno *et al.* 2000, Mariani *et al.* 2003, Salvioni *et al.* 2003), IVS3 + 1G/T (Wallace *et al.* 1999), IVS5 + 1G/A (Steiner *et al.* 2002), P160ΔC, and V68ΔT (Pointon *et al.* 2000)), but the frequency of these mutations in the general population and HH probands seems to be very low.

The penetrance of *HFE* gene mutations is currently a topic for intensive debate. Penetrance depends on the limits and definitions that are selected. It can be defined as biochemical penetrance (the proportion of subjects who will develop biochemical signs of the disease out of all individuals with the mutations) or as clinical penetrance (the proportion of subjects who will develop clinical manifestations of the disease out of all mutant individuals). A number of reports have shown that biochemical penetrance is high (80%-100%) in C282Y homozygotes (Burt *et al.* 1998, Olynyk *et al.* 1999, Deugnier *et al.* 2002, Phatak *et al.* 2002, Ryan *et al.* 2002). The clinical penetrance of this genotype, nevertheless, has remained a matter of controversy. C282Y homozygosity has naturally been demonstrated also to possess the highest clinical penetrance within the various *HFE* categories. It was previously thought that most C282Y homozygotes will eventually develop clinical manifestations of hemochromatosis (Edwards *et al.* 1998), but it has since been realized that the penetrance of C282Y homozygosity is unlikely to exceed 50% (Burke *et al.* 2000). Beutler *et al.* (2002b) even suggested that only less than 1% of C282Y homozygotes develop frank clinical hemochromatosis. This study has been criticized in many other reports, pointing out that the penetrance of C282Y homozygosity is likely to be higher (Allen *et al.* 2002, Cox *et al.* 2002, Poullis *et al.* 2002). The exact clinical penetrance of this genotype is, however, impossible to assess based on current knowledge. The biochemical and clinical penetrances of the other prevalent *HFE* genotypes associated with HH are substantially lower than that of C282Y homozygosity. It has been estimated that the risk of C282Y and H63D compound heterozygotes for iron loading is nearly 200 times lower than that of C282Y homozygotes (Risch 1997). Furthermore, it has been concluded that H63D homozygotes do not develop clinically significant iron overload (Gochee *et al.* 2002). Multiple environmental and so far unknown genetic factors may contribute to iron loading in HH and modulate the penetrance of *HFE* mutations (Levy *et al.* 2000).

2.2.7 Clinical features

The symptoms of HH depend directly on the amount and duration of iron excess in the body. Some individuals with a genetic predisposition are asymptomatic, whereas others may have severe and life-threatening manifestations (Niederau *et al.* 1996). Early signs and mild symptoms of the disease are common and nonspecific, but many classical clinical features may appear in the course of time. The clinical features of HH are presented in the tables 2 and 3. The most common symptoms are weakness, fatigue, and arthralgia. Other frequent symptoms include abdominal pain, loss of libido, and impotence. Clinical examinations of patients with HH may reveal such conditions as

arthritis, hepatomegaly, cirrhosis of the liver, hepatocellular carcinoma, diabetes mellitus, skin pigmentation, cardiac failure, and/or arrhythmia. (Adams *et al.* 2000a, Brandhagen *et al.* 2002, Powell 2002.)

Joint involvement may often occur in the early stage of the disease. The most characteristic feature is arthralgia/arthritis of the second and third metacarpophalangeal joints, but basically any joint can be affected, and the findings are very similar to that seen in osteoarthritis. The direct effect of iron is the most plausible cause of arthropathy in HH, even though arthropathy, once initiated, tends to progress even after the excess iron has been removed. (Adams *et al.* 2000a, Ines *et al.* 2001, von Kempis 2001, Powell 2002.)

The excessive iron present in the body accumulates first in the parenchymal cells of the liver, which may induce elevation of the liver enzymes and eventually lead to fibrosis and cirrhosis of the liver in many untreated patients (Bacon & Britton 1989, Andrews 2000). Furthermore, hepatic cirrhosis may lead to carcinoma of the liver in up to 30% of these patients (Witte *et al.* 1996). Iron accumulation may also occur in cardiac myocytes and pancreatic acinar cells, causing cardiac and pancreatic involvement (Andrews 2000). Hepatic cirrhosis, diabetes mellitus, and heart failure are usually present only in the fully established form of the disease. However, it is noteworthy that 5% of the patients with cirrhosis of the liver are asymptomatic at the time of diagnosis (Adams *et al.* 2000a).

Iron deposition in the hypothalamic-pituitary axis leads to insufficiency of pituitary gonadotrophic secretion. This is the main cause of hypogonadism and hypogonadism-related symptoms, such as impotence, loss of libido, sparse body hair, gynecomastia, and amenorrhea, though primary testicular impairment may also be involved in some cases. Adrenal gland insufficiency, hypothyroidism, and hypoparathyroidism may also result from the impairment of hypothalamic-pituitary function or direct glandular involvement. (Hash 2001, Powell 2002.)

Several intrinsic and extrinsic factors influence HH morbidity. The clinical picture is progressive, and the disease manifestations typically occur only after 40 years of age, rarely before the age of 20. Disease expression is often seen earlier in men than in women, and it is estimated that the manifestations are at least 5 times more frequent in males than in females. This gender difference in disease presentation is due to the blood loss of women through menstruations and pregnancies. The progression of the disease is related to the degree of dietary iron intake and possibly to previous blood donations. Furthermore, alcohol abuse and hepatitis viruses B and C are considered to accelerate the clinical expression of the disease, and additional genetic factors may be involved in the pathogenesis of HH, thus modulating the penetrance of the *HFE* mutations (Merryweather-Clarke *et al.* 2003, Jacolot *et al.* 2004). (Adams *et al.* 2000a, Powell 2002.)

Clinical manifestations of HH are usually reversible and preventable with early diagnosis and proper treatment, but when the diagnosis is delayed, irreversible conditions, such as cirrhosis of the liver, hepatocellular carcinoma, diabetes mellitus, and cardiomyopathy, may occur (Adams *et al.* 2000a).

Table 2. Symptoms in patients with hereditary hemochromatosis (Powell 2002).

Symptoms	
General symptoms	
Weakness	Apathy
Fatigue	Weight loss
Lethargy	
Organ-specific symptoms	
Joints: arthralgia	Heart: arrhythmias
Liver: abdominal pain	Endocrine: diabetes, loss of libido, impotence, amenorrhea, gynecomastia, loss of body hair

Table 3. Findings in patients with hereditary hemochromatosis (Powell 2002).

Findings	
Clinical findings	
Joints: arthritis, joint swelling	Heart: dilated cardiomyopathy, congestive heart failure
Liver: hepatomegaly	Spleen: splenomegaly
Skin: hyperpigmentation	Endocrine: diabetes, testicular atrophy, hypogonadism, hypothyroidism
Laboratory findings	
Blood sample: increased iron parameters (s-Fe, s-TS and/or s-ferritin), increased liver transaminases (s-ALT and/or s-AST)	Liver biopsy: increased stainable iron, HIC and/or HII, fibrosis, cirrhosis, HCC

HCC, hepatocellular carcinoma; HIC, hepatic iron concentration; HII, hepatic iron index; s-ALT, serum alanine aminotransferase; s-AST, serum aspartate aminotransferase; s-Fe, serum iron concentration; s-ferritin, serum ferritin concentration; s-TS, serum transferrin saturation.

2.2.8 Diagnosis

Diagnosis in the early stages of HH is difficult due to the insidious onset combined with common and nonspecific clinical features. After the symptoms and signs have evoked suspicion, however, the diagnostics is relatively simple. It is based on screening for values of serum iron parameters by rapidly and widely available inexpensive laboratory tests detecting iron overload (Brandhagen *et al.* 2002, McCullen *et al.* 2002, Powell 2002). Liver biopsy is usually no longer necessary for the diagnosis of HH, and the definitive diagnosis can be confirmed by *HFE* genotyping (Adams *et al.* 2000a, Brissot *et al.* 2000).

The body iron overload can be detected and evaluated with measurements of serum transferrin saturation (s-TS), iron (s-Fe), and ferritin (s-ferritin) concentrations. Screening based on serum iron concentration alone may fail to detect the majority of affected

subjects. Serum iron concentration also varies throughout the day and may be considerably affected by the ingested food or some diseases. (McCullen *et al.* 2002.) Not only the sensitivity but also the specificity of the detection of HH is well enhanced by dividing the serum iron concentration with the total iron-binding capacity (TIBC). Elevated serum transferrin saturation is usually the earliest sign of HH (Hash 2001), and it is commonly considered the most reliable classical laboratory parameter in the diagnostics of HH. Serum ferritin concentration is generally a good indicator of body iron stores. Although serum ferritin is rated as a highly sensitive measure of iron overload, it is usually elevated later than serum transferrin saturation, and an abnormal value does not necessarily indicate the presence of iron overload. Serum ferritin is an acute phase protein, and its concentration is elevated in various infections and inflammations. Elevated levels can also be seen in patients with alcoholic liver disease or cancer. (Hash 2001, Brandhagen *et al.* 2002.) Even so, a normal concentration of serum ferritin basically excludes clinically significant iron overload in the body (Adams *et al.* 2000a, McCullen *et al.* 2002). At the moment, it can be recommended that subjects with transferrin saturation greater than 45% should be referred for further investigations (Bacon *et al.* 1999b, Adams *et al.* 2000a, Hash *et al.* 2001, Powell 2002). Unsaturated iron-binding capacity (UIBC) has also been introduced as a potential instrument for the detection of HH. UIBC decreases early in the course of disease progression, and it seems to be an even more cost-effective method than the serum transferrin saturation test. However, the measurement of UIBC is not as widely available as serum transferrin saturation. (Adams *et al.* 2000b, Hickman *et al.* 2000, McCullen *et al.* 2002, Murtagh *et al.* 2002.)

HFE genotyping may confirm the diagnosis in patients with iron overload, and it has recently become a popular diagnostic tool in many countries. It is a DNA-based test that only requires a single blood sample to determine the *HFE* genotype, being thus a safe and convenient method for the patient. The mutation analysis usually includes at least the determination of C282Y and H63D mutations. One important advantage of this method is that the outcome remains the same for the rest of the individual's life regardless of the treatment given. It has been widely agreed that the diagnosis of HH cannot be based exclusively on elevated iron parameters or the results of *HFE* genotyping. Instead, the diagnostic criteria now include both abnormal biochemical parameters and mutant genotypes. In general terms, the diagnosis can be ascertained if serum transferrin saturation >45% is associated with homozygosity for C282Y mutation or compound heterozygosity for C282Y and H63D mutations (Brissot *et al.* 2000, Powell 2002).

Liver biopsy has previously been the gold standard in the diagnostics of HH. The quantity of iron in the liver can be evaluated with the hepatic iron concentration (HIC). This quantitative determination reflects the degree of iron overload in the body. Hepatic iron index (HII) is derived from HIC and the individual's age (HIC/age), and it is useful for distinguishing HH from other iron overload conditions. HII is usually >1.9 in patients with HH. (Witte *et al.* 1996, Bacon *et al.* 1999b, Adams *et al.* 2000a.) Typical histological findings in HH include periportal iron deposition in hepatocytes with a decreasing gradient towards the centrilobular zone. Iron deposition into Kupffer cells is relatively sparse in HH, contrary to secondary iron overload disorders. (Brunt *et al.* 2000.) Liver biopsy is an invasive maneuver associated with increased morbidity and mortality, and the utilization of serum iron parameters combined with the gene test has

replaced liver biopsy in most cases in the diagnostics of HH. (Witte *et al.* 1996, Adams *et al.* 2000a.) Serum iron parameters and *HFE* genotyping cannot, however, provide reliable and accurate information on the liver status, and excess hepatic iron can be evaluated with magnetic resonance imaging (MRI) or dual-energy computed tomography only if these special and costly devices are available (Chapman *et al.* 1980, Gandon *et al.* 1994). Thus, a liver biopsy is still needed for the determination of the degree of hepatic iron and for the evaluation of the severity of liver injury, which can provide valuable prognostic information (Adams *et al.* 2000a). The generally accepted indications for liver biopsy are age >40 years, serum ferritin >1000 µg/l, or abnormal level of liver enzymes (Powell 2002). Liver biopsy has also remained as a diagnostic tool for iron-loaded patients without the typical genetic profile (Adams 2000).

2.2.9 Family and population screening

Genetic screening of C282Y and H63D mutations is highly recommended for the first-degree relatives of HH patients. Body iron status should be further determined if the family member of a proband turns out to be positive for the causative mutations of the *HFE* gene. (Powell 2002.) This strategy enables early diagnosis and treatment of several affected individuals.

There is no general agreement as to whether population screening for HH is justified. Population-based screening programmes could be justified by the following facts: i) The prevalence of the causative mutations for iron overload in the *HFE* gene is high in Caucasian populations. ii) The disease may lead to severe clinical manifestations and reduced life expectancy if untreated. iii) There is a recognized latent or early symptomatic phase of the disease, and suitable non-invasive tests are available for early diagnosis. iv) Safe, effective, and inexpensive treatment (phlebotomies) is widely available. v) Early diagnosis and proper treatment prevent morbidity and improve survival. (Tavill 1999, Brissot *et al.* 2000, Brissot 2001, Adams 2002.) On the other hand, the essential questions have remained unresolved. One overwhelming concern has been the penetrance of HH, because population screening would become less cost-effective as penetrance decreases (Adams *et al.* 2000a, Bomford 2002). Other major uncertainties have been linked with possible genetic discrimination and the psychosocial effects on especially asymptomatic subjects with a positive test result. There is a chance for discrimination in employment and insurance, but this can be avoided by amended legislation that disallows genetic discrimination (Adams 2002). Although insurance companies should recognize that they would, in fact, benefit from early detection and treatment of HH (McCullen *et al.* 2002), HH patients without severe disease manifestations have reported difficulties in the acquisition of insurance in the U.S.A. (McDonnel *et al.* 1999, Shaheen *et al.* 2003a). However, the overall proportions of those with active health insurance and those employed among HH patients without end organ damage seem to be normal (Shaheen *et al.* 2003a). It is conceivable that positive test results may have some negative psychosocial effects, but no such effects have been reported, and the quality of life of HH patients without end organ damage appears to be

similar to that of their unaffected siblings (Power and Adams 2001, Shaheen *et al.* 2003a). Additional studies in this area still need to be done.

It has been well established that the use of *HFE* genotyping as an initial test for population screening may not be prudent, although genetic testing has an advantage over phenotypic screening to detect individuals at risk of developing iron overload. Genetic screening is not considered to be cost-effective, and unlike phenotypic screening, genetic screening would not detect iron-loaded patients without typical *HFE* mutations and individuals with iron deficiency. (Adams & Valberg 1999, Adams 2002.) Therefore, the optimal algorithm for population screening would probably include phenotypic screening with s-TS or UIBC followed by genetic testing (Adams *et al.* 2000a). These two phenotypic screening tests are estimated to have equal reliability in screening for HH (Murtagh *et al.* 2002), but UIBC seems to be even more cost-effective and useful in simultaneous detection of iron deficiency (Hickman *et al.* 2000).

Several arguments support population screening for HH, but there is insufficient evidence to prove that the benefits of population screening would outweigh the potential disadvantages. Indeed, further information is required to show whether population-based screening for HH is advisable, and whether the screening programmes should focus specifically on limited groups (e.g. subject with liver disease, diabetes, or endocrinopathies). The results of ongoing screening studies, appropriate medical education, and rationalization of the diagnostics of HH in general practice may prove to constitute an appropriate and maximally effective approach. (Adams *et al.* 2000a, Bomford 2002, Pietrangelo 2003.)

2.2.10 Treatment

Recurrent therapeutic phlebotomies are the most effective treatment of HH. The treatment schedule has to be designed separately for each patient, and it should usually be based on the serum ferritin level and also initiated for asymptomatic patients with iron overload. The currently recommended thresholds for phlebotomies are serum ferritin levels of ≥ 300 $\mu\text{g/l}$ in men and serum ferritin levels of ≥ 200 $\mu\text{g/l}$ in women. (Barton *et al.* 1998.) For the majority of patients, induction therapy includes 400-500 ml (contains 200-250 mg of iron) phlebotomies on a weekly basis until the excess iron has been removed from the body (until serum ferritin level < 50 $\mu\text{g/l}$ and transferrin saturation $< 30\%$). The removal of blood may induce anemia, and hemoglobin concentration should hence be controlled during phlebotomy therapy. Other health problems may also set limits for phlebotomies and the volume of blood removed in each time. (Witte *et al.* 1996, Adams *et al.* 2000a, Brissot *et al.* 2000.)

With the exception of a few elderly patients, maintenance therapy is needed to prevent the recurrence of iron overload after the additional iron has once been removed. This usually requires 2-4 phlebotomies per year. (Witte *et al.* 1996.) Nowadays, it is accepted that the blood obtained by phlebotomies from HH patients can be utilized for transfusions (Adams *et al.* 2000a), providing that the serum iron parameters and hemoglobin are

within the reference range, and that the subjects have no organic manifestations of the disease.

If phlebotomies are contraindicated, iron chelation therapy with deferoxamine is recommended. This therapy is rarely needed, but must be used for patients suffering from, for instance, severe anemia, hypoproteinemia, or heart manifestations (Adams *et al.* 2000a, Brissot *et al.* 2000, Hash 2001).

A low-iron diet is unlikely to have any substantial therapeutic effect (Witte *et al.* 1996, Brissot *et al.* 2000). Patients with HH should not be recommended to avoid iron-rich food, although some think that refraining from red meat and liver could be beneficial (Witte *et al.* 1996, Barton *et al.* 1998, Adams *et al.* 2000a, Brissot *et al.* 2000). It is known that vitamin C facilitates and tea reduces iron absorption, but adjustments for neither the dietary vitamin C intake nor the consumption of tea need to be made (Witte *et al.* 1996, Barton *et al.* 1998, Hash 2001, Temme & Van Hoydonck 2002). Patients with HH should, however, abstain strictly from iron-containing supplements (Witte *et al.* 1996, Adams *et al.* 2000a, Hash 2001) and restrict their use of vitamin C supplements within reasonable limits (≤ 500 mg/d) if not entirely (Barton *et al.* 1998, Herbert 1999). It is also advisable to avoid mineral supplements containing cobalt, manganese, zinc, or lead because of the increased absorption of these metals in HH (Barton *et al.* 1998). Alcohol may enhance the hepatotoxic effect of iron, and consumption of alcohol should therefore be minimized (Adams & Agnew 1996, Fletcher *et al.* 2002). Furthermore, iron overload predisposes to *Vibrio vulnificus* infection, and this highly invasive marine bacterium can be transmitted via uncooked seafood, which should be avoided (Wright *et al.* 1981, Linkous & Oliver 1999, Gerhard *et al.* 2001).

Long-term survival of HH patients with iron overload is normal if phlebotomies are initiated in asymptomatic or symptomatic patients without cirrhosis of the liver or diabetes mellitus (Niederau *et al.* 1985, Niederau *et al.* 1996, Barton *et al.* 1998, McCullen *et al.* 2002). Also, life expectancy is better in patients already suffering from severe clinical manifestations and treated with phlebotomies than in untreated patients (Witte *et al.* 1996).

3 Aims of the research

This research was mainly focused on *HFE* genotyping. The specific aims were:

- To assess the utilization of *HFE* genotyping in the diagnostics of HH in the service of public health care.
- To help to outline appropriate criteria for referring patients for *HFE* genotyping.
- To investigate the association between *HFE* mutations and various hematological disorders.
- To investigate the association between *HFE* mutations and IDCM, and to examine the modifying impact of these mutations on IDCM.

4 Materials and methods

4.1 Subjects (I, III, IV)

In study I, doctors at health clinics or general hospitals in various parts of Finland consecutively referred 137 subjects for *HFE* genotyping over the period 1999-2001. The study population consisted of 85 (62.0%) men (mean age, 49.0 years; 95% confidence interval (95% CI), 46.1 to 51.9) and 52 (38.0%) women (mean age, 54.2; 95% CI, 50.0 to 58.5). Serum iron, ferritin, and transferrin saturation were measured at the health clinics or hospitals before *HFE* genotyping (n=30) or assayed in the laboratory of Oulu University Hospital at the same time as the genetic testing (n=86). 5 patients in the control group, 6 in the CY/CY group, and 1 in the CY/HD group had been treated with venesections before the laboratory results had been obtained. Previous medical records were available for 120 subjects.

The samples for study III were collected from 232 patients (106 men, 126 women) with various hematological disorders during their routine diagnostic and treatment evaluations at Oulu University Hospital between 1987 and 2000. These patients were diagnosed as having acute lymphoblastic leukemia (ALL, n=43), acute myeloid leukemia (AML, n=53), chronic myeloid leukemia (CML, n=16), essential thrombocythemia (ET, n=37), myelodysplastic syndromes (MDS, n=35), multiple myeloma (MM, n=33), or polycythemia vera (PV, n=15). ALL and AML were classified on the basis of their lymphoid or myeloid morphological appearance, respectively, according to the French-American-British (FAB) criteria (Bennet *et al.* 1976, Bennet *et al.* 1985). In addition, the diagnoses of acute leukemia were based on the immunophenotype as assessed by flow cytometry using standard lineage immunophenotype markers. The diagnosis of MDS was also based on the FAB criteria (Bennet *et al.* 1982), while that of PV was based on the criteria of the PV Study Group (Wasserman 1971), and that of CML on the presence of the Philadelphia chromosome translocation (9;22) (Rowley 1973). The diagnosis of ET was based on the criteria described by Hoffman (2000), and those of MM on the criteria described by Kyle (1992). The investigation was approved by the Ethics Committee of Oulu University Hospital.

Paper IV covers 91 consecutively recruited and evaluated patients with idiopathic dilated cardiomyopathy (IDCM) from Eastern and Southern Finland (mean age 46.2, standard deviation (SD) 12.4, 64 male and 27 female). IDCM diagnosis was based on the commonly accepted diagnostic criteria for DCM (left ventricular ejection fraction (LVEF) < 45% and left ventricular end-diastolic diameter (LVEDD) > 27 mm/m²), and the secondary causes of DCM were excluded (Manolio *et al.* 1992). The patients were evaluated in terms of a personal and family history, physical examination, 12-lead electrocardiography, chest x-ray, and transthoracic echocardiography (M-mode, two-dimensional and Doppler). Diagnostic coronary angiography was performed for 88% of the patients. The mean follow-up period of IDCM patients from the diagnosis was 6.0 years (SD 4.7). The control group in this study consisted of 102 healthy subjects. The investigation conforms with the principles outlined in the Declaration of Helsinki.

4.2 HFE genotyping (I, III, IV)

In the papers I, III, and IV, the C282Y and H63D mutations were determined for each patient. In addition to these mutations, the S65C mutation was determined for patients and controls in paper IV. DNA was extracted either from peripheral blood (I, III, IV) or from bone marrow (III) using a blood kit (Nucleospin, Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. Sets of primers described by Feder *et al.* (1996) and Jeffrey *et al.* (1999) (I, IV) or by Smillie (1997 & 1998) (III) were used to amplify parts of the *HFE* gene by polymerase chain reaction (PCR). The control primers designed by Steffensen *et al.* (1998) ensured that the PCR was working properly (III). The amplification products were digested with the restriction enzymes SnaB I (New England BioLabs, Beverly, MA, USA) and Bcl I (New England BioLabs) in the papers I and IV and Hinf I (New England BioLabs) in paper IV. The interpretation of the data was based on the facts that the C282Y mutation creates a restriction site for SnaB I, while the H63D and S65C mutations abolish the restriction sites for Bcl I and Hinf I, respectively (I, IV). The PCR products were resolved on a 1.5% (I, III) or 1.2% (IV) agarose gel containing ethidium bromide (I,III) or nucleic acid gel stain (IV) (GelStar, BioWhittaker Molecular applications, Rockland, ME, USA) and visualized by UV light illumination.

4.3 Antibodies (II)

The polyclonal rabbit antiserum against HFE and TfR2 has been previously produced and characterized by Parkkila *et al.* (1997a). The monoclonal antibodies against human TfR1 and CD42b were purchased from Zymed Laboratories (San Francisco, CA, USA) and Novocastra Laboratories (Newcastle, UK), respectively. The production of polyclonal antibody against human carbonic anhydrase I has been characterized earlier by Parkkila *et al.* (1993).

4.4 Immunocytochemistry (II)

In paper II, human platelet concentrates obtained from the Finnish Red Cross include approximately 69×10^9 platelets per unit. The maximum numbers of contaminant cells are: white cells 0.01×10^9 (0.00001%) and erythrocytes 0.11×10^9 (0.15942%). Platelets were further enriched by centrifugation. After fixation and permeabilisation by a fixation and permeabilisation kit (Caltag Laboratories, Der Grub Bio Research GmpH, Hamburg, Austria), the platelets were spread onto microscope slides.

Immunofluorescence staining was performed by employing the following protocol:

1. Pre-treatment of platelets with 0.1% bovine serum albumin (BSA) and 0.05% saponin in phosphate-buffered saline (PBS) (BSA-PBS-saponin) for 30 min.
2. Incubation for 1 h with the primary anti-HFE, anti-TfR1, or anti-TfR2 antibody (10 μ g IgG per microscope slide) in BSA-PBS-saponin.
3. Washing three times for 5 min with BSA-PBS-saponin.
4. Incubation for 1 h with fluorescein isothiocyanate (FITC)-conjugated swine F(ab')₂ anti-rabbit IgG (Dakopatts, Glostrup, Denmark), goat F(ab')₂ anti-mouse IgG (Dakopatts) diluted 1:30, Alexa Fluor 488-coupled goat anti-rabbit F(ab') IgG-diluted 1:200 (Molecular Probes Europe, Leiden, the Netherlands), or Alexa Fluor 568-coupled goat anti-mouse F(ab') IgG diluted 1:200 (Molecular Probes Europe) in BSA-PBS-saponin.
5. Washing twice with BSA-PBS-saponin and once with PBS for 5 min.
6. Covering the samples with mounting medium (Inova Diagnostics, San Diego, USA) and cover slips.

All the steps were carried out at room temperature. The immunostained platelets were examined under a confocal laser-scanning microscope (Zeiss, Göttingen, Germany).

4.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (II)

For paper II, platelet concentrates treated with lysis buffer (Amersham Pharmacia Biotech, Buckinghamshire, UK) were subjected to SDS-PAGE under reducing conditions according to the method of Laemmli (1970). Protein standards for SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond, CA, USA). The electrophoreses were performed in a Novex Xcell SureLock electrophoresis unit (Invitrogen Corp/ Novex, Carlsbad, CA, USA) using Novex NuPAGE 10% Bis-Tris gels. The proteins were transferred electrophoretically from the gel on to a nylon membrane (Millipore, Bedford, MA, USA) in a Novex Blot Module. These membranes were first incubated for 30 min with TBST buffer (10mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% Tween-20) containing 10% cow colostrum whey followed by the first antibody diluted either 1:2000 (anti-TfR2), 2 μ g IgG/ml (anti-HFE), or 1 μ g IgG/ml (anti-TfR1) in TBST buffer for 1 h. The membranes were washed four times for 5 min with TBST buffer and then incubated with

alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (Bio-Rad Laboratories) diluted 1:3000 in TBST buffer for 30 min. After washing three times for 5 min in TBST buffer, the polypeptides were visualized by a chemiluminescence substrate (Bio-Rad Laboratories). All the incubations and washings were carried out at room temperature.

4.6 Statistical analyses (I, III, IV)

The data were analyzed using SPSS for Windows software (version 10.1 (I, III) or 11.5 (IV)). Measurements for continuous variables are presented in the form of means (I, IV), SD (I, IV), 95% confidence interval (95% CI) (I, IV), medians (I), and quartiles (I). Statistical differences between the prevalences of *HFE* genotypes in the patients and the controls were analyzed using Fisher's exact probability test (III, IV) and χ^2 -test (IV).

In paper I, the positive trend in the rate of detection of the most typical mutations responsible for HH over the years 1999, 2000, and 2001 was analyzed using the Chi-squared test for trends. Mann-Whitney U-test was used to evaluate the statistical significances of differences in s-TS, s-Fe, and s-ferritin and Student's *t*-test and χ^2 -test to assess the statistical significances of the differences in age and gender between the various categories of *HFE* genotypes, respectively.

In paper IV, the associations of age, gender, and medication with LVEDD and LVEF were assessed using correlation coefficients and Student's two-sample *t* test. The differences in medication between the various categories of *HFE* genotypes were analyzed with χ^2 -test and Fisher's exact probability test. Based on these analyses, the impacts of various *HFE* genotypes and confounding factors on LVEDD and LVEF were evaluated with a two-way analysis of variance. Multinomial logistic regression analysis (Agresti 1990) was performed to evaluate the effect of various *HFE* genotypes on the New York Heart Association (NYHA) classes and analysis of variance with repeated measurements on the alterations in LVEDD and LVEF within the follow-up period. The effect of the duration of follow-up was adjusted using two-way analysis of variance and analysis of repeated measurements.

5 Results

5.1 HFE genotyping in public health care (I)

A total of 137 subjects were referred for *HFE* genotyping during the period 1999-2001, of whom 104 [69 males (mean age, 50.8 years; 95% CI, 53.2 to 62.1), 35 females (mean age, 57.6 years; 95% CI, 53.2 to 62.1)] were referred on account of laboratory and clinical findings and 33 (mean age, 44.3 years; 95% CI, 38.4 to 50.2) were asymptomatic. The asymptomatic subjects were referred as first- (n=30) or second-degree (n=3) relatives of *HFE*-genotyped HH patient. The frequencies of various categories of *HFE* genotypes are presented in Table 4.

Table 4. Genotypic frequencies of C282Y and H63D mutations in subjects referred for HFE genotyping during the years 1999-2001.

Genotyping result	Count	%
C282Y/C282Y	23	16.8
C282Y/H63D	7	5.1
H63D/H63D	2	1.5
C282Y/WT	25	18.2
H63D/WT	21	15.3
WT/WT	59	43.1
Total	137	100

The reasons that contributed to the doctors' decision to refer subjects for *HFE* genotyping were variable, and some cases included more than one reason for referral. These included several characteristic and some quite unusual reasons. The most common and important referral indications were elevated serum iron parameters (s-Fe and/or s-ferritin and/or s-TS) (n=47, 39.2%), being a first-degree relative of a *HFE*-genotyped HH patient (n=30, 25%), and phenotypically diagnosed hemochromatosis (n=19, 15.8%). Common reasons contributing to doctors' decision also included elevated liver enzymes [serum alkaline phosphatase (s-ALP) and/or serum alanine aminotransferase (s-ALT) and/or serum

aspartate aminotransferase (s-AST)] (n=19, 15.8%), diabetes mellitus (n=13, 10.8%), family history of non-genotyped hemochromatosis (n=9, 7.5%), cirrhosis of the liver (n=8, 6.7%), and elevated serum iron parameters resulting from alcoholism (n=8, 6.7%). In addition, other referral indications included elevated iron status resulting from multiple RBC transfusions (n=7), cardiomyopathy (n=6), hemosiderosis (n=6), arthralgia (n=5), being a second-degree relative of a *HFE*-genotyped HH patient (n=3), arthritis (n=2), family history of liver disease (n=2), heart failure (n=2), hepatomegaly (n=2), nonspecified liver disease (n=2), skin hyperpigmentation (n=2), gastralgia (n=2), hepatopathy (n=1), increased iron in bone marrow (n=1), and panhypopituitarism (n=1). Table 5 shows the frequency of C282Y homozygotes and C282Y/H63D compound heterozygotes among the subjects with the common reasons that contributed to doctors' decisions to refer patients for *HFE* genotyping.

Table 5. Proportion of C282Y homozygotes and C282Y/H63D compound heterozygotes in the different subject categories.

The most common referral indications	Genotyping result (CY/CY or CY/HD)
Hemochromatosis (not genotyped)	12 of 19 (63.2%)
1st degree relative of HH patient (CY/CY or CY/HD)	8 of 30 (26.7%)
Abnormal iron status (s-Fe and/or s-ferritin and/or s-TS)	8 of 47 (17.0%)
Elevated laboratory parameters (s-ALP and/or s-ALT and/or s-AST)	3 of 19 (15.8%)
Diabetes	1 of 13 (7.7%)
Family history of hemochromatosis (not genotyped)	0 of 9
Cirrhosis of the liver	0 of 8
Alcoholism, abnormal iron status	0 of 8

Note that some subjects had more than one reason for referral in the medical records.

The detection ratio for C282Y homozygotes and C282Y/H63D compound heterozygotes combined over the years 1999-2001 is illustrated in Figure 8. The rate of positive findings for the most typical mutations responsible for HH steadily increased during this period ($p=0.004$).

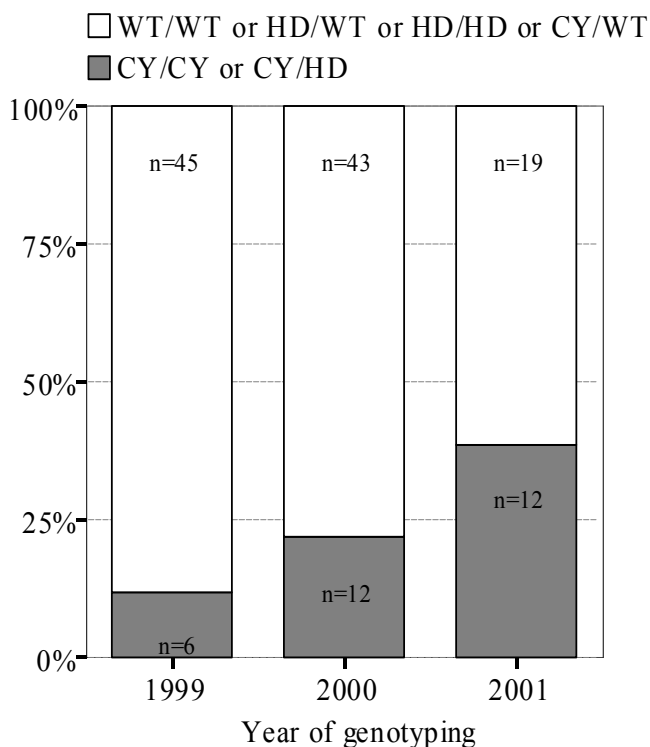


Fig. 8. Positive trend in the detection rate of the most typical mutations responsible for HH during the years 1999-2001.

Serum iron parameters varied in accordance with each *HFE* genotype category. The highest values were found for C282Y homozygotes with the median (quartiles) of 35.0 $\mu\text{mol/l}$ (20-40, $n=20$) for s-Fe, 499 $\mu\text{g/l}$ (45-1672, $n=20$) for s-ferritin, and 78% (32-97, $n=17$) for s-TS. The corresponding figures for C282Y/H63D compound heterozygotes were 26.0 $\mu\text{mol/l}$ (7-38, $n=7$), 51 $\mu\text{g/l}$ (31-997, $n=7$), and 33% (25-56, $n=7$) and those for a control group consisting of WT/WT, HD/WT, or CY/WT genotypes 20.0 $\mu\text{mol/l}$ (15-25, $n=77$), 333 $\mu\text{g/l}$ (94-537, $n=77$) and 31% (24-41, $n=73$), respectively. Analyses of serum iron parameters revealed statistically significant differences between C282Y homozygotes and the control group for s-TS ($p=0.001$) and s-Fe ($p<0.001$), where age and sex were not confounding factors.

5.2 HFE and transferrin receptors in human platelets (II)

Body iron status may have implications for platelet count, the size of platelets and their activation (Berger & Brass 1987, Soff & Levin 1988, Kellar *et al.* 1990, Caliskan *et al.* 1999, Loo & Beguin 1999, Pratico *et al.* 1999, Yang *et al.* 1999, Kurekci *et al.* 2000). Only scant information, however, is available about the expression of the proteins

regulating platelet iron homeostasis. Western blotting and immunofluorescent staining were used to investigate the expression and localization of HFE, TfR1, and TfR2 in human platelets. Positive western blotting reactions were observed for HFE (54 kDa) and TfR2 (87 kDa), while no reaction was observed for TfR1. Immunocytochemistry showed that HFE and TfR2 proteins are expressed at the platelet plasma membrane. The staining for TfR1 remained completely negative. CD42b (platelet glycoprotein Ib) was used in double immunofluorescence staining as a specific marker for platelets. HFE and TfR2 were expressed in CD42b-positive cells, further confirming that these proteins are present in human platelets.

5.3 HFE mutations and various hematological disorders (III)

The C282Y and H63D mutations were determined from 232 patients with various hematological disorders. Parkkila *et al.* (2001b) previously reported the frequencies for a control group from the same hospital district (n=128) to be 10.2% and 20.3% for the C282Y and H63D mutations, respectively. Patients with AML (3.8%, 2 of 53) had a slightly lower and those with ET (16.2%, 6 of 37) a slightly higher tendency for having the C282Y mutation. The C282Y mutation frequencies in ALL (7.0%, 3 of 43), CML (6.3%, 1 of 16), MDS (11.4%, 4 of 35), MM (6.1%, 2 of 33), and PV (6.7%, 1 of 15) were very similar to that of the control group. The frequencies of H63D heterozygous patients were 14.0% (6 of 43) in ALL, 15.1% (8 of 53) in AML, 25.0% (4 of 16) in CML, 16.2% (6 of 37) in ET, 25.7% (9 of 35) in MDS, 12.1% (4 of 33) in MM, and 20.0% (3 of 15) in PV. Additionally, one H63D homozygote was found in both the AML and MDS patient groups. Even though the C282Y and H63D mutation frequencies showed slight changes in various hematological disorders, the differences were not statistically significant in any of the patient categories compared to the control group from the same hospital district.

5.4 HFE mutations and IDCM (IV)

The C282Y, H63D, and S65C mutations were determined for 91 patients with IDCM and 102 control subjects in study IV. The *HFE* mutation frequencies did not show any significant deviations between these two groups. The frequency of C282Y heterozygotes was 13.2% (n=12) in the group of IDCM patients and 9.8% (n=10) in the control group, and that of H63D heterozygotes 22.0% (n=20) and 14.7% (n=15), respectively. In addition, the control group included 3 (2.9%) H63D homozygotes, which means that the frequency of the H63D allele was also very similar to that of the control group. The frequency of S65C heterozygotes in the IDCM group was 2.2% (n=2) and that in the control group 3.9% (n=4).

The influences of the C282Y and H63D mutations on the characteristic echocardiographic changes of IDCM and the severity of symptoms in these patients were

analyzed by comparing the measurements of LVEDD, LVEF, and NYHA functional classes between the various *HFE* categories. The confounding effects of age, gender, medication, and follow-up period were considered in these statistical analyses. The values of LVEDD in the C282Y heterozygous patients with IDCM were higher than those of the wild types. This difference did not reach statistical significance at the time of diagnosis (mean 70.3 mm, SD 7.7 in C282Y heterozygotes vs. mean 65.2 mm, SD 7.5 in wild types, $p=0.106$), but a statistically significant difference was observed at the end of the follow-up period (mean 71.8 mm, SD 9.8 in C282Y heterozygotes vs. mean 64.8 mm, SD 10.0 in wild types, $p=0.037$). The differences and changes in LVEF between the various *HFE* categories during the follow-up were not statistically significant, although LVEF improved only slightly in the C282Y heterozygotes compared to the wild type patients during this period. The differences in NYHA functional classes were statistically non-significant, and the changes in LVEDD during the follow-up period were small between the various categories of *HFE* genotypes.

6 Discussion

Although iron is an essential requirement for human life, an excessive amount of this element in the body is known to be harmful for health (Emerit *et al.* 2001, Fraga & Oteiza 2002). Even though the scientific literature contains thousands of reports on the regulation of iron homeostasis, this issue has been particularly challenging for scientists, because the molecular mechanisms involved have turned out to be complex. Previous extensive studies on HFE protein have explicitly established it as one of the major regulatory factors affecting the body iron homeostasis. Indeed, greater iron stores have been seen in subjects with *HFE* mutations compared to those without these mutations (de Valk *et al.* 2000a, Rossi *et al.* 2000a, Raddatz *et al.* 2003, Sánchez *et al.* 2003). The vast majority of HH patients are C282Y homozygotes, and a few are C282Y and H63D compound heterozygotes (Feder *et al.* 1996). It was recently found that C282Y homozygotes treated with venesections maintain high levels of potentially harmful serum non-transferrin-bound iron (NTBI), and elevated NTBI levels were also found in heterozygotes (de Valk *et al.* 2000b). Not only the potential toxic effects of iron but the fact that HFE is structurally homologous to MHC class I molecules have aroused active interest in the possible associations between *HFE* mutations and various diseases other than HH. Studies investigating these associations have also made it possible to evaluate whether there is a need for *HFE* genotyping in certain groups of patients and provided further information on the effects of iron in the development of various diseases.

6.1 HFE genotypes in cancer

Iron seems to be a carcinogenic metal, and elevated body iron stores may increase the risk of cancer. The main mechanism by which iron may promote carcinogenesis is thought to include induction of oxidative stress. Iron can also stimulate carcinogenesis by activating oxidative responsive transcription factors involved in hypoxia signaling. The other possible mechanisms include suppression of the immune system and promotion of tumor growth. (Toyokuni 1996, Weiss 2002, Huang 2003.) There is a lot of epidemiological data supporting the hypothesis that abundant body iron stores increase

the risk of cancer (e.g. liver cancer, colorectal cancer, and lung cancer) (Toyokuni 1996, Nelson 2001). Because *HFE* mutations are associated with increased body iron stores, it has been hypothesized that these mutations may increase the incidence of cancer by affecting body iron homeostasis. Thus, several recent studies have investigated the association between various cancers and *HFE* mutations.

Liver cirrhosis irrespective of its etiology is considered the most important risk factor for hepatocellular carcinoma (HCC) (Colombo & Sangiovanni 2003). It is known that elevated iron stores in untreated HH may lead to fibrosis and cirrhosis of the liver and, consequently, to HCC. Contradictory pieces of evidence have been published for and against the claim that C282Y homozygosity further increases the risk of HCC in patients with cirrhosis (Fracanzani *et al.* 2001, Boige *et al.* 2003). More than 50% of HCC patients without cirrhosis of the liver have mild iron overload (Huang 2003). Interestingly, a previous study revealed an increased C282Y mutation frequency in HCC patients without cirrhosis of the liver and with iron overload compared to those with HCC in non-cirrhotic liver without iron overload (Blanc *et al.* 2000). It has recently been concluded that C282Y homozygotes have an approximately 20-fold risk to develop liver cancer (Cauza *et al.* 2003, Elmberg *et al.* 2003), which is 10 times lower than the previous estimates (Niederau *et al.* 1985). Although there are also reports of increased frequencies of one or more *HFE*-mutated alleles in HCC patients (Pirisi *et al.* 2000), *HFE* genotypes other than C282Y homozygosity do not seem to significantly increase the risk of HCC (Racchi *et al.* 1999, Cauza *et al.* 2003). It is noteworthy that alcohol abuse and chronic hepatitis C infection typically produce excessive iron accumulation in the liver (Lieu *et al.* 2001), and it is hence possible that the C282Y allele increases the hepatotoxic effect of iron in these situations. It has been reported that C282Y heterozygosity together with hepatitis virus infection or alcohol abuse may increase the risk to develop cirrhosis and, subsequently, liver cancer (Fargion *et al.* 2001, Lauret *et al.* 2002). In addition, an increased risk of liver cirrhosis and HCC was found in subjects carrying the C282Y allele together with TfR Ser 142 homozygosity. The risk was further increased in subjects homozygous for C282Y or compound heterozygous for the C282Y and H63D mutations combined with TfR Ser 142 homozygosity. (Beckman *et al.* 2000.) Taken together, these observations support a role for iron in the pathogenesis of HCC.

The evidence has shown an increased risk of HCC in C282Y homozygotes, but the association between *HFE* mutations and extrahepatic cancers has been unclear (Geier *et al.* 2002, Barton *et al.* 2003, Barton *et al.* 2004). It has been postulated that HH heterozygotes may have an increased incidence of colorectal cancer and hematological malignancies (Nelson *et al.* 1995). Several studies, however, have reported that C282Y and H63D mutations do not increase the risk of colorectal cancer (Altés *et al.* 1999, Macdonald *et al.* 1999, van der A *et al.* 2003), while only one has concluded that *HFE* mutations may correlate positively with colon cancer (Shaheen *et al.* 2003b). Shaheen *et al.* (2003b) found very similar C282Y and H63D allele frequencies in patients with colon cancer (11% and 4.6%, respectively) compared with those in control subjects (9% and 4.4%, respectively). They reported that, after controlling for confounding factors, the risk of colon cancer was elevated in subjects with the *HFE* mutations. This risk in subjects with *HFE* mutations appeared to become higher with increasing age and total iron intake, thus suggesting that the elevated risk of colon cancer in subjects with *HFE* mutations may reflect iron accumulation in the body among the elderly.

The hypothesis that *HFE* mutations are associated with an increased incidence of hematological malignancies was further strengthened, when it was reported that C282Y heterozygosity increases the risk for childhood ALL in males (Dorak *et al.* 1999). In contrast, our study did not show any such association between C282Y mutation and childhood ALL. We found a normal C282Y mutation frequency in ALL patients (7.0%, 3 of 43), and none of the male childhood ALL patients (n=14) carried this mutation. One study has also reported an increased incidence of *HFE* mutations in MDS (Varkonyi *et al.* 2003). In contrast, we did not find an increased prevalence of the *HFE* mutations in MDS in agreement with another study (Speletas *et al.* 2003). Nor did our results support any significant association between *HFE* mutations and other hematological malignancies. In agreement with other studies, the *HFE* mutation frequencies observed in our study were not increased in AML and MM (Van Landeghem *et al.* 1998, Gimferrer *et al.* 1999, Veneri *et al.* 2003). The prevalences of the *HFE* mutations in PV and CML were also normal. (paper III.)

The high frequency of at least one C282Y allele (36.6%) recently observed among 41 patients with breast cancer is intriguing (Kallianpur *et al.* 2004), but it is noteworthy that the C282Y mutation alone showed no association with the incidence of breast cancer earlier in a larger study population (n=165) (Beckman *et al.* 1999). Although Beckman *et al.* (1999) found no difference in the incidence of the C282Y and H63D mutations in colorectal cancer, breast cancer, and MM, an increased frequency of the C282Y allele together with TfR Ser 142 homozygosity was observed among these patients. The risk for malignancies seemed to further increase in subjects homozygous for C282Y or compound heterozygous for C282Y and H63D combined with TfR Ser 142 homozygosity. This seems to support the hypothesis that the increased uptake of iron may have an effect on carcinogenesis.

The C282Y and H63D mutations were also evaluated in a recent study, which included 87 patients with lung cancer, 89 patients with head and neck cancer, and 390 matched control subjects. The C282Y allele seemed to be a risk factor for lung cancer in females but not in males, and *HFE* mutations were not associated with an increased risk of head and neck cancer. (Rodriguez-Paris *et al.* 2003.) A high frequency of C282Y mutation has also been reported in males with HIV-related Kaposi's sarcoma (14.5%, n=131) compared to HIV-positive controls (3.0%, n=132) (Dorak *et al.* 2003). An increased occurrence of H63D mutations has been found in patients with malignant gliomas. The H63D allele frequencies in these patients were 19.5% (n=174) and those in the controls 12.5% (n=144) (p=0.0169) (Martinez di Montemuros *et al.* 2001). Even though the difference in the frequency of H63D mutation between patients with malignant gliomas and control subjects was statistically significant, it was relatively small. More extensive studies are still needed to show or exclude the association between *HFE* mutations with lung cancer, HIV-related Kaposi's sarcoma, or malignant gliomas.

6.2 HFE genotypes in platelet disorders

In paper III, we investigated *HFE* mutation frequencies in patients with ET. The C282Y mutation frequency was slightly elevated (16.2%, 6 of 31) and the H63D mutation frequency normal (16.2%, 6 of 31) in these patients. The frequencies of the C282Y and H63D mutations have also been pointed out to be very similar between patients with venous thrombosis and controls (Xie *et al.* 1998, Brown *al.* 1999, MacLean *et al.* 1999). An increased C282Y mutation frequency was found in thrombosis patients with the factor V Leiden (FVL) mutation in one study (Xie *et al.* 1998), but not in two other studies (Brown *al.* 1999, MacLean *et al.* 1999). It has been reported that the C282Y mutation frequency may, nevertheless, be increased in patients with both the FVL mutation and a family history of thrombosis (MacLean *et al.* 1999).

HFE protein can influence body iron status, which, in turn, may alter platelet count, platelet size, and platelet activation (Berger & Brass 1987, Soff & Levin 1988, Kellar *et al.* 1990, Caliskan *et al.* 1999, Loo & Beguin 1999, Pratico *et al.* 1999, Yang *et al.* 1999, Kurekci *et al.* 2000). In paper II, we observed that HFE protein is expressed in human platelets. TfR2 protein expression, but not TfR1, was also detected in human platelets. This is consistent with the previous results, because no TfR1 expression has been found in platelets or megakaryocytes (Parmley *et al.* 1983), and TfR2 expression has been observed in large bone marrow cells, which probably represented megakaryocytes (Kawabata *et al.* 2001). It has been reported that HFE can physically bind to TfR1, but not to TfR2 (West *et al.* 2000). Hence, HFE may serve a role other than modulating iron homeostasis in platelets. Interaction between HFE and TfR2 was recently suggested, however, based on the observed co-localization of HFE and TfR2 in human intestinal crypt cells and cultured Caco-2 cells (Griffiths & Cox 2003). In either way, our results point to a possibility that mutations in *HFE* or *TfR2* gene may have functional implications for platelets.

6.3 HFE genotypes in cardiovascular diseases

Iron has been proposed to contribute to the development of cardiovascular diseases. It can promote lipid peroxidation, and iron-induced free radicals may directly damage arterial endothelium (Shah & Alam 2003). Several epidemiological studies have supported the association between elevated iron parameters and the increased risk of cardiovascular diseases (Tuomainen *et al.* 1998, de Valk & Marx 1999, Yuan & Li 2003), while several others have failed to show evidence of increased iron stores in these conditions (Fox *et al.* 2002, Knuiman *et al.* 2003, Shah & Alam 2003). The association between *HFE* mutations and cardiovascular diseases has been intensively examined, but only a few studies have supported such interaction. Tuomainen *et al.* (1999) suggested that the risk for first acute myocardial infarction (MI) is increased among men with C282Y mutation. Another study revealed an elevated risk of cardiovascular death, including increased mortality for MI, cerebrovascular disease, and other cardiovascular diseases in C282Y heterozygous women (Roest *et al.* 1999). Additionally, Rasmussen *et al.* (2001) reported that the

C282Y mutation may increase the risk of coronary heart disease (CHD). A large cross-sectional study (n=30 916) found no association between C282Y or H63D heterozygosity and CHD. An increased prevalence of CHD was detected only in male C282Y/H63D compound heterozygotes compared to males without the *HFE* mutations after adjustment for cardiovascular risk factors. This modest increase of CHD prevalence in male C282Y/H63D compound heterozygotes was not explained by the serum ferritin or TS levels, and the findings are thus not consistent with the hypothesis that *HFE* mutations increase the risk of CHD by influencing the body iron status. (Waalén *et al.* 2002b.) The majority of studies have shown that *HFE* mutations do not increase the risk for MI or CHD (Nassar *et al.* 1998, Annichino-Bizzachi *et al.* 2000, Battiloro *et al.* 2000, Calado *et al.* 2000, Hetet *et al.* 2001b, Bozzini *et al.* 2002, Claeys *et al.* 2002, Fox *et al.* 2002, Campbell *et al.* 2003, Candore *et al.* 2003a, Gunn *et al.* 2004). It has also been demonstrated that *HFE* mutations do not seem to relate to brain infarction, either (Hetet *et al.* 2001b, Njajou *et al.* 2002a). Arterial wall thickness has been found to increase before the onset of cardiovascular complications and reverse by iron depletion therapy in genetic hemochromatosis patients (Failla *et al.* 2000). Hetet *et al.* (2001b) observed that atherosclerotic plaques were frequent in H63D mutation carriers, but not in persons with C282Y. Other studies have not shown any evidence of an association between *HFE* mutations and atherosclerosis (Franco *et al.* 1998, Battiloro *et al.* 2000, Calado *et al.* 2000, Rossi *et al.* 2000b, Hetet *et al.* 2001b, Njajou *et al.* 2002a).

Since cardiomyopathy is known to be one of the most severe complications of HH, we set out to study the prevalence of *HFE* mutations in patients with IDCM. In such patients, Mahon *et al.* (2000) had previously reported an increased frequency of H63D mutation. In contrast, our study showed a similar H63D mutation frequency in IDCM patients compared to controls (paper IV), consistent with two other reports (Hetet *et al.* 2001a, Pereira *et al.* 2001). No increase in the C282Y mutation frequency has been observed in IDCM patients (Mahon *et al.* 2000, Hetet *et al.* 2001a, Pereira *et al.* 2001, paper IV). We demonstrated that the S65C mutation frequency in these patients seems to be normal as well (paper IV). Pereira *et al.* (2001) reported an increased frequency of C282Y heterozygosity among patients with cardiomyopathy of ischemic etiology (10.4%, 6 of 58) compared to another group of patients with nonischemic etiology (2.3%, 6 of 261). The C282Y mutation, however, was not present in any of the Israeli patients with dilated cardiomyopathy of ischemic etiology (n=117). In this context, it should be remembered that the C282Y frequency seems to be low in the general Israeli population. (Goland *et al.*, in press).

6.4 HFE genotypes in other diseases

Iron-induced oxidative stress has been suggested to contribute to the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). In these conditions, iron accumulation has been observed in the brain regions that are most seriously affected due to excessive neuronal death. (Lieu *et al.* 2001, Sipe *et al.* 2002.) Apolipoprotein E (APOE) is a known risk factor for AD (Jellinger 2002). Moalem

et al. (2000) proposed that *HFE* mutations may predispose to familial AD in APOE- ϵ 4-negative males. Sampietro *et al.* (2001) did not observe differences in the C282Y or H63D allele frequencies between sporadic late-onset AD patients and controls. Interestingly, they found that patients with a mutated H63D allele had an earlier disease onset than those who did not carry this allele. Combarros *et al.* (2003) reported that the H63D mutation results in an earlier onset of sporadic AD in APOE- ϵ 4 carriers, but not in APOE- ϵ 4-negative patients. Pulliam *et al.* (2003) showed an increased frequency of C282Y homozygotes and C282Y and H63D compound heterozygotes among subjects with AD/mild cognitive impairment (MCI) and among those with AD-like pathological changes without dementia. The increased lipid peroxidation marker (F(2)-Isoprostane) levels seen in subjects with any *HFE* mutation further suggest that these mutations may induce oxidative stress (Pulliam *et al.* 2003). On the contrary, two recent studies argue against any association between *HFE* mutations and sporadic AD. Normal *HFE* mutation frequencies in AD were observed, and *HFE* mutations did not alter the age at disease onset. The other study also showed that age at the onset of cognitive or AD symptoms were not affected by *HFE* mutations, and the frequencies of these mutations were not increased in MCI. The presence or absence of APOE- ϵ 4 did not have any significant effect on the results of these two studies. (Candore *et al.* 2003b, Berlin *et al.* 2004.) It was also recently reported that the C282Y allele together with the C2 variant of the transferrin gene increased the risk of AD, and the risk is further increased when these two alleles are combined with APOE- ϵ 4 (Robson *et al.* 2004). Conflicting results have also been obtained in studies investigating the association between *HFE* mutations and PD. Borie *et al.* (2002) found that the frequency of mutated *HFE* alleles in PD patients was not different from that in controls. Additionally, one study reported a decreased frequency of the C282Y allele among PD patients (Buchanan *et al.* 2002), while another study found an increased frequency of C282Y mutation in PD and in other forms of parkinsonism (Dekker *et al.* 2003).

Diabetes mellitus is a recognized complication of HH. It has been shown that abundant iron stores may increase the incidence of type 2 diabetes (Salonen *et al.* 1998). Conte *et al.* (1998) observed that the frequency of genetic hemochromatosis was higher in patients with type 2 diabetes, but not in type 1 diabetes, compared to controls. They based the diagnosis of genetic hemochromatosis on the HII and the exclusion of secondary iron overload. Kwan *et al.* (1998) further demonstrated a C282Y mutation frequency of 21.9% in patients with type 2 diabetes (n=105) and 11.7% in those with type 1 diabetes (n=103). An increased frequency of C282Y homozygotes was, nevertheless, found among patients with late-onset type 1 diabetes (Ellervik *et al.* 2001). Salonen *et al.* (2000) found that the carriers of C282Y allele develop type 2 diabetes more frequently (11%, 4 of 35) than those without this allele (5%, 23 of 473). In addition to these studies, only one has supported the increased risk of type 2 diabetes in carriers of the C282Y allele (Moczulski *et al.* 2001), while several others have failed to detect an increased C282Y mutation frequency in type 2 diabetes (Braun *et al.* 1998, Dubois-Laforgue *et al.* 1998, Dubois-Laforgue *et al.* 2000, Sampson *et al.* 2000, Acton *et al.* 2001, Kankova *et al.* 2002, Njajou *et al.* 2002b, Van Lerberghe *et al.* 2002, Halsall *et al.* 2003, Malecki *et al.* 2003). The H63D mutation frequency has been shown to be normal in type 2 diabetes (Braun *et al.* 1998, Dubois-Laforgue *et al.* 1998, Dubois-Laforgue *et al.* 2000, Sampson *et al.* 2000, Acton *et al.* 2001, Kankova *et al.* 2002, Njajou *et al.* 2002b, Van Lerberghe *et*

al. 2002, Malecki *et al.* 2003), but Malecki *et al.* (2003) detected an increased frequency of this mutation in male patients with type 2 diabetes and in those diagnosed at an age over 49 years. It has also been suggested that C282Y heterozygotes may have an increased risk of proliferative diabetic retinopathy and H63D mutation carriers an increased risk of diabetic nephropathy in type 2 diabetes (Moczulski *et al.* 2001, Peterlin *et al.* 2003). Therefore, it is impossible to conclusively exclude the possibility that even heterozygosity for *HFE* mutations has a modifying effect on the pathogenesis of type 2 diabetes mellitus.

Insulin resistance is likely to play a central role in the development of nonalcoholic steatohepatitis (NASH), and oxidative stress is probably another factor involved in its progression. Hence, iron overload has been proposed to influence the pathogenesis of NASH. (Chitturi & George 2003.) Four studies out of five reported an increased C282Y allele frequency in NASH, but only one study reported an increased frequency of H63D (George *et al.* 1998, Bonkovsky *et al.* 1999, Chitturi *et al.* 2002, Deguti *et al.* 2003, Valenti *et al.* 2003). The C282Y mutation could theoretically affect the development of hepatic fibrosis in NASH by inducing iron accumulation in the liver, but such mechanism is currently not supported (Chitturi & George 2003). Even though most studies so far have observed high frequencies of C282Y mutation in NASH, more detailed studies are still required in this area.

Sporadic porphyria cutanea tarda (PCT) is caused by reduced activity of uroporphyrinogen decarboxylase (URO-D) in the biosynthesis of heme. It is commonly characterized by iron overload and hepatic siderosis, and iron may inhibit URO-D in the hepatocytes of PCT patients. Additionally, removal of excessive iron with recurrent phlebotomies has been shown to reverse the decreased activity of URO-D. (Sampietro *et al.* 1999.) The association between *HFE* mutations and PCT has been studied intensively after the discovery of the *HFE* gene. Most studies have found an increased frequency of C282Y mutation among patients with PCT (Roberts *et al.* 1997, Bonkovsky *et al.* 1998, Stuart *et al.* 1998, Christiansen *et al.* 1999, Bulaj *et al.* 2000, Martinelli *et al.* 2000, Dereure *et al.* 2001, Tannapfel *et al.* 2001, Cruz-Rojo *et al.* 2002, Egger *et al.* 2002, Lamoril *et al.* 2002, Chiaverini *et al.* 2003, Nagy *et al.* 2004), while only a few studies have not been able to find such association (Santos *et al.* 1997, Sampietro *et al.* 1998, Furuyama *et al.* 1999, Ivanova *et al.* 1999). The frequency of H63D mutation in PCT has been found to be increased in some studies (Santos *et al.* 1997, Bonkovsky *et al.* 1998, Sampietro *et al.* 1998, Christiansen *et al.* 1999, Bulaj *et al.* 2000, Dereure *et al.* 2001, Egger *et al.* 2002), but not in others (Roberts *et al.* 1997, Stuart *et al.* 1998, Ivanova *et al.* 1999, Martinelli *et al.* 2000, Cruz-Rojo *et al.* 2002, Lamoril *et al.* 2002, Chiaverini *et al.* 2003, Nagy *et al.* 2004). The S65C mutation alone does not increase the risk of PCT (Christiansen *et al.* 1999, Dereure *et al.* 2001, von Ahsen *et al.* 2001, Egger *et al.* 2002, Lamoril *et al.* 2002, Chiaverini *et al.* 2003). Based on the current knowledge, *HFE* mutations seem to increase the risk of PCT at least in some populations. The differences in the C282Y and H63D mutation frequencies in PCT may be explained by the different ethnic origins of the populations studied (Hift *et al.* 2002). The increased incidence of C282Y homozygotes and C282Y/H63D compound heterozygotes found in several studies supports the role of iron in the pathogenesis of PCT (Roberts *et al.* 1997, Bonkovsky *et al.* 1998, Stuart *et al.* 1998, Bulaj *et al.* 2000, Tannapfel *et al.* 2001, Egger *et al.* 2002,

Lamoril *et al.* 2002). Furthermore, it was recently reported that *HFE* mutations may decrease the response to chlorocine treatment in PCT (Stolzel *et al.* 2003).

Joint involvement is considered to occur frequently at the early stages of HH progression (Ines *et al.* 2001). The prevalence of the C282Y mutation seems to be normal in rheumatoid arthritis and spondylarthritis, but an increased frequency of H63D mutation has been found in rheumatoid arthritis (Li *et al.* 2000, Rovetta *et al.* 2002). Willis *et al.* (2002) reported that the C282Y and H63D mutation frequencies are not increased in patients with inflammatory arthritis (n=1000). Ross *et al.* (2003), however, recently observed an increased prevalence of C282Y mutation in a population with hand osteoarthritis (12.5% vs. 7.8%).

6.5 Algorithm for HH diagnostics

Overall, excessive iron in the body seems to contribute to the pathogenesis of certain diseases. *HFE* mutations may increase iron accumulation in the liver, inducing hepatic injury. These mutations may hence be enriched in various liver diseases, and a high number of previously undiagnosed C282Y homozygotes has actually been found among subjects who visit liver clinics (Moodie *et al.* 2002). These patients probably benefit from the removal of excessive iron by phlebotomies, which may also prevent the development of liver cirrhosis and HCC. The detection of causative mutations for HH may prevent not only the development of irreversible liver injury but also other iron-induced non-hepatic manifestations. Therefore, screening for *HFE* mutations should be recommended in various liver diseases. It may, however, be reasonable to determine s-TS before referral for *HFE* genotyping. Iron overload and *HFE* mutations are frequent in PCT, and *HFE* mutations may affect the selection of its treatment. Thus, screening for *HFE* mutations should be included in the diagnostics of PCT. Even though screening programmes might also be beneficial in some other patient groups, these cannot be considered necessary, since there is not enough evidence of a significantly increased incidence of *HFE* mutations in any other condition. Future studies may still reveal some groups of patients, such as type 2 diabetic patients, in whom routine *HFE* screening may turn out recommendable.

HFE genotyping is a simple and effective tool in the diagnostics of HH, enabling early diagnosis of the disease (I). It is commonly agreed that determination of s-TS is the best single laboratory test when selecting subjects for *HFE* genotyping (I). It should, nevertheless, be remembered that a number of young adult C282Y homozygotes who subsequently develop significant liver fibrosis can have normal s-TS in their 20- to 30-year age range and show an increase later (Olynyk *et al.* 2004). Based on current knowledge, large-scale population screening for HH cannot be recommended (Galhenage *et al.* 2004). Instead, *HFE* genotyping is highly advisable for first-degree relatives of HH patients (Powell 2002), since it provides a highly valuable laboratory parameter for the detection of subjects who have a significantly increased risk for HH (I). The lack of clinical penetrance of *HFE* mutations is now a recognized fact, but HH may also be a seriously underdiagnosed disease, and it can be argued that a large number of patients

even with several characteristics of HH continue to escape detection (I). Although our data suggest that the knowledge of HH among Finnish clinicians has generally improved during the years 1999-2001 (I), it has recently been reported that many clinicians do not have adequate knowledge about the diagnostics and treatment of hemochromatosis (Acton *et al.* 2002). Proper education of clinicians and the rationalization of diagnostics of HH in general practice may improve the detection of symptomatic and presymptomatic subjects (Adams *et al.* 2000a). A diagnostic algorithm for HH is presented in Figure 9.

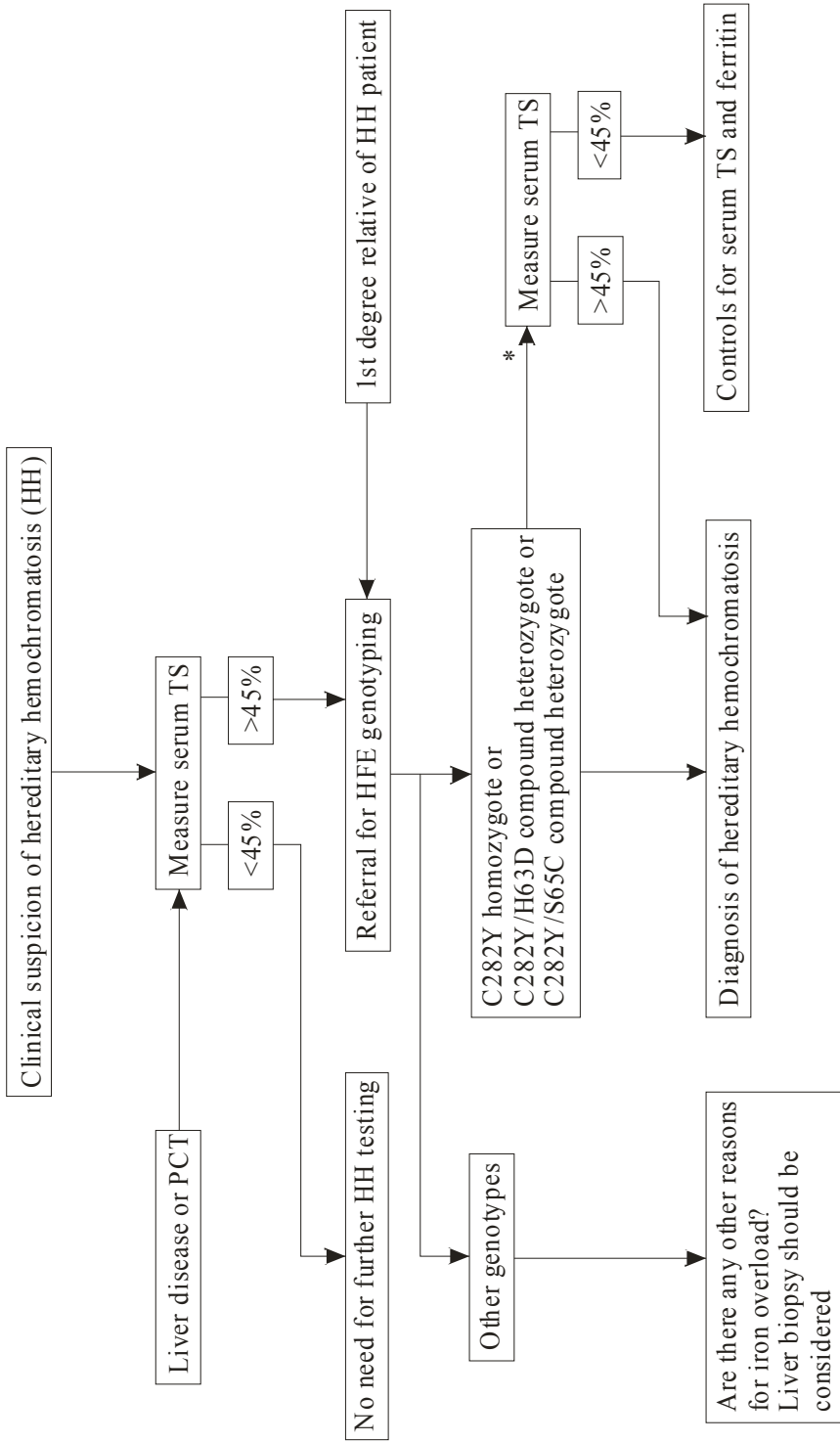


Fig. 9. Diagnostic strategy for detecting individuals with HH. *If serum transferrin saturation has not been measured prior to HFE genotyping. PCT, porphyria cutanea tarda; TS, transferrin saturation. Modified from Parkkila and Niemelä (2001).

7 Conclusions

- Active testing for the *HFE* gene mutations C282Y and H63D is recommended in the diagnostics of HH in health care. Based on the previous and present findings, serum transferrin saturation can be considered the most suitable laboratory test to select individuals for *HFE* genotyping and the definitive diagnosis can be confirmed by *HFE* genotyping.
- HFE and TFR2, but not Tfr1, are expressed in human platelets. Mutations in the *HFE* and *Tfr2* genes may thus have implications for platelet count, size, and/or activation.
- The C282Y and H63D mutations do not seem to increase the risk of various hematological disorders or IDCM. The frequency of S65C mutation in IDCM appears to be normal. *HFE* genotyping cannot be considered necessary for patients with various hematological disorders or IDCM. The C282Y mutation may, nevertheless, have a modifying effect on LV dilation and remodeling in patients with IDCM.

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