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Diagnosis of Hereditary Haemochromatosis using Non-Invasive Methods

P. Nielsen[a] R. Fischer[a] R. Engelhardt[a] J. Düllmann[b]

[a] Abteilung Molekulare Zellbiologie, Institut Medizinische Biochemie und Molekularbiologie,
[b] Abt. für Neuroanatomie, Universitätsklinikum Hamburg-Eppendorf, Germany

Key Words

Hereditary haemochromatosis · Iron overload · Serum ferritin · Liver iron concentration · *HFE* gene · C282Y mutation · H63D · S65C · SQUID biosusceptometry · MRI

Summary

Hereditary (idiopathic, genetic) haemochromatosis is a genetic disease in which food iron absorption is inadequately increased and not balanced to the body iron stores. As a consequence, untreated patients have a risk to develop iron overload which can give rise to various iron-induced organ damages, leading in some cases to multi-organ dysfunction (liver cirrhosis, diabetes, cardiomyopathy). Diagnosis in this iron loading disease was formerly based on highly increased transferrin saturations, elevated serum ferritin concentrations and on excessive iron deposits in biopsy material from liver. After the identification of the *HFE* gene and its most common mutations, a genetic diagnosis independent from iron stores has become available. Because of individual variations in the loading process, diagnostic criteria are still necessary to characterise the risks of a given patient to develop iron-induced lesions and clinical symptoms. This review describes the current knowledge of the molecular pathology and discusses criteria for diagnosis and screening of hereditary haemochromatosis. Non-invasive liver iron quantification could represent the optimal complement to the *HFE* gene testing. Data on our experience with SQUID biosusceptometry, which makes liver biopsy unnecessary in most cases, are presented.

Schlüsselwörter

Hereditäre Hämochromatose · Eisenüberladung · Serum Ferritin · Lebereisen · *HFE*-Gen · C282Y-Mutation · H63D · S65C · SQUID-Biosuszeptometrie · MRI

Zusammenfassung

Die hereditäre Hämochromatose ist eine genetische Erkrankung, bei der die Regulation der Nahrungseisenabsorption außer Funktion gesetzt ist, mit der Konsequenz, dass ständig Eisen über den Bedarf aufgenommen wird. Unbehandelte Patienten haben ein hohes Risiko, eine Eisenüberladung zu entwickeln, die im Einzelfall zu schweren Organschäden (Leberzirrhose, Diabetes, Kardiomyopathie) führen kann. Die klassische Diagnose orientierte sich im Wesentlichen an einer stark erhöhten Transferrinsättigung und Serum-Ferritin-Konzentration sowie am Nachweis einer exzessiven Eisenspeicherung in Leberbiopsie-Proben. Seit der Identifizierung des *HFE*-Gens auf Chromosom 6 im Jahre 1996 steht eine von der Eisenspeicherung unabhängige genetische Diagnostik zur Verfügung. Bedingt durch den sehr unterschiedlichen Ausprägungsgrad der Eisenüberladung, bleiben jedoch auch zukünftig diagnostische Kriterien wichtig, die es erlauben, die Eisenüberladung am Patienten individuell zu beurteilen. Der vorliegende Artikel gibt einen Überblick über den aktuellen Stand der Diagnostik bei Hämochromatose. Die nichtinvasive Lebereisenquantifizierung könnte die optimale Ergänzung zur *HFE*-Gen-Analytik darstellen. Über eigene Erfahrungen mit der SQUID-Biosuszeptometrie, die eine Biopsie in vielen Fällen überflüssig machen kann, wird berichtet.

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Fax +49 761 4 52 07 14
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PD Dr. Dr. Peter Nielsen
Abteilung Molekulare Zellbiologie
Institut für Medizinische Biochemie und Molekularbiologie
Universitätsklinikum Hamburg-Eppendorf
Martinistraße 52, D-20246 Hamburg
Tel. +49 40 428 03–33 89, Fax -47 97, E-mail nielsen@uke.uni-hamburg.de

Iron Homeostasis in Humans

Iron is essential to life in almost all biological systems [1]. When complexed, most often with porphyrin, and inserted into appropriate proteins, iron binds oxygen reversibly. As a transitional metal with two stable valence states, Fe^{2+} and Fe^{3+}, iron participates in a broad repertoire of vital redox reactions.

However, iron as a heavy metal can also induce a variety of cytotoxic reactions in biological systems, and iron overload can result in clinical relevant organ damages. Therefore, under physiological conditions the whole body iron homeostasis is precisely maintained producing a steady state which is called normosiderosis [2–4]. This is achieved by adjusting the intestinal iron absorption (normal, 1–2 mg; at maximum, 5 mg Fe/day) to the need of iron. In the last 5 years, the mechanism of iron absorption has been clarified in some but still not all details [5].

Uptake of Fe^{2+} through a divalent metal transporter *DCT1/DMT1* in the apical cell membrane is the first step, followed by an intracellular processing of iron in an exchangeable iron pool, and finally transfer of the iron by efflux across the basolateral membrane via the iron exporter *IREC1/ferroportin* [5–7]. The non-absorbing crypt cells play the key role in the adaptation of iron absorption to the need of iron in the body [8]. Crypt cells are supplied with iron from the basolateral side and are thereby informed about the iron balance in the body. In iron deficiency, the iron concentration in crypt cells is low, and these cells begin to upregulate the iron transporter proteins DMTI and IREC1 and migrate and differentiate into absorbing villus cells (fig. 1). The recently found peptide *leap1*/*hepcidin* may represent the predicted 'storage iron regulator' [9], which informs the absorbing enterocytes about the body iron status [10–12]. The search for the hepcidine receptor is currently an important task. It is speculated that malfunction of this protein may represent the pathomechanism for juvenile haemochromatosis.

A chronic positive iron balance, induced by i) malregulation of intestinal iron absorption in hereditary haemochromatosis or ii) by multiple blood transfusions in secondary haemosiderosis leads to storage of excessive iron, which can induced cell and organ damage.

Secondary Haemosiderosis

Secondary forms of iron overload can only be mentioned in this review in short form. In different diseases such as β-thalassaemia major, Blackfan-Diamond anaemia and myelodysplastic syndrome patients are dependent from regular blood transfusions. About 250 mg of iron are added to the body from each unit of blood. For the long-term survival of iron-loaded patients, early and well adjusted treatment with iron chelators is of crucial importance, especially in children [13, 14]. Basis of an adequate treatment are appropriate diagnostic parameters which are capable to monitor the range of the individual iron burden [15].

Hereditary Haemochromatosis

The term 'haemochromatosis' was introduced by the German pathologist von Recklinghausen in 1889 [16] who described the excessive iron storage in a cirrhotic liver. The combination of liver cirrhosis, diabetes and skin pigmentation as symptoms of a new disease was first described by Sheldon in 1935 [17]. It was long thought that chronic alcohol abuse is the important factor in the pathogenesis of haemochromatosis until Simon et al. [18] found an association between the disease and HLA A3B7 and the autosomal recessive mode of inheritance. With the identification of the *HFE* gene in 1996 by Feder et al. [19], the close genetic linkage of haemochromatosis to the HLA complex on chromosome 6 was confirmed.

The HFE protein is a 343-residue type 1 transmembrane glycoprotein which has structural similarity to MHC class I proteins [20]. It has three extra-cellular domains (α1–α3) from which α3 binds β_2 microglobulin non-covalently. The most important mutation, a G to A transition at nucleotide 845, substitutes a tyrosine for a cysteine at position 282 (C282Y). The mutated HFE protein has a structural change in the α3 domain and cannot bind β_2 microglobulin. The HFE protein binds at the transferrin receptor (TfR) close to the transferrin binding side and decreases the binding affinity of the TfR for iron-rich transferrin. HFE which is impaired in hereditary haemochromatosis thus influences the transferrin iron-mediated intracellular iron delivery into crypt cells (fig. 1) [21]. The concept of hepcidine/leap1-mediated information of the crypt cell about the body iron status fits with the finding that a haemochromatosis-like phenotype is performed in knockout-mice lacking either HFE, β_2 microglobulin, or hepcidine/leap1 [12, 22, 23].

Hereditary haemochromatosis is a very frequent disease. The allele frequency for the C282Y mutation in the Caucasian population is about 0.063 [24]. A plausible explanation for such a high gene frequency is that carriers of the C282Y mutation had a better change in the evolution to avoid severe iron deficiency in times of long hunger periods or frequent pregnancies.

In Northern Europe, including Germany [24–27], Australia and USA, between 90 and 100% of the haemochromatosis patients are homozygous for the C282Y mutation, whereas in Southern European countries only about 60–70% of all patients show this specific mutation [28].

Some patients have a substitution of aspartic acid for histidine at position 63 (H63D) [19]. This mutation alone has little effect on iron metabolism, leading to only slightly increased blood iron parameters in H63D homozygotes. When combined with the C282Y mutation in different alleles, however,

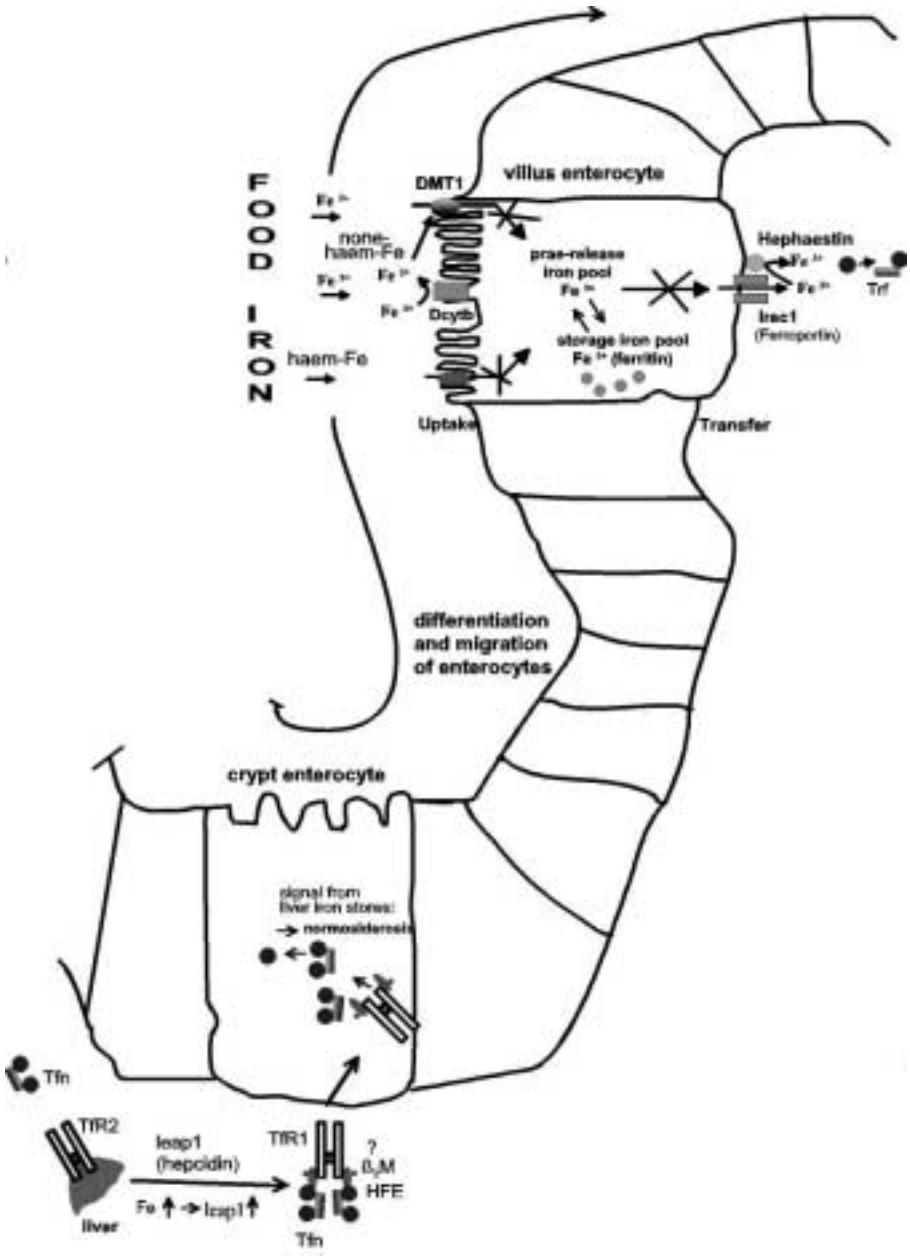

Fig. 1. Regulation of iron absorption. The 25 amino acid peptide leap1 (liver expressed antimicrobial peptide, also named hepcidin, hepatic bactericidal protein) is synthesised in the liver and seems to interact with the β_2 microglobulin HFE transferrin receptor1 complex which is involved in the basolateral transport of iron into crypt cells. This cells then migrate and differentiate into absorbing villus enterocytes before exfoliation at the top of the villus occurs.
Iron-sufficient crypt cells become villus enterocytes in which iron absorption from food iron mediated by the DMT1 receptor is down-regulated. In case of iron deficiency, the expression of the 'iron storage regulator' leap1 is low and iron is absorbed as far as triggered (iron-deficient) crypt cells become absorbing villus cells. In haemochromatosis, the *HFE* gene product is absent, the β_2 microglobulin HFE transferrin receptor1 complex cannot be formed, and the crypt cells become iron-deficient and start to up-regulate iron transporters such as DMT1.

this compound heterozygosity can also produce a clinical relevant iron overload in some but not all patients. Other mutations in the *HFE* gene have been found in single patients [29], but these mutations are rare and have little diagnostic relevance. Some other forms of haemochromatosis-like iron overload diseases, not related to *HFE*, have been characterised as HFE2–HFE4 (table 1) [30–35]. In Sicily, a Y250X mutation in the *transferrin receptor 2* gene on chromosome 7 was found in a few patients [32]. Recently, a large family was described in which iron overload was inherited by an autosomal-dominant trait not linked to *HFE* and characterised by an early iron accumulation in reticuloendothelial cells [33]. The gene is encoding ferroportin (SLC11A3), a transmembrane iron export protein.

So far, the large number of non-*HFE* gene haemochromatosis cases found in Italy cannot be explained genetically, indicating that still unknown proteins exist which influence iron homeostasis [34]. This is also true for an iron overload disease in Sub-Saharan Africans, which is also not connected to the *HFE* gene [35]. This form of iron overload is not identical to the well-known 'Bantu-siderosis', a nutritional intoxication with iron-enriched traditional African beer [1].

About 100 cases with *neonatal haemochromatosis* have been reported in the literature. Although excess storage of iron with accompanying hepatic damage can be the end result of many processes affecting mother and infant such as hereditary tyrosinaemia and of maternal viral infections, it appears that certain sub-types of this fatal disease are heritable [36]. The *HFE* gene seems not to be affected, details of a genetic cause of the disease are not known to date.

Clinical Symptoms

The intensity of iron accumulation and thus the frequency and severity of clinical symptoms vary markedly in haemochromatotic patients. The underlying reasons are not quite clear and so far cannot be explained sufficiently by non-genetic factors such as age, sex, different food iron intake, individual iron loss and alcohol intake. It seems more likely that unknown genetic factors may modify the malregulation of intestinal iron absorption.

Many studies have evaluated the occurrence of clinical symptoms in persons with haemochromatosis [37–39]. Studies in clinical settings may have overestimated the disease burden because only subjects affected by iron overload were included. This is indicated by the fact that the number of patients with severe clinical symptoms in more recent studies is much lower than in older ones (table 2), also demonstrating the progress in early diagnosis of haemochromatosis in the last years. On the other hand, screening studies in healthy populations [40, 41], for example in blood donors, may theoretically exclude sick persons and therefore tend more to underestimate the number of clinically affected haemochromatosis patients. The actual view on the penetrance of the disease is that the morbidity of haemochromatosis can only be low because two large studies in unselected populations have found only a very low number (about 1%) of clinically affected subjects [42, 43]. However, due to the high frequency of homozygous C282Y mutation (1:100 to 1:400 in Northern Europe), haemochromatosis remains still a significant diagnostic and clinical problem.

Early unspecific symptoms include weakness, fatigue and joint pain [1, 37–39]. Arthropathy especially in small joints is found in about 30% of all patients, independent from the stage of iron loading. In some patients, arthropathy is still progressing or even starts under or after the initial phlebotomy treatment. Advanced complications are related to damage of the liver, pancreas and heart. Some patients have a long history of increased liver enzymes in blood. However, complete cirrhosis of the liver is found only in a few patients, whereas the majority of patients are diagnosed only with a pronounced liver fibrosis [37–39].

Diabetes is the most severe endocrine disorder in iron overload. According to our experience, this complication is found mainly in heavily iron-loaded patients with liver cirrhosis. Iron-induced liver cirrhosis is a known risk for the development of hepatocellular carcinoma [44]. Therefore, early diagnosis of hepatocellular carcinoma is important in haemochromatosis, especially in those patients with known chronic liver disease. Cardiac involvement manifested as arrhythmia or cardiomyopathy is also restricted only to cases with advanced iron overload. Congestive heart failure is the main cause of death in iron overload diseases.

It should be noted that a large number (the majority?) of C282Y-homozygote subjects, identified in family and population screening studies, show almost no clinical symptoms of haemochromatosis, some are even not iron-overloaded at all as judged by a normal liver iron content [27].

Table 1. Different forms of hereditary haemochromatosis

Type	Characteristics
HFE	iron overload associated with the C282Y mutation in the *HFE* gene on chromosome 6; the H63D mutation in combination with heterozygous C282Y may also cause haemochromatosis; prevalence in Caucasian populations: 1:100 to 1:400 [19, 24–27]
HFE2	juvenile haemochromatosis, most severe form of primary iron overload, gene locus 1q, gene not known [30, 31]; possibly 2 different genes
HFE3	rare form in the Italian population, gene locus 7q22, mutation in the *TfR2* gene [32]
HFE4	rare form, 2q32, mutation in the *IREC1/Ferroportin* gene, autosomal dominant form [33]

Table 2. Clinical symptoms in hereditary haemochromatosis at the time of diagnosis

Clinical symptoms	Occurrence, % of cases		
	Niederau et al., 1985 [37]	Adams et al., 1997 [39]	Nielsen, 1998 [27]
Liver cirrhosis	69	22	10
Diabetes		14	5
Arthropathy	43	29	35
Impotency	55	40	5
Brown-grey skin colour	75	38	22
Asymptomatic		27	35
No iron overload			15

Diagnosis of Hereditary Haemochromatosis

Excess iron, derived from hyperabsorption of iron in the gut, is predominantly be deposited within parenchymal liver cells. Later in the natural history of haemochromatosis, endocrine glands and myocytes are involved in iron accumulation. Organ damages producing clinical symptoms are found in advanced stages and become irreversible in single patients when iron depletion therapy is not started in time. Therefore early diagnosis based on genetics is urgent. The diagnosis of hereditary haemochromatosis as an autosomally transmitted disease of life-long intestinal hyperabsorption of iron is now mostly a prophylactic one. Today affected infants can be identified before a clinically relevant iron overload is present.

Indirect diagnostic parameters are affected in patients as the consequence of iron loading. Direct parameters characterise the gene defect or quantitate the tissue concentration of excessive iron (table 3).

Table 3. Diagnostic parameters in haemochromatosis

Genotype
HFE-gene testing ==> C282Y, H63D mutation
Phenotype
Indirect parameters
Serum iron
Non-transferrin-bound iron
Transferrin iron saturation
Total iron binding capacity
Serum ferritin
Serum ferritin iron
Direct parameters
Invasive
Liver biopsy
– Histological iron distribution
– Liver iron concentration
Biopsy stomach/duodenum
Non-invasive
Liver iron concentration (SQUID, MRI)

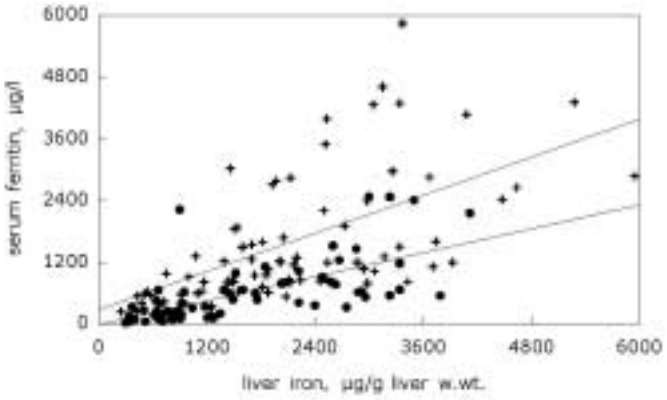

Fig. 2. Correlation between serum ferritin and liver iron in haemochromatosis patients. + = males; ● = females. Lines represent linear correlation curves. w.wt. = Wet weight.

Serum Iron, Transferrin Iron Saturation

For the phenotypical characterisation, indirect parameters such as serum iron and transferrin iron saturation are sensitive parameters, which are increased very early in the progress of iron loading. These parameters alone or in combination with serum ferritin are therefore very useful and also cost effective in screening studies to detect patients with iron overload.

However, there is no correlation between one of these parameters and the amount of the individual storage iron. Transferrin saturation of more than 100% in advanced stages of iron overload may indicate substantial fractions (up to 10 µ*M*) of non-transferrin-bound iron in plasma [45]. The structure of non-transferrin-bound iron is not yet clear. It probably represents a rather inhomogeneous fraction of low-molecular-weight iron in the plasma and could play an important role in the pathophysiology of iron-induced organ damages.

Single values of serum iron or transferrin saturation are not always reliable indicators in the diagnosis of iron overload because these parameters can be altered in both directions, thus producing false-negative and false-positive results. For example, serum iron can be decreased in the presence of febrile illness or malignant tumour even in iron-loaded patients, whereas acute liver cell necrosis can increase serum iron by the release of iron from liver cells.

Serum Ferritin

Serum ferritin is as important for the diagnosis of iron overload as it is for iron deficiency. In contrast to the serum iron level and transferrin saturation, there are fewer false-positive and no false-negative results, and little or no diurnal variations. In addition, the serum ferritin level reflects total body iron stores [46] in a certain range. The 'quantitative' relation (iron store (mg) = serum ferritin (µg/l) × 8 mg × l/µg) is frequently used to assess iron stores. However, a large interindividual variation has to be taken into account both in primary and secondary haemochromatosis, and therefore it is almost impossible to predict individual iron stores from given serum ferritin values (fig. 2). Moreover, even in the presence of normal serum ferritin levels a substantial liver siderosis (liver iron concentration about 1 mg iron/g wet weight) can already be present [47].

Serum ferritin is increased unspecifically (false-positive results) during infections, in some malignancies (e.g. leukaemia), or as a consequence of acute liver cell damage when ferritin is released from the liver cell. Therefore, in a given patient, an increased serum ferritin value alone is not sufficient to establish a clinical relevant iron overload disease. Nevertheless, serum ferritin is a valuable parameter in screening studies and is also frequently used, despite its shortcomings, for monitoring disease and therapy in primary or secondary siderosis.

The iron content of serum ferritin can also be measured, and the diagnostic benefit was reported to be superior to that of ferritin protein [48]. However, we could not corroborate the diagnostic significance of this parameter in patients with iron overload [49].

Direct Parameters

Storage of iron in the heart is of special importance in advanced stages of iron overload, because heart failure is the most frequent cause of death in haemochromatosis [37]. In endomycard samples from autopsy or biopsy from patients with haemochromatosis a significant storage of iron in myocytes was found, inducing oedema, atrophy and fragmentation of cells [50]. The detailed mechanism of iron toxicity in the heart is not known. Because of the invasive character of such an in-

vestigation, the indication for a heart biopsy in haemochromatosis is very limited.

In former times, a duodenal biopsy was part of routine diagnostic procedures in some centres. In contrast to post-transfusional siderosis, there is a lack of iron storage in macrophages and an increased iron deposition in plasma cells of the duodenal mucosa in hereditary haemochromatosis [51].

So far the gold standard in the diagnosis and quantification of iron overload was liver biopsy [52]. In early haemochromatosis, a distinct increase of storage iron demonstrated by Prussian Blue staining is found in parenchymal cells of the liver, whereas Kupffer cells and sinus endothelial cells are almost free of iron deposits. With progression of iron storage other cells types such as Kupffer cells, sinus lining endothelia and bile duct epithelia contain excess iron. A semiquantitative histochemical determination of liver storage iron is possible using scoring systems [53]. More precise is the chemical determination of iron in the biopsy material [52, 54]. The liver iron concentration is the most reliable parameter to characterise the individual intensity of iron loading in haemochromatosis. Taking age into account, a hepatic iron index is useful to separate patients with homozygous haemochromatosis from heterozygous carriers for C282Y or from subjects with chronic alcohol abuse [52].

Since the *HFE* mutation diagnostic is available, liver biopsy is no more essential for the diagnosis of hereditary haemochromatosis. Therefore many centres do not recommend invasive liver biopsy any longer for the majority of patients although a biopsy is useful in clinically severe cases to assess liver architecture and to measure liver iron in clinically not affected cases.

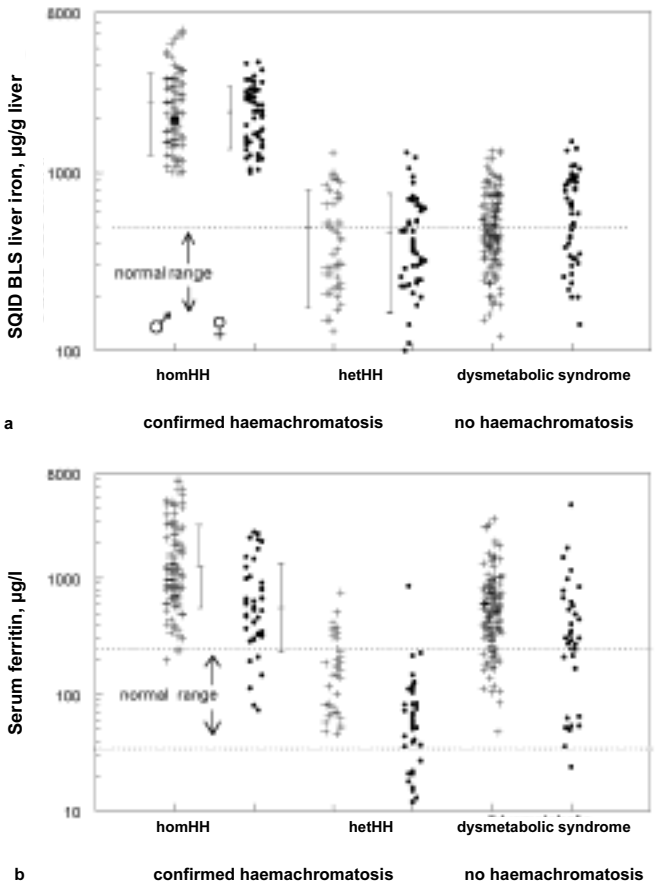

Fig. 3. **a** Liver iron concentration; **b** serum ferritin concentration in groups of subjects with homozygous (homHH), heterozygous C282Y mutation (hetHH) or in patients with chronic liver disease and wild-type HFE (liver disease).

Non-Invasive Measurements of Liver Iron

The result of large screening studies in different populations was that the majority of the patients diagnosed today showed only a sub-clinical haemochromatosis stage [42, 43]. Therefore a non-invasive technique for liver iron quantification is very helpful to i) identify patients with heavy iron overload who have a higher risk in the future to develop (more) severe clinical symptoms and ii) to characterise patients with rather low liver iron concentration who are in a low-risk group and need less intensive follow-up diagnostics. Such a technique would be the optimal supplement to *HFE* mutation analysis [55].

Three non-invasive techniques have been investigated for their use in the diagnosis of iron overload diseases: computed tomography (CT) [56], nuclear magnetic resonance imaging (MRI) [57–60], and SQUID biomagnetic susceptometry [61–63]. In CT, the liver density in Houndsfield units is clearly correlated to the liver iron concentration in vivo, however, the sensitivity in the diagnostically relevant range was found to be too low to differentiate for example between patients with chronic alcohol abuse and those with idiopathic haemochromatosis [56]. Dual energy technique was suggested to overcome these difficulties, but this could so far not be documented.

As far as MRI is concerned, Kaltwasser et al. [57] were the first to show that a special MRI technique using short echo time is able to quantify liver iron overload in patients with idiopathic haemochromatosis. This procedure was confirmed by others [58–60]. The advantage of MRI technique is the availability of the equipment in nearly every town. However, the quantitation of liver iron is a task more or less outside the mainstream of applications for MRI, and a special software and expertise is necessary. In addition, each MRI system needs a calibration with liver biopsies from a number of iron-loaded patients. Up to now, this technique has been used only within studies and is no routine method.

We have evaluated the clinical use of SQUID biomagnetometry in a large group of haemochromatosis patients [47, 61–63] as well as in patients with secondary siderosis [15]. This method needs a special equipment which is so far only available in Hamburg, New York, NY, Turin and in Oakland, CA. This technique can be offered for routine investigations for all

patients visiting our diagnostic centre in Hamburg. The costs are carried in most cases by the respective insurance companies (for info see *http://home.t-online.de/home/PNielsen*).
Figure 3 shows a comparison between liver iron and serum ferritin concentration in discriminating different groups of patients (C282Y homozygotes, C282Y heterozygotes, patients with liver disease). It is obvious that strongly increased serum ferritin values are not only found in haemochromatosis but also in subjects with various liver diseases (hepatitis, cirrhosis, fatty liver), whereas a substantial liver siderosis (> 1 mg iron/g wet weight) is restricted mainly to homozygous subjects with haemochromatosis.

HFE Gene Mutation Analysis

Although >30 allelic variants of the *HFE* gene have been reported, in Northern European population only the C282Y and the H63D have diagnostic relevance [25]. This holds also for the S65C mutation which is part of the diagnostic program in some laboratories in Germany following the positive results in French patients [64]. However, among 500 subjects suspected to have iron overload and tested also for the S65C mutation, we found only 12 carriers (including 1 H63D/S65C constellation). None of these subjects was substantially iron-overloaded. The diagnostic relevance of the S65C mutation in patients from Germany is therefore not obvious.
Different commercial or 'homemade' test systems for the C282Y and H63D mutation which should all be rather sensitive and specific are described in the literature. In Germany, a regular participation in a co-operative test is available and recommended, in order to guarantee the technical quality of the respective analytical method.
We have compared results from the system used in our laboratory with the commercial test system 'GenoType® Hereditäre Hämochromatose' (Hain Lifescience GmbH, Nehren, Germany) (see below).

PCR Test System for the Molecular Analysis of HFE Mutations (C282Y, H63D, S65C)

Genomic DNA was isolated from –20 °C-frozen EDTA whole-blood samples using the QIAamp Blood Kit (QIAGEN, Hilden, Germany). Purified DNA was stored in aqueous solution at –20 °C. Gene fragments surrounding the respective *HFE* gene mutations were amplified by polymerase chain reaction (PCR) using a 'Personal Cycler' (Biometra, Göttingen, Germany). Specific primers for the C282Y mutation were forward 5′-GTC ACC TCT TCA GTG ACCand backward 5′-AAT GAG GGG CTG ATC CAG; for the H63D mutation, the primers were forward 5′-ATG GGT GCC TCA GAG CAG; backward 5′-AGT CCA GAA GTC AAC AGT; and for S65C, forward ACA TGG TTA AGG CCT GTT GC; backward GCC ACA TCT GGC TTG AAA TT. The PCR mixture (100 µl) contained 1× buffer (10 m*M* Tris-HCl, pH 8.8 at 25 °C; 1.5 m*M* $MgCl_2$, 50 m*M* KCl,), 0.2 m*M* dNTP, 0.2 µ*M* of the respective set of primers, 2.5 U Taq DNA polymerase (Primezyme oder Dynazyme, Biometra, Göttingen, Germany) and 0.3–0.4 µg DNA. A 'touchdown' PCR with hot start between 72 °C and 50 °C annealing temperature (20 cycles) followed by 14 cycles at 50 °C annealing temperature was performed for all mutations. 10 µl of the amplified product were incubated with restriction enzymes *Sna*BI (MBI Fermentas, St. Leon-Rot, Germany) for C282Y mutation, *Bcl*I (MBI Fermentas) for H63D and HinfI (MBI, Fermentas) for the S65C mutation according to the protocols of the respective manufacturer. The digested DNA products were separated on pre-formed 4% NuSieve 3:1 agarose gels (FMC, Rockland, ME, USA) in TBE buffer (0.089 *M* Tris-Borate, pH 8.3, 2 m*M* EDTA, Sigma, München, Germany) at 150 V for 0.5–1.5 h.
The C282Y mutation forms a new cut sequence for *Sna*BI. Digestion of PCR products from mutated DNA with *Sna*BI forms two fragments of 197 and 40 bp, whereas the uncut DNA represents a 237 bp band. The H63D (S65C) mutation removes a cut sequence for the *Bcl*I (*Hinf*I) digestion from a 210 bp (208 bp) PCR product of mutated DNA, whereas wild-type DNA is cut into two fragments of 129 bp and 81 bp (or 147 and 61 bp).
Results from 233 subjects with different *HFE* constellations (34 C282Y homozygotes, 48 C282Y heterozygotes, 34 H63D homozygotes, 40 H63D heterozygotes, 47 compound heterozygotes and 30 with neither mutation) were analysed with the PCR technique described above and compared with results obtained using the commercial test system 'GenoType® Hereditäre Hämochromatose'. This test system, based on the Hain DNA·Strip® Technology was applied in its automated version with a Profiblot II T30 system (Tecan, Hombrechtikon, Switzerland). A complete match of the results was found for all samples. We found this test system very fast and convenient, especially when a large sample number has to be analysed.

Population-Based Screening for Haemochromatosis: Yes or No?

In populations of North European descent, the prevalence for C282Y homozygosity is estimated 1 of 250. Haemochromatosis meets most of the criteria of the WHO for population screening [62]. In Australia, a genetic population screening in all men > 40 years will therefore start in 2002/2003. This strategy will clearly identify a relevant number of patients with already severe clinical symptoms, who were so far not diagnosed. An even higher number of subjects will be detected who are homozgous for C282Y but who are at the time of diagnosis not affected in a clinical sense. These subjects would represent a group of optimal blood donors [41].

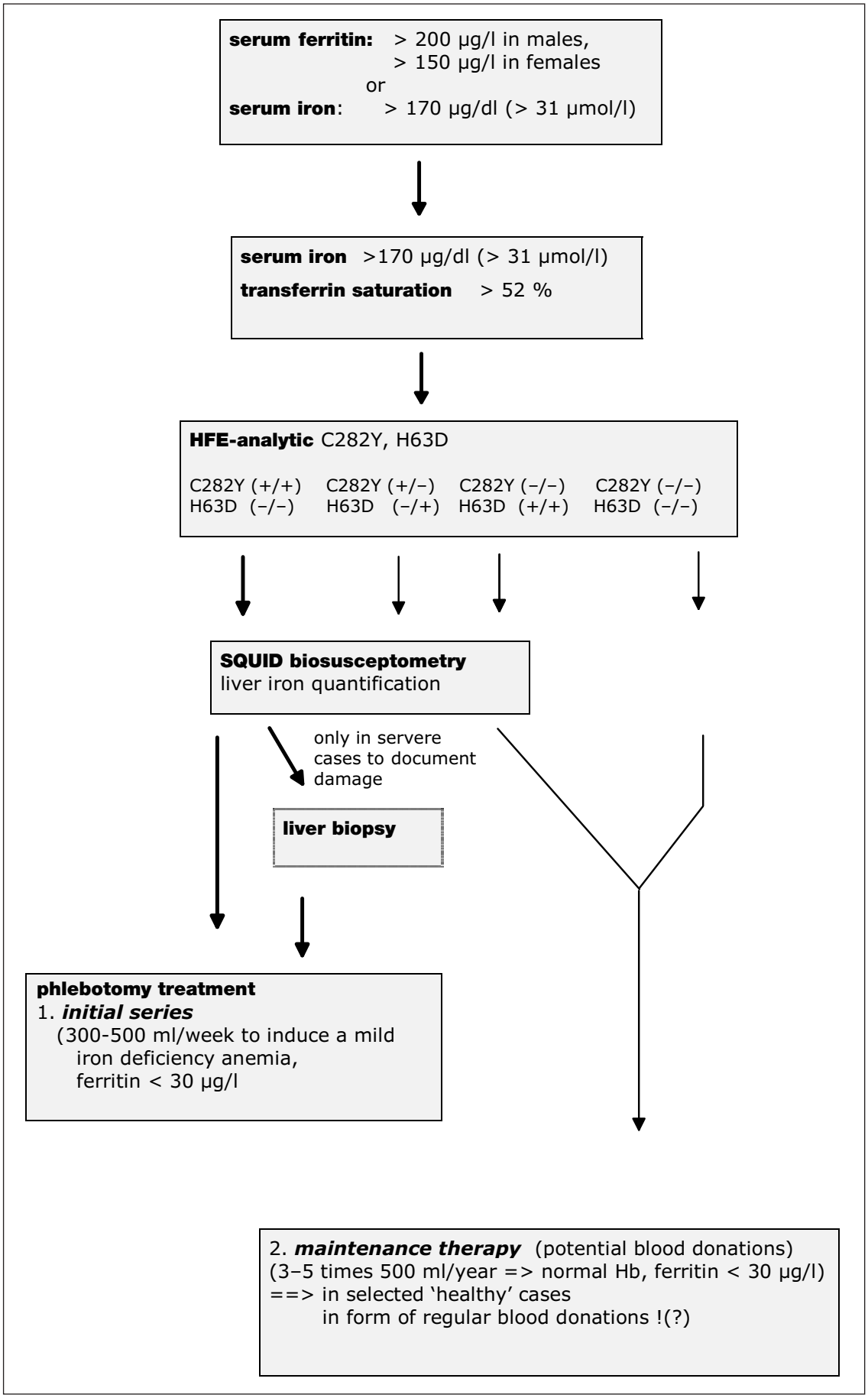

Fig. 4. Model for the diagnosis of haemochromatosis using a non-invasive method (SQUID biosusceptometry) for liver iron quantification. Without this technique available, we would recommend a classical liver biopsy only in severe cases in which organ damage has to be documented. The advantage of liver iron quantification for the patient is to get an information on the range of the respective excessive iron stores (how many phlebotomies necessary, high-risk, low-risk group).

On the other side, it should also be noted that questions about the penetrance of clinical expression, the efficacy of early treatment and the screening accuracy are yet not finally answered [65]. Genetic testing alone raises concerns about stigmatisation, discrimination and diminished self-worth of the positively tested individuals which may in part get never sick [66, 67].

Some experts therefore favour the phenotypic screening approach using for example serum ferritin, serum iron or transferrin iron saturation measurements in a first filter. It has been demonstrated that screening for hereditary haemochromatosis has also a favourable cost-effectiveness ratio over a wide range of assumptions [68]. Recently, in the large Norwegian study the estimated cost was USD 390.– per newly discovered subject with hereditary haemochromatosis [43]. The blood parameters mentioned above should be used routinely in patients coming into hospitals or visiting out-doctors and should also be involved in the initial testing in prospective blood donors. The gene testing should then be performed in a second step only in those subjects with increased blood values.

For both strategies, genotypic or phenotypic screening, we recommend a non-invasive technique (MRI, SQUID biosusceptometry) to finally characterise the individual iron load at the time of diagnosis. This is important to estimate the individual risks for a given patient with hereditary haemochromatosis. A classical liver biopsy is only advisable in patients with severe liver damage in order to document the liver status before treatment. A stepwise diagnostic procedure is outlined in figure 4.

Concerning transfusion medicine, it should be pointed out that haemochromatosis is also a significant topic in every blood transfusion centre. In larger units with for example 16,000 regular blood donors there must exist about 40–60 homozygous subjects. The greatest chance to detect these subjects is at beginning when the iron parameter is not affected by the following regular blood donations. The still ongoing debate in Germany is whether or not 'healthy' subjects with hereditary haemochromatosis are accepted as regular blood donors not in the initial phlebotomy series but during the following maintenance period (fig. 4).

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