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Review and actualizations of Molecular Genetic Diagnosis, Symptoms, and diagnostic strategies of Hereditary Hemochromatosis

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Summary

The hemochromatosis term describes a group of diseases caused by excess iron in the body. Hemochromatosis is the inborn error of metabolism most frequent (80%) and is considered a potentially serious illness due to cell damage that occur in different organs such as liver, heart, joins, skin or pancreas because for much of the life can develop without symptoms. Hereditary Hemochromatosis (HH) is an autosomal recessive disorder characterized by iron overload. Liver cirrhosis, diabetes, cardiomyopathy, arthritis, hypogonadism and skin pigmentation can be caused by iron overload. Most patients with hemochromatosis are p.Cys282Tyr homozygous or p.Cys282Tyr/p.His63Asp compound heterozygous. In addition to *HFE* gene, mutations in the genes that encode Hemojuvelin (HJV),Hepcidin (HAMP), Transferring Receptor 2 (TFR2) and Ferroportin (SLC40A1) have been associated with regulation of iron homeostasis and development of HH. The aim of this paper is to review the main gene mutations involved in the pathogenesis of HH type 1 to 4 and their genetic testing indication. With diverse diagnostic tests are reporting practices in use, there is a clear need for establishing a review of hemochromatosis genetic testing and diagnostic strategies ofHH. Most importantly, early and effectiveness diagnosis and treatment of HH prevents complications and results in a normal life expectancy. Review of algorithms of diagnostic strategies in HH is presented in this work.

Keywords: Hereditary hemochromatosis; HFE; non-HFE; Iron overload; Genetic testing; Genetic counselling

Introduction

The hemochromatosis term was originally used by von Recklinghausen in 1889 to describe tissue injury caused by iron overload. A current definition of hemochromatosis describes it as an inherited disorder of iron metabolism, characterized by inappropriately high absorption of iron by the gastrointestinal mucosa, leading to excessive storage of iron (particularly in the liver, skin, pancreas, heart, joints and testes) and ultimately resulting in impaired organ structure and function [1,2].

Hereditary Hemochromatosis (HH) is a common genetic disease with autosomal recessive inheritance that occurs predominantly in populations of northern and western European descent (prevalence: 1-4/1,000). Excessive digestive absorption of iron, leading to its progressive accumulation in different tissues of the body (notably liver, pancreas, and heart) and then to alteration of the structure and function of these organs is the principal characteristic of this disease. Hereditary hemochromatosis includes three stages: latency, biologic expression (appearing rarely < 20 years) and corresponding to increased iron parameters, such as serum iron, Transferring Saturation (TS), and Serum Ferritin (SF), and clinical expression. The first clinical symptoms appear generally at about age 40 years in males and later in females, approximately age 50 years, because of the protective effects of menstrual blood loss and of pregnancies. These clinical symptoms are nonspecific and include fatigue, skin pigmentation, hepatomegaly, arthritis, diabetes, and cardiomyopathy [1,3].

Hereditary hemochromatosis is one of the genetic diseases benefit from simple and efficient therapy when detected at an early. Treatment relies on regular therapeutic venesection, generally weekly, until iron depletion occurs (i.e., normalization of iron parameters) and is followed by a maintenance treatment. Hereditary hemochromatosis will have a poor prognosis and therefore could evolve toward irreversible damage, such as heart failure or hepatocellular carcinoma if treatment is not implemented early [1,4,5].

In 1996, a candidate gene for HH was cloned on chromosome 6, at position 6p21.3. This gene, *HFE,* encodes the HFE protein, which is a transmembrane glycoprotein implied in modulation of iron uptake. Figure 1 shows normal structure of HFE protein.HFE protein is an integral membrane protein of 343 amino acids that belongs to the family of major histocompatibility complex class Ib. This protein is localized in the gastrointestinal mucosa, liver, brain, lung, heart and regulates cellular iron uptake via the transferring and beta 2 microglobulin. HFE protein has three domains: a. Extracellular alpha domain: alpha 1, alpha 2, and alpha 3, on the extracellular surface, one of which (alpha 3) is coupled to the alpha 2 microglobulin. There is non-covalent association of alpha 3 domain with beta 2 microglobulin for complex structural assembly and exposure to the membrane, and are essential several disulfide bonds in alpha 2 and alpha 3, b. transmembrane region; c. cytoplasmatic tail.

The *HFE* gene contains a main mutation, p.Cys282Tyr (C282Y), corresponding to substitution of a Tyrosine (Tyr) for a Cysteine (Cys) at amino acid 282 and preventing formation of a disulfide bond. This mutation, whose allelic frequency varies between 0.5 and 10 percent in Caucasian populations, is present in a homozygous state in 80-95 percent of patients from northern Europe. In some US populations,

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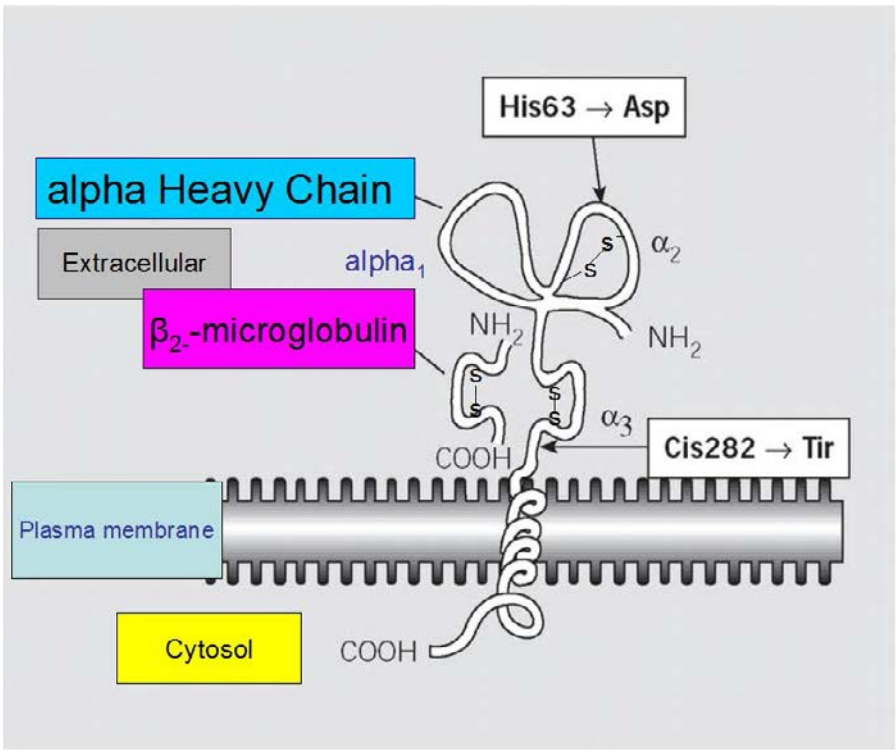

Figure 1: Normal structure of HFE protein [2].

only 60 percent of hemochromatosis patients are homozygous for this C282Y mutation. Other susceptibility factors associated with milder forms of HH (p.His63Asp (H63D) and p.Ser65Cys (S65C)) and about ten rare mutations have been identified in the *HFE* gene [1,5,6].

Discovery of the *HFE* gene has enabled a better understanding of the physiopathologic mechanisms implied in HH. However, this pathology remains complex and presents a large phenotypic heterogeneity. The different mutations identified in the *HFE* gene do not have the same penetrance, and rapidity of the evolution of iron overload can be modified by factors that may reduce the iron stores (blood donation) or increase them (intake of iron). Severity of HH can vary in patients with similar degrees of iron overload. For example, the risk of cirrhosis is increased by excessive alcohol consumption or the presence of viral hepatitis, while the risk of cardiomyopathy is increased by high intake of vitamin C, which potentiates iron uptake. Phenotypic expression of HH can therefore be influenced by environmental factors and is the result of interactions between the gene and modifying factors. The factors that influence phenotypic expression of HFE-related HH are not clearly defined. The reduced prevalence of iron overload related disease in female with p.Cys282Tyr mutation in homozygous state reflects a lower iron burden due to physiological blood loss, but also possibly sex-related disease modifier genes [1,7].

Clinical Presentation and Phenotypic Expression

The most common symptoms of HH are non-specific, such as lethargy, fatigue and arthralgia (Figure 2). In cases of significant iron overload, patients could present with organ specific symptoms such as those related to chronic liver disease. Arthralgia or arthritis related to hemochromatosis typically involves the second and third metacarpophalangeal joints. Endocrine dysfunction can take the form of diabetes or hypogonadism. Occasionally patients could present symptoms related to cardiomyopathy, either with symptomatic cardiac failure or arrhythmias. It is now quite rare to see the classic triad of bronzed skin, diabetes and cirrhosis, probably because of earlier diagnosis and management [4,8].

Commonly, patients are diagnosed with HH subsequent to the discovery of abnormal laboratory serum parameters (SF and TS), suggestive of iron overload as part of standard health checks. Iron overload may be identified as part of the evaluation of an asymptomatic individual with abnormal liver function tests. Additionally, an increasing number of cases are detected as a result of family studies [8,9].

Early non-specific symptoms include abdominal pain, weakness, lethargy and weight loss. Untreated individuals may develop hepatic fibrosis or cirrhosis and hepatocellular carcinoma develops in 25% of patients with established cirrhosis. In addition, untreated individuals could also develop progressive increase in skin pigmentation, diabetes mellitus, congestive heart failure and/or arrhythmias, arthritis and hypogonadism [9]. Symptomatic patients have biochemical evidence of iron overload (elevated TS and SF) and such altered biochemical parameters is found even in the absence ofsymptoms [4,6].

Most orthopaedic surgeons meet patients with undiagnosed HH on a yearly basis and an early diagnosis and treatment is important in order to avoid cirrhosis of the liver. Also, the risk of developing hepatocellular cancer is at least 20-fold higher than in their first-degree relatives. The mechanism behind the arthropathy in HH is unknown. In HH, the sensitivity to cartilage damage is presumably increased and/or reparative capacity is reduced. The presence of long-standing joint pain and/or osteoarthritis in a person below the age of 55-60 years should thus arouse suspicion of HH if the symptoms cannot be related to another specific disease, e.g. seropositive rheumatoid

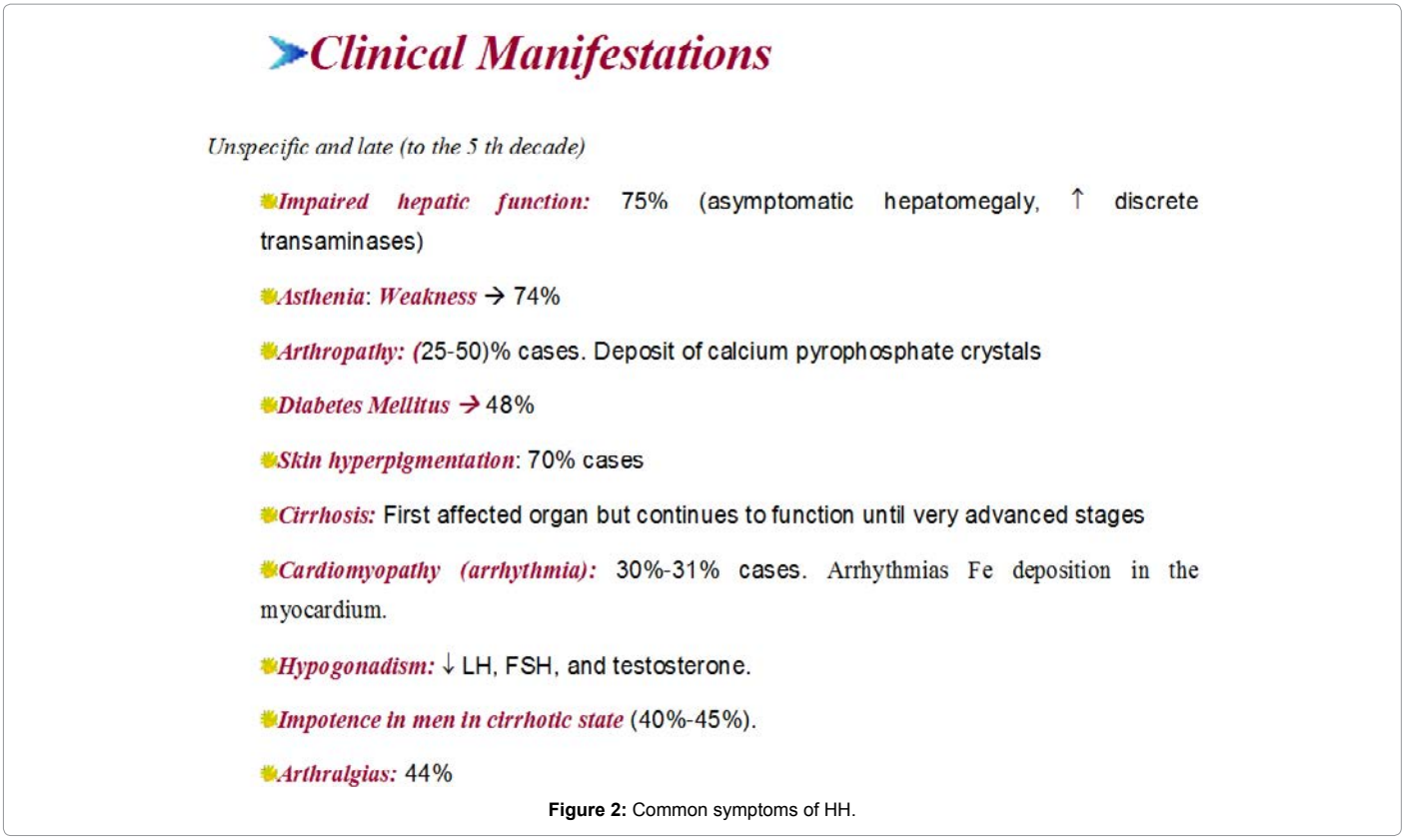

Figure 2: Common symptoms of HH.

arthritis, psoriasis, or arthritis urica. If more than once major joint is involved-notably bilateral ankle arthropathy without previous trauma-the suspicion is strengthened. In such cases, iron concentration in plasma or serum, total iron-binding capacity (TIBC), and SF should be analyzed. An iron-saturation level above 50% or an increased ferritin value should be followed by genetic testing. If this confirms, that the patient is homozygous, or has a so-called compound heterozygoty for HH, the individual should be referred to a gastroenterologist for further examination. Joint symptoms do not appear to be influenced by early diagnosis and treatment, but awareness of the condition and positive screening may prevent patients and their relatives from undergoing the more serious consequences of HH by regular phlebotomy [4,10,11].

Other Pathologies and Gene-Environment Interactions: Association with HH

Alcohol consumption relationship with expression in homozygous C282Y patients

The pathology of HH is multifactorial so it is interesting to analyze the complexity of gene-gene and gene-environment interactions. Phenotypic expression of HH is influenced by exogenous risk factors such as alcohol consumption.

Occult celiac disease (CD) relationship with hemochromatosis

Iron deficiency anemia in patients with HH rarely discovered. The genotype is done only in patients with fasting TS >45%. However, rare cases have been described and could be associated with disorders leading to duodenal atrophy with iron malabsorption [12].

More than 90% of patients with HH present C282Y mutation in homozygous state, but the majority of patients, who have been identified as homozygous for the C282Y mutation, have no evidence of iron overload. The current estimate of penetrance for C282Y mutation in homozygous state is that less than 1% of homozygotes may develop clinical symptoms. In contrast, another studies found a higher prevalence of 50%, but these data are difficult to interpret because clinical findings are not matched to a control group. Overall population prevalence for clinical HH and HH-related death appears to be far less than expected from C282Y allele frequency and heterozygosity frequency [12].

Normal HFE protein is expressed in cryptal enterocytes of the small intestine and acts to facilitate the iron-sensing function of these cells by a physical association with the Transferring Receptor (TfR1) at a strategic site to influence TfR1-mediated iron transport. This effect is lost by C282Y mutant HFE resulting in a "relative" iron deficiency of enterocytes and an uncoupling of iron uptake regulation. This uncoupling leads to increased expression of the Divalent-Metal Transporter 1 (DMT1) and Ferroprotein 1 (FP1). Whereas DMT1 represents a transmembrane transporter in the apical membrane of duodenal enterocytes which facilitates iron uptake from the intestinal lumen into the enterocytes. FP1 is involved in basolateral iron export into the portal circulation [12].

Iron transport across the basolateral plasma membrane of villus enterocytes involves intact epithelia with a sufficient amount of basolateral and apical transport proteins. Atrophy of the duodenal mucosa in chronic inflammatory disorders of the small bowel such as CD leads to iron deficiency [12].

CD and hemochromatosis are common HLA defined conditions with surprisingly high frequencies in population of Northern Europe commonly attributed to survival advantages. A genetic association

between CD and the Human leukocyte antigen (HLA)-D locus has emerged and it has been shown that over 95% of patients express the DQα1*0501 DQß1*0201 heterodimer (HLA DQ2). This locus on chromosome 6p is in close proximity to the *HFE*locus. Recently, *HFE* gene mutations have been found to be common and in linkage disequilibrium with different HLA alleles in CD patients compared with controls. A disease specific haplotype that carries both the p.Cys282Tyr *HFE* gene mutation and HLA DQ2 has been suggested but the origins of the genetic linkage still remain to be investigated in detail. The *HFE* gene may have spread due to the protection of heterozygotes against iron deficiency and the same might be true for CD which diminishes iron overload [12].

Occult CD could prevent increased DMT1 expression in a specific subset of individuals with homozygous C282Y mutations in the *HFE* gene thus contributing to the low penetrance of HH [12].

Mutations in HFE causing Hemochromatosis, association with primary hypertriglyceridemia

HFE mutations causing HH are approximately five times more frequently found in subjects with primary hypertriglyceridemia (HTG) than in controls. *HFE* locus is associated with primary HTG, in approximately 5% of these hyperlipidemias, the *HFE* genotype could play a role in their pathogenesis. Iron overload is present in subjects with primary HTG with a higher prevalence rate than in control groups. This is due to: 1) a high prevalence of HH genetic predisposition genotypes [C282Y homozygotes and compound heterozygotes (C282Y/H63D)]; 2) a high penetrance of the phenotype; and 3) iron storage is commonly increased in familial combined hyperlipidemia (FCH) and in familial HTG (FHTG) independently of the *HFE*genotype [13].

Molecular genetic diagnosis of type I hereditary hemochromatosis

HH is an autosomal recessive disorder characterized by enhanced intestinal absorption of dietary iron. The major mutation that has been associated with disease is the p.Cys282Tyr in the *HFE* gene that occurs in approximately 80% of HH cases. In addition, a high proportion of the remaining patients are compound heterozygous for the *HFE* p.Cys282Tyr and the common *HFE* p.His63Asp mutation. In Northern European populations, the *HFE* p.Cys282Tyr homozygous genotype is particularly common (1 in 200-300 healthy subjects) and the *HFE* 282Tyr allele frequency is high (5.1 to 8.2%) [2]. In contrast, in countries with radical/ethnic heterogeneity from South America, Asia and Africa a lower prevalence of HH have been observed, and an increased number of patients with primary iron overload do not carry the p.Cys282Tyr/p.Cys282Tyr or p.Cys282Tyr/p.His63Asp genotypes (for example, a minor allele frequency of p.Cys282Tyr allele of 2.3% is observed in Brazilian blood donors) [2,4].

Apart from the compound heterozygous state for C282Y and the widespread p.His63Asp (H63D) variant allele, other rare *HFE* mutations can be found *in trans* on chromosome 6 [14].

In addition to the *HFE*gene, mutations in the genes that encode Hemojuvelin *(HJV),*Hepcidin *(HAMP),*Transferring Receptor 2 *(TFR2)* and ferroportin *(SLC40A1)* have been associated with regulation of iron homeostasis and development of HH [2,6,15]. Genetic testing applied to HH can, in addition to performing the differential diagnostic with secondary iron overload, lead to more adequate and faster treatment.

Types of HH, Related Genes and Main Mutations

According to OMIN (Online Mendelian Inheritance in Man, www.ncbi.nlm.nih.gov/omin) five types of HH have been identified on the basis of clinical, biochemical, and genetic characteristics (Table 1). The classic hemochromatosis is most often caused by a mutation in a gene designated *HFE* on chromosome 6p21.3. Nonetheless, in minor frequency, there are four additional disorders of primary iron overload: Juvenile Hemochromatosis (JH) or type 2 hemochromatosis, which is divided into two forms: type 2A JH, caused by mutations in the *HJV* gene on chromosome 1q21, and type 2B JH, caused by mutations in the*HAMP* gene chromosome 19q13. HH types 3 and 4 are caused by mutations in the *TFR2* and *SLC40A1*genes on chromosomes 7q22 and 2q32, respectively (Table 1) [2,16].

HFE

HFE related-HH (OMIM 235200), classified as type I, is the most frequent form of the disease and the most common autosomal recessive disorder in Northern European populations. *HFE* gene (613609), constituted by 6 exons, encodes a membrane protein that is similar to major histocompatibility class I-like proteins, called *HFE* [2].

Most HH patients present p.Cys282Tyr/p.Cys282Tyr or p.Cys282Tyr/p.His63Asp genotypes. Besides the missense mutation at position 282, where cysteine is replaced by tyrosine (p.Cys282Tyr, c.845G>A, rs1800562) and the common substitution of histidine for aspartic acid at position 63 (p.His63Asp, c.87C>G, rs1799945), a third mutation is also commonly assessed: the substitution of cysteine for serine at amino acid position 65 (p.Ser65Cys, c.193A>T, rs1800730). However, recent reports have suggested that rare *HFE*variants, such as p.Gly43Ala, p.Leu46Trp, p.Val53Met, p.Gly93Arg, p.Ile105Thr, p.Gln127His, p.Asp129Asn, p.Glu168Gln, p.Glu168del, p.Leu183Pro, p.Glu277Lys, p.Gln283Pro, p.Val284Met, p.Arg330Met, and a deletion in the 6p chromosome region containing *HFE* could also be linked to HH thus contributing to genetic and phenotypic heterogeneity of the disease [2].

The first proposed pathogenic mechanism for explaining HH was the disruption of a disulfide bond in HFE that is critical for its binding to ß2 microglobulin. This complex interacts with transferring receptor 1, decreasing the affinity with transferring and consequently modulating iron absorption in enterocytes. However, in recent years, evidences indicating HFE protein as a hepcidin modulator have emerged. The functional loss of HFE in mice and humans has been shown to reduce hepcidin synthesis and that HFE loss seems to be associated with blunted signalling responses to BMP6 (bone morphogenetic protein 6), a key regulator of hepcidin, *in vitro* and *in vivo* [2].

After the identification of the *HFE* gene in 1996 it became apparent that not all cases of hemochromatosis are caused by mutations in *HFE*. HFE-associated HH (HFE-HH) or type 1 HH is the most common form, especially in populations of Northern European origin, where the C282Y mutation has a high allele frequency. Hemochromatosis that is unrelated to mutations in the *HFE* gene is collectively referred to as non-HFE hemochromatosis. Non-HFE hemochromatosis occurs in populationsworldwide and makes up a larger proportion of HH cases in areas where the C282Y mutation is less common, such as Southern Europe and Asia. Non-HFE HH could be further differentiated according to the gene mutated. There are four main types of non-HFE HH. The molecules mutated in all forms of HH are related in pathways involved in the regulation of iron homeostasis [2].

HJV and HAMP

Juvenile hemochromatosis (JH), also classified as type 2, is a rare autosomal recessive disorder of iron overload that leads to organ damage before the age of 30, and usually causes cardiomyopathy,

HH types	Phenotype MIM number	Gene MIM member	Location/ Gene Map Locus	Inheritance	Gene productfunction	Clinicalfeatures	Laboratoryfinfings	Liverpathology	Functionalconsequences of mutations
1	235200	*HFE,* (HLA-H) 613609	6p21.3	AR	Involved in hepcidin synthesis way BMP6, interaction with TFR1	Fatigue, lethargy, arthropathy, skin pigmentation, liver damage, diabetes mellitus,endocrine dysfunction, cardiomyopathy, hypogonadotropic, hypogonadism	↑serum ferritin ↑transferrin saturation	Hepatocyte iron loading, fibrosis cirrhosis	Impaired hepcidin regulation by iron leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2A	602390	Hemojuvelin *HJV,* (HFE2) 608374	1p21	AR	Involved in hepcidin synthesis, BMP co-receptor	As for HFE Earlier onset (<30 yr). Cardiomyopathy and hypogonadism more prevalent	↑serum ferritin ↑transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Loss of hepcidin regulation, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2B	613313	Hepcidin *HAMP,* 606464	19q13	AR	Downregulation of iron efflux from enterocytes	As for HFE Earlier onset (<30 yr). Cardiomyopathy and hypogonadism more prevalent	↑serum ferritin ↑transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	No/inactive hepcidin, leading to maximal iron absorption and release of iron from reticuloendothelial cells
3	604250	Transferrin Receptor 2 *TFR2,* 604720	7q22	AR	Involved in hepcidin synthesis, interaction with transferrin	As for HFE	↑serum ferritin Normal transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Impaired hepcidin regulation by iron, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
4	606069	Ferroportin *SLC40A1*, 604653 IREG1, MTP1	2q32	AD	Duodenal ironexport	Typical presentation: as for HFE, except generally milder. May have mild anemia and lower tolerance to phlebotomies	↑↑serum ferritin ↑transferrin saturation	Predominant Kupffer cell iron loading, fibrosis	Reduced ferroportin iron transport ability, leading to accumulation of iron in reticuloendothelial cells
						Atypical: as for HFE	↑serum ferritin ↑transferrin saturation	Predominant hepatocyte iron loading, fibrosis, cirrhosis	Loss of ferroportin regulation by hepcidin, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells

MIM: Mendelian Inheritance in Man; TFR1: transferring receptor 1, *HFE*: encodes HFE protein; *HJV*: encodes hemojuvelin; *HAMP*: encodes hepcidin; *TFR2*: encodes transferring receptor 2; *SLC40A1*: encodes ferroportin; BMP6: bone morphogenetic protein 6; AR: Autosomal recessive; AD: autosomal dominant [2,4,16,17,18,19]

Table 1: Types of HH: genetic, clinical characteristics and laboratory features.

hypogonadotrophic hypogonadism, liver damages and endocrine dysfunctions. Types 2A (OMIM 602390) and 2B (OMIN 613313) are caused by mutations in *HJV* and *HMAP* genes, respectively [2].

HJV (608374) gene, constituted by 4 exons, was identified in 2004 and encodes a protein called hemojuvelin. Patients with type 2A JH and knockout mice models demonstrate low hepcidin levels implying that hemojuvelin is involved in the hepcidin synthesis. *HAMP* gene (606464), constituted by 3 exons, encodes hepcidin, a peptide known as iron hormone. Hepcidin is produced by hepatocytes and it plays a role absorption related to ferroportin degradation if the enterocytes [2].

Several HJV mutations have been found: p.Arg54del, p.Cys80Arg, p.Ser85Pro, p.Gly99Arg, p.Gly99Val, p.Leu101Pro, p.Gly116del, p.Cys119Phe, p.Ile222Asn, p.Arg131fs, p.Asp149fs, p.Leu165del, p.Ala168Asp, p.Phe170Ser, p.Asp172Glu, p.Arg176Cys, p.Trp191Cys, p.Asn196Lys, p.Ser205Arg, p.Ile222Asn, p.Lys234del, p.Asp249His, p.Gly250Val, p.Asn269fs, p.Ile281Thr, p.Arg288Trp. p.Cys321Trp, p.Cys321del, p.Arg326del, p.Ser328fs, p.Cys361fs, and p.Arg385del. However, the *HJV* p.Gly320Val is the most frequent mutation and has been reported in JH patients in several populations around the world [20]. Mutations in *HAMP* are a very cause of JH: p.Met31fs, p.Met50fs, p.Arg56del, p.Arg59Gly, p.Cys70Arg, p.Gly71Asp, and p.Cys78Thr. In addition, some studies support the concept that digenic inheritance of *HFE* and *HJV* or of *HFE* and *HAMP*mutations could lead to iron overload or may aggravate the phenotype [2].

For both type 2A and 2B JH, it is well established that the cause of iron overload could be explained by decreases in the synthesis and, consequently depressed hepcidin levels (Figure 2). Cell-surface expression of hemojuvelin was associated with increased expression of hepcidin; likewise, loss of hemojuvelin expression, as in juvenile hemochromatosis, was associated with reduced hepcidin expression [2,16].

HJV seems to play a role in iron absorption and release from cells and has anti-inflammatory properties. HJV acts as a BMP co-receptor and signals via the SMAD pathway to regulate hepcidin expression. A BMP6 dependent signalling pathway has been shown to play a key role in regulation of hepcidin expression (Figure 3). BMPs bind to type I and type II serine threonine kinase receptors, which phosphorylate specific intracellular SMAD proteins (SMAD 1, 5, 8). Phosphorylated SMAD 1, 5, 8 (P-SMAD 1, 5, 8) binds to the common mediator SMAD4, and the SMAD complex translocates to the nucleus to affect transcription of target genes *HAMP* (encoding hepcidin) is transcriptionally up-regulated by BMPs. Impaired hepatic signalling through mutations in genes encoding either the ligand BMP6, the BMP co-receptor hemojuvelin or SMAD4 leads to low hepcidin levels and iron overload

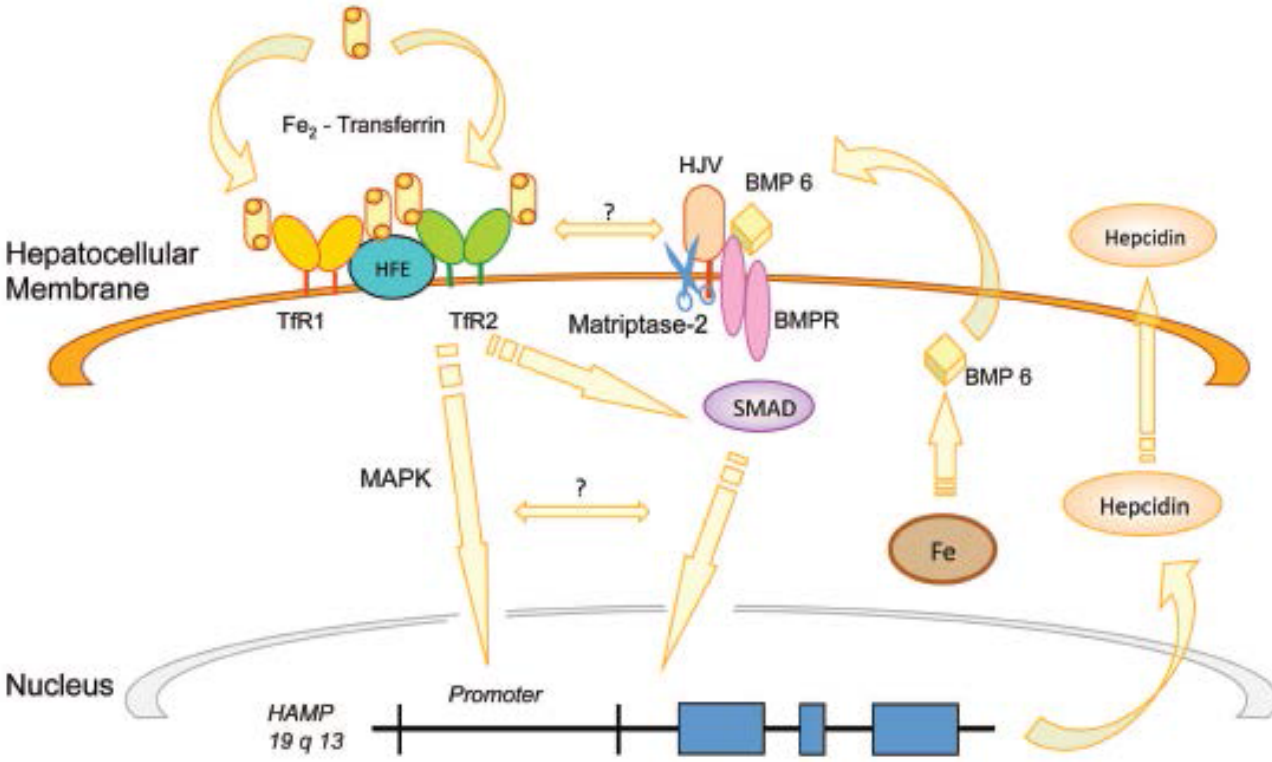

Figure 3: Iron regulatory pathways of hepatocyte hepcidin synthesis [15].

in mice. Collectively, these data show that BMP-SMAD signalling is an important regulatory pathway for hepcidin expression and thus iron metabolism [2,16].

Cardiomyopathy and hypogonadism are more significant features of JH, hypogonadism being the most common symptom at presentation. The rapid accumulation of iron in subjects with JH could be fatal, and the death usually is the resul from heart failure.

TFR2

Type 3 HH (OMIN 604250) is an autosomal recessive disease caused by mutations in *TFR2* gene (604720), constituted by 18 exons, and encodes Transferring Receptor 2 protein (TFR2) [2, 21]. TFR2 is involved with uptake of transferrin bound iron by hepatocytes and it is also involved in the hepcidin synthesis. One possibility is that it operates in the pathway discussed above for HFE (or in a parallel pathway of its own) facilitating the BMP/SAMD signalling that activates hepcidin expression [2]. Another possibility is that TFR2, which is also able to interact with HFE, forms an iron-sensing complex that modulates hepcidin expression in response to blood levels of diferric transferring (Figure 2) [15,16].

Few *TFR2* mutations have been reported: p.His33Asn, p.Glu60del, p.Arg105del, p.Met172Lys, p.Tyr250del, p.Gln317del, p.Arg396del, p.Ala444Thr, p.Arg455Gln, p.Arg481His, p.Leu490Arg, p.Val561del, p.Gln690Pro, and p.Gly792Arg. In both animal models and patients with TFR2 related-HH decreased hepcidin levels were observed [2].

This proposed model depicts two iron signals to hepcidin, one mediated by intracellular iron stores (Fe) and the other by Ferri-Transferrin (Tf-Fe_2) Hepatocellular stores increase the expression of bone morphogenetic protein 6 (BMPG6), which serves as an autocrine factor by interacting with surface BMP receptors. Hemojuvelin (HJV) is a BMP co-receptor which augments BMP binding. The consequent activation of intracellular SAMD proteins transduces a signal to increase hepcidin transcription. Under low iron conditions matriptase-2 (scissors) cleaves HJV from the cell surface, weakening the BMP6 signal. Extracellular Tf-Fe_2 mediates a second iron signal, in this schema Tf-Fe_2 displaces HFE from TfR1. HFE is then freed to interact with transferring receptor 2 (TfR2). The HFE-TfR2 complex activates hepcidin transcription via MAPK and/or BMP/ SMAD signalling. These pathways have recently been reviewed [15].

SLC40A1

Type 4 HH (OMIN 606069) has an autosomal dominant pattern and it is caused by mutations in the *SLC40A1* gene. This rare disease can present peculiar clinical features such as high SF levels plus low or normal TS values until the end stage of the disease. It may also be the presence of a mild iron-deficient anemia in the initial stage a reduced tolerance to therapeutic phlebotomy. *SLC40A1* (604353) gene, constituted by 8 exons, encodes a membrane transporter called ferroportin that modulates iron efflux. SLC40A1 mutations, such as p.His32Arg, p.Tyr64Asn, p.Val72Asp, p.Ala77Asp, p.Gly80Val, p.Arg88Thr, p.Asn144His, p.Asp157Gly, p.Asp157Asn, p.Val62Asp, p.Asn174Ile, p.Arg178Gly, p.Ile180Thr, p.Asp181Val, p.Gln182His, p.Asn185Asp, p.Gln248His, p.Gly267Asp, p.Gly323Val, p.Cys326Ser, p.Cys326Tyr, p.Gly330del, p.Ser338Arg, p.Arg489Ser, p.Gly490Asp, and p.Gly490Val were associated with type 4 HH. Two hypotheses have been proposed to account for this disease: The trapping of iron in macrophages that are unable to export iron and the failure to be degraded by interaction with hepcidin [2].

In conclusion, are four main genes implicated in non-HFE

hemochromatosis? The study of these disorders has led to a greater understanding of how the body regulates iron homeostasis. All the genes implicated in the different forms of hemochromatosis are involved in the regulation and maintance of iron homeostasis. Hepcidin is at the centre of the iron regulatory pathway. Its expression in the liver could be regulated by the activities of HFE, TfR2 and HJV. Hepcidin itself could regulate the activity of the iron exporter ferroportin. Mutations in any one of these genes can disrupt the regulation of iron homeostasis and lead to iron overload [2, 6].

Biochemical assays for evaluating hemochromatosis

The most common biochemical tests performed in routine for iron overload analysis are serum iron, TIBC, TS, which is a ratio between serum iron and TIBC expressed as percentage, and SF. SF is a highly sensitive test for iron overload in HH, but it has low specificity, being also elevated in patients with inflammatory process, diabetes, alcohol consumption, and liver damage [2].

Typically, TS values could be a helpful tool as a marker of iron overload. Some studies reported that TS values are frequently elevated in 50% in females and 60% in males with iron overload caused by genetic alterations [2].

It can be established five stages according with values of biochemical parameters and clinical symptoms [14] (Table 2).

Serum Indices of Iron Stores

The appropriate interpretation of TS and SF results is essential in the diagnosis of iron overload. Fasting TS (the ratio of serum iron to TIBC) is the most sensitive initial phenotypic screening test. A cut-off value of ≥ 45% will detect almost all affected C282Y homozygotes. An early morning sample is recommended as serum iron concentration could be misleading elevated in the postprandial state and also by circadian rhythm. However one large study has suggested that the use of fasting TS had no advantage over the use of random samples in a primary care population. Unsaturated Iron Binding Capacity (IBC) has also been used and is a valid alternative to TS.

SF reflects body iron stores and generally rises later in the progression of iron overload. Excess of iron increases the hepatic production and release of ferritin. Interestingly, the role of ferritin in the blood remains unclear. There are a number of confounding causes of hyperferritinemia that warrant consideration (Table 3). These include alcohol abuse, the metabolic syndrome, inflammatory states and acute or chronic hepatitis. There are secondary iron overload and other pathologies that cause elevated levels of TS and SF (Table 3). In the absence of these conditions, SF is a good marker greater than 1000 µg/L indicate a greater risk of cirrhosis or advanced fibrosis and have been used, irrespective of age and transaminase levels, as an indication for liver biopsy. The negative predictive value of a normal TS and SF is 97%. In this situation, no further testing is recommended.

Hematologic values

Recent studies have found higher mean corpuscular volume (MCV) and haemoglobin levels in patients with C282Y homozygous state with increased TfS at the time of diagnosis than in patients with other HFE genotypes or control subjects. This is likely attributable to increased iron uptake by erythroid precursors. Among C282Y homozygotes, the adjusted mean MCV and haemoglobin levels in women, but not in men, were higher than in HFE wt (wild type)/wt controls.

This effect was observed even for female C282Y homozygotes with SF in the reference range, suggesting that an as-yet-unidentified influence of the HFE C282Y/C282Y genotype is a significant determinant of MCV and haemoglobin in women, in addition to the effects of elevated TfS and SF [17].

Genetic testing and technical assays for molecular diagnostic of HH

*HFE*testing for the two common mutations (p.Cys282Tyr and p.His63Asp) should be performed in all subjects with unexplained increased TS and/or SF values. In these cases, the molecular diagnostic of *HFE* related-HH is usually associated with the presence of p.Cys282Tyr/p.Cys282Tyr, (p.[Cys282Tyr];[Cys282Tyr]) and p.Cys282Tyr/p.His63Asp (p.[Cys282Tyr];[His63Asp]) genotypes. However, p.His63Asp homozygous (p.[His63Asp];[His63Asp]) and p.His63Asp/p.Ser65Cys (p.[His63Asp]:[Ser65Cys]) compound heterozygous genotypes have been associated with HH phenotype [2, 7].

In the absence of the mentioned *HFE* genotype combinations, other HH type could be considered. When there is genetic iron overload in a patient with less than 30 years and cardiac or endocrine manifestations, JH diagnostic is suggestive. Thus, the evaluation of the p.Gly320Val mutation in the *HJV* gene must be the molecular test of choice [20]. According to several studies, this procedure would

Stages	*Biochemical Parameters and clinical symptoms*
Stage 0	No biochemical and clinical abnormalities
Stage 1	Increased TS (>45%), normal serum ferritin No clinical symptoms
Stage 2	Increased TS, increased serum ferritin (>200µg/L in females and >300 µg/L in males) No clinical symptoms
Stage 3	Abnormalbiochemical values Initial clinicalsymptoms (fatigue, arthritis, impotence, skin hyperpigmentation)
Stage 4	Abnormal biochemical values Clinical symptoms manifesting organ damage (cirrhosis, diabetes, hypogonadism, or cardiomyopathy)

Scale proposed by the Haute Autorité de Santé as clinical recommendations on the HH management [14]

Table 2: Stages in HH.

HH	HFE related HH (C282Y/C282Y, C282Y/H63D)
	Non-HFE related HH Juvenile Hemochromatosis Hemojuvelin related Hepcidin related Transferrin receptor-2 related HH
	Ferroportin related HH
Secondary Iron Overload	Iron loading anemia Thalassemia major Sideroblastic anemia Chronic haemolytic anemia
	Parenteral iron overload (multiple transfusions)
Others	Metabolic syndrome
	Chronic liver disease Hepatitis C Alcoholic liver disease Non-alcoholic steatohepatitis Porphyria cutanea tarda
	African Iron Overload
	Very low levels of ceruloplasmin
	Very low levels of transferrin
	Neonatal iron overload

Table 3: Classification of Iron Overload [6,8].

confirm the majority of JH cases. Early diagnosis is paramount. If result is negative, sequencing should be performed to evaluate the *HJV* and *HAMP* genes [2].

Mutations in the *TFR2* and *SLC40A1* genes are rare compared with *HFE* mutations and they have also been reported in children, adolescents, and adults. These genes should be sequenced after negative results for other genes. Nowadays, the costs for sequencing have come down, especially if it evaluates the number of bases per dollar of the next generation sequencings. However, for the most part of clinical practice around the world, screening of *HFE,HJV, HAMP, TFR2* and *SLC40A1* through direct sequencing is not widely available. This approach is usually reserved for scientific studies and for very specific cases such as patients who are not responsive to treatment and had more severe complications due to iron overload. In addition and for the most part of cases, the treatment is not dependent on molecular diagnosis [2].

The study of p.Cys282Tyr and p.His63Asp mutations is one of the most requested molecular tests in the genetic laboratories (Figure 3). The analysis of single nucleotide polymorphisms, for example *HFE* p.Cys282Tyr, *HFE* p.His63Asp, and *HJV* p.Gly320Val, could be performed by several available methods for genotyping, such as restriction fragment length polymorphisms (RFLP) allele-specific amplification analysis real time-polymerase (RT-PCR), denaturing HPLC, sequencing strategies, TaqMan assay, multiplex amplification followed by reverse hybridization, allele-specific oligonucleotide assay (ASO), reverse hybridization line-probe assay (LiPA) [7]. A larger number of tests are used by laboratories: Most use a combination of PCR and restriction enzyme digest for one or both mutations (Figure 4 and Table 4). Other methods employed include real time PCR (LightCycler), allele-specific PCR, heteroduplex analysis and PCR-SSCP, real-time PCR followed by melting curve analysis using fluorescence resonance energy transfer (FRET) probes (also called hybridization probes) [22]. The European Molecular Genetics Quality Network (EMQN) and United Kingdom National External Quality Assessment Service (UKNEQAS) assessors have commented on the wide range of different technological approaches used. It is important to note that the S65C variant (A193T), which is just six bases away from the H63D mutation, has been reported to interference with H63D analysis for some methods such as the LightCycler and the Stott duplex method, such that H63D/S65C compound heterozygotes appear as

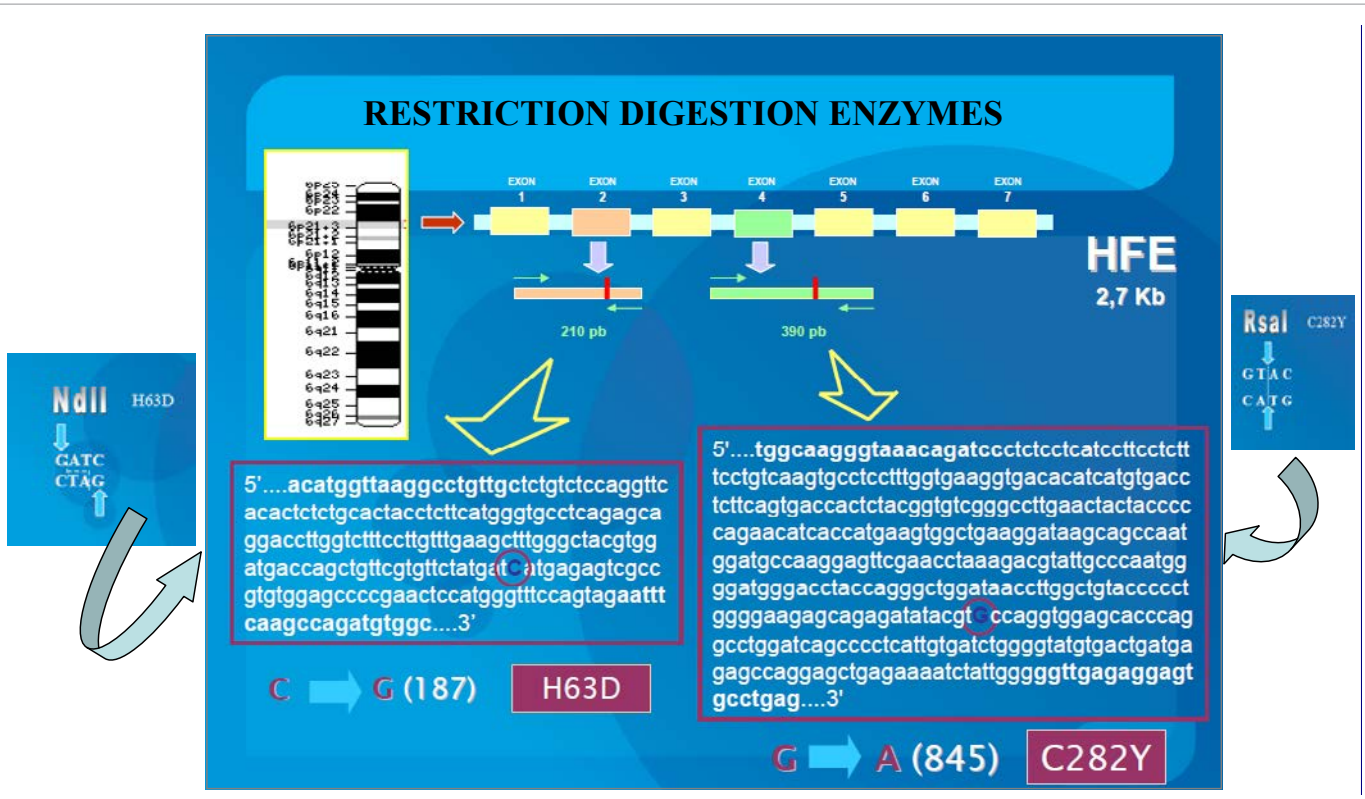

Figure 4: Restriction enzyme digest. Genotyping of *HFE* p.Cys282Tyr and p.His63Asp mutations.

Test method	Mutations Detected	% of Individuals [in populations of European origin] with HH	Genotype
Targeted mutation analysis	HFE mutations: p.C282Y, p.H63D	~60-90	p.C282Y/p.C282Y
		3-8	p.C282Y/p.H63D
		~1	p.H63D/p.H63D[1]
Sequence analysis	HFE sequence alterations	Unknown	Unknown (compound heterozygotes for p.C282Y allele and rare HFE mutation)

[1]There is no evidence that p.H63D/p.H63D (homozygous for H63D in HFE: p.[His63Asp];[His63Asp]) is associated with a hemochromatosis phenotype in the absence of another cause of iron overload

Table 4: Molecular Genetic Testing Used in HFE-HH [7].

H63D homozygotes. Therefore, apparent H63D homozygous detected by such methods must be subjected to further analysis to out rule interference by S65C giving an erroneous result [2].

The clinical criteria required for molecular testing to proceed depend on local guidelines. Suggested biochemical criteria include elevated, fasting, serum TS and persistently raised SF concentration. Numerically, local biochemical testing methodologies will dictate what constitutes elevated TS but a result of 45% would generally be accepted for genetic testing, particularly to facilitate early detection of iron overload. A value exceeding the local upper limit of normal constitutes an elevated concentration of ferritin. However, an elevated ferritin is not specific for HH and therefore should be considered in conjunction with indicators of inflammation and liver disease. Iron studies are not necessary for predictive testing but such cases may benefit from a referral to a clinical genetics service [6].

HFE Gene Mutation Testing

Only those who are homozygous to develop significant iron overload related to *HFE* gene mutations. Testing for *HFE* gene mutations is generally indicated in those with an iron overload phenotype and those with a family history of *HFE*-related HH.

Confirmatory diagnostic testing currently involves molecular genetic testing for the p.Cys282Tyr and p.HisH63Asp mutations in the *HFE* gene or histological assessment of hepatic iron stores on liver biopsy. Most individuals with HFE related HH are either homozygous for the C282Y mutation (80-93%) or compound heterozygous for the mutations C282Y and H63D (<5%). Genetics testing for non-HFE related hemochromatosis is not widely available and diagnosis may have to be based on liver biopsy findings [4].

Predictive testing for at-risk relatives (e.g. siblings of genetically confirmed HH individuals) may be requested as well as carrier testing for family members (Figure 5). Pre-natal diagnosis is not usually offered as the condition is a treatable, adult onset condition.

Population screening is not currently recommended primarily due to the penetrance issue surrounding the C282Y mutation [23].

Carrier testing is routinely offered to close relatives of confirmed cases or of confirmed carriers. When devising a carrier testing policy, it is worth considering whether more distant relatives have carrier risks elevated above that of the general population [4].

Subjects with p.Cys282Tyr/p.Cys282Tyr and p.Cys282Tyr/p.His63Asp mutations are known to be predisposed to the development

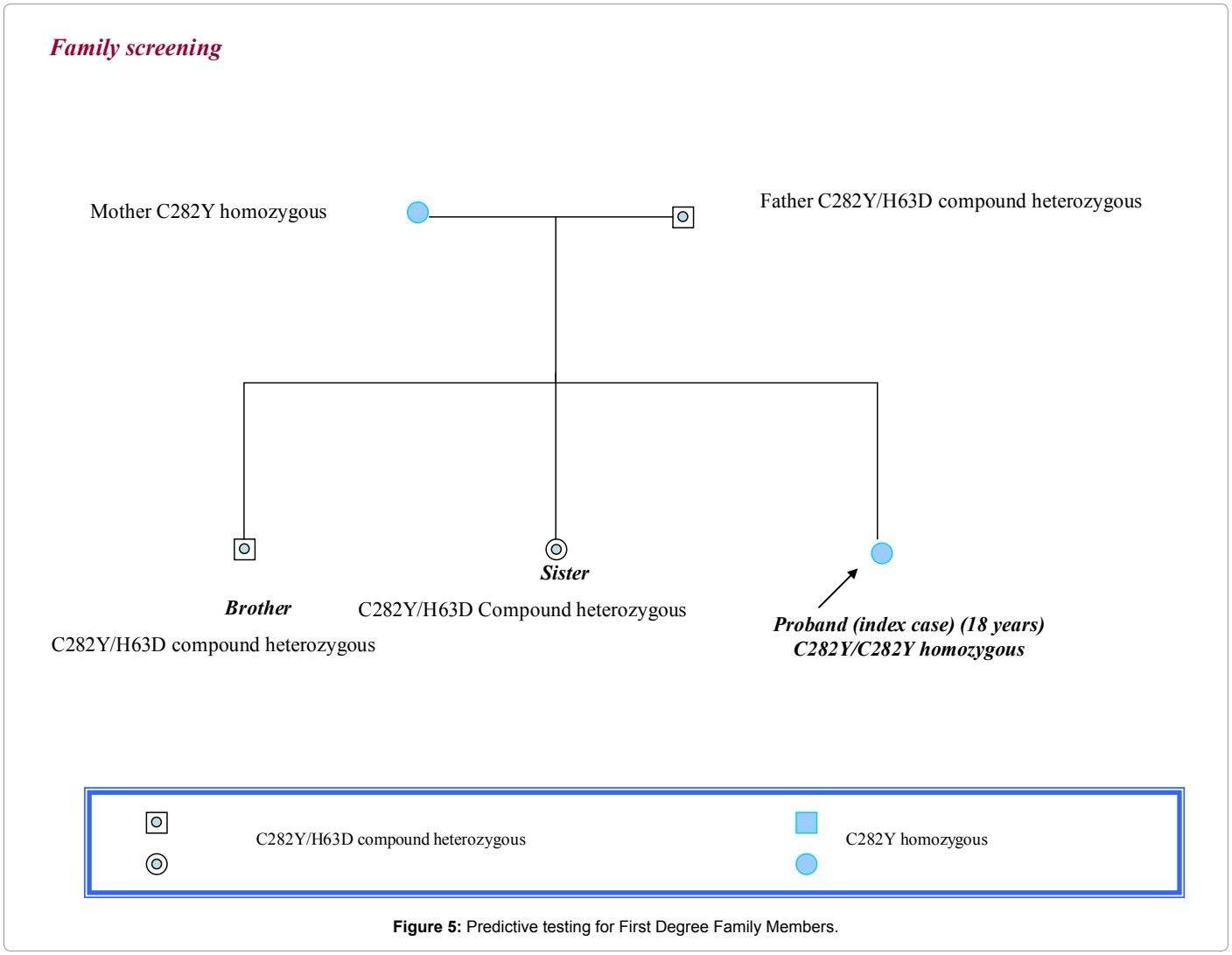

Figure 5: Predictive testing for First Degree Family Members.

of HH. As the significance of H63D in the homozygous state is unclear, H63D testing is currently only clinically useful in individuals heterozygous for C282Y. This would seem to imply that, ideally, C282Y should be tested for initially and H63D analysis performed on C282Y heterozygotes only as a reflex test. However, when there is a family history of HH, both mutations should be tested for, even if the case is homozygous for C282Y and especially if extensive screening has not been performed in the family. The reason is that, due to the high carrier frequency of H63D (25% in the general population), it is possible for both C282Y and H63D to be present in the same family and therefore for a family member to be a compound heterozygote at risk of iron overload. H63D testing is appropriate when carrier status is required due to family history or for a spouse of a homozygote or heterozygote. Where a method detects both mutations concurrently, there is an obligation to report the complete result [4].

With respect to testing for mutations/variants other than C282Y and H63D, there is little published evidence that warrants such testing for diagnostic purposes. If a test picks up S65C, a genetic laboratory may have an obligation to report the presence of this variant if it occurs in a compound heterozygote state with C282Y. However, in the absence of any clinical symptom, it would be best to avoid detecting S65C and hence the obligation to report on it is removed [4].

Reporting Results of Genetic Testing

Each laboratory could have its own reporting format

Symptomatic-diagnostic test

Diagnostic referral (i.e. affected individual) and genotype C282Y homozygous: Reports should state, at a minimum, that this genotype is consistent with a diagnosis of HH. Additional comments could refer to implications of the result for other family members, offer carrier testing and suggest that genetic counselling be considered. If iron overload is present, lifelong venesection is required. Cirrhosis is unlikely if the ferritin level is <1000 μg/L, the aspartate aminotransferase (ALT) level is normal, and there is no hepatomegaly. Liver biopsy may be performed to establish or exclude the presence of cirrhosis if blood tests are suggestive of cirrhosis.Iron biochemical studies could be done every 2-5 years in subjects without iron overload. Testing of all first degree family members is recommended once they have reached the age of 18 years. Those who are p.Cys282Tyr/p.Cys282Tyr genotype, who wish to test offspring under the age of 18 years, should first test their partner, as offspring will not be affected by HH if the partner does not carry the C282Y mutation. There have been no reports of iron overload from C282Y homozygosity occurring before the age of 18 years, therefore children should be tested after they have turned 18 years and can self-manage any preventive measure [4].

Diagnostic referral (i.e. affected individual) and genotype C282Y/H63D compound heterozygous: Some subjects with this genotype could have iron overload but to a lesser degree than patients with C282Y mutation in homozygous state. Therefore, the genotype is consistent with the presence of iron overload and may be diagnostic of HH once all other reasons for iron overload have been excluded (e.g. alcohol consumption, hepatitis C, hyperferritinemia). Another comment could refer to implications of the result for other family members, offer carrier testing and suggest that genetic counselling be considered. About one to two percent of subjects with this genotype develop hemochromatosis. Monitoring biochemical parameters every 2-5 years should be indicated. Elevated SF may also be due to acute phase reactants and therefore other causes should be investigated if SF is persistently elevated over 6 months in the absence of elevated fasting TS [4].

Diagnostic referral (i.e. affected individual) and genotype C282Y heterozygous: This genotype makes a diagnosis of HH unlikely and such a diagnosis could only be made on a clinical basis. About 25% of patients with C282Y mutation in heterozygous state could exhibit mild to moderately raised indices of iron overload but complications are rare and may be influenced by additional factors, both genetic and environmental. In addition, other forms of iron overload and other types of hemochromatosis exist; therefore, a referral to a specialist unit could be suggested. Additional comments may refer to implications of the result for other family members and suggest that genetic counselling be considered (Figure 6,7). It is not considered appropriate to state that all relatives should be tested. The result should not state that a diagnosis of hemochromatosis is excluded [4].

Diagnostic referral (i.e. affected individual) and genotype H63D homozygous: This genotype is present in about 2% of the population and its significance remains uncertain. Subjects with H63D homozygous state have a slight risk of iron overload and therefore a diagnosis of hemochromatosis cannot be excluded and must be made on a clinical basis. Additional comments may offer carrier testing for other family members and suggest that genetic counselling be considered. It is not considered appropriate to state that all relatives must/should be tested [4].

Diagnostic referral (i.e. affected individual) and genotype H63D heterozygous: This genotype makes a diagnosis of HH very unlikely and such a diagnosis can only be made on a clinical basis. Additional comments may refer to implications of the result for other family members and suggest that genetic counselling be considered (Figure 6,7). It is not considered appropriate to state that all relatives must be tested. The report should not state that a diagnosis of hemochromatosis is excluded [4].

Diagnostic Referral (i.e. affected individual) and a Normal Genotype

This genotype makes a diagnosis of HH very unlikely and such a diagnosis can only be made on a clinical basis. Other forms of iron overload and other types of hemochromatosis exist therefore a referral to a specialist unit may be suggested [4].

Asymptomatic-predictive test

Predictive referral (i.e. individual currently unaffected) and genotype C282Y homozygous: The individual is at risk of developing iron overload/HH and it is recommended that the indices of iron overload should be regularly monitored. Suggested frequency for biochemical monitoring of individuals with this genotype is yearly. Additional comments may suggest a referral to a specialist. The report could refer to implications of the result for other family members and suggest that genetic counselling be considered. It is not considered appropriate to state that all relatives must be tested. Reported penetrance values for C282Y homozygosity range from 50% to 96% depending on the definition of iron overload used in the studies. Since penetrance is age related and gender influenced, these factors must be incorporated into any genotype/phenotype correlation. Some studies demonstrated that the penetrance of C282Y homozygosity in males over 40 years is 95% for iron overload, when corrected for age and gender. For males under 40 years, the value is 80% with additional symptoms present in 12% of males in this age group. In the over 40 year females age group, 80% of C282Y homozygotes have iron overload

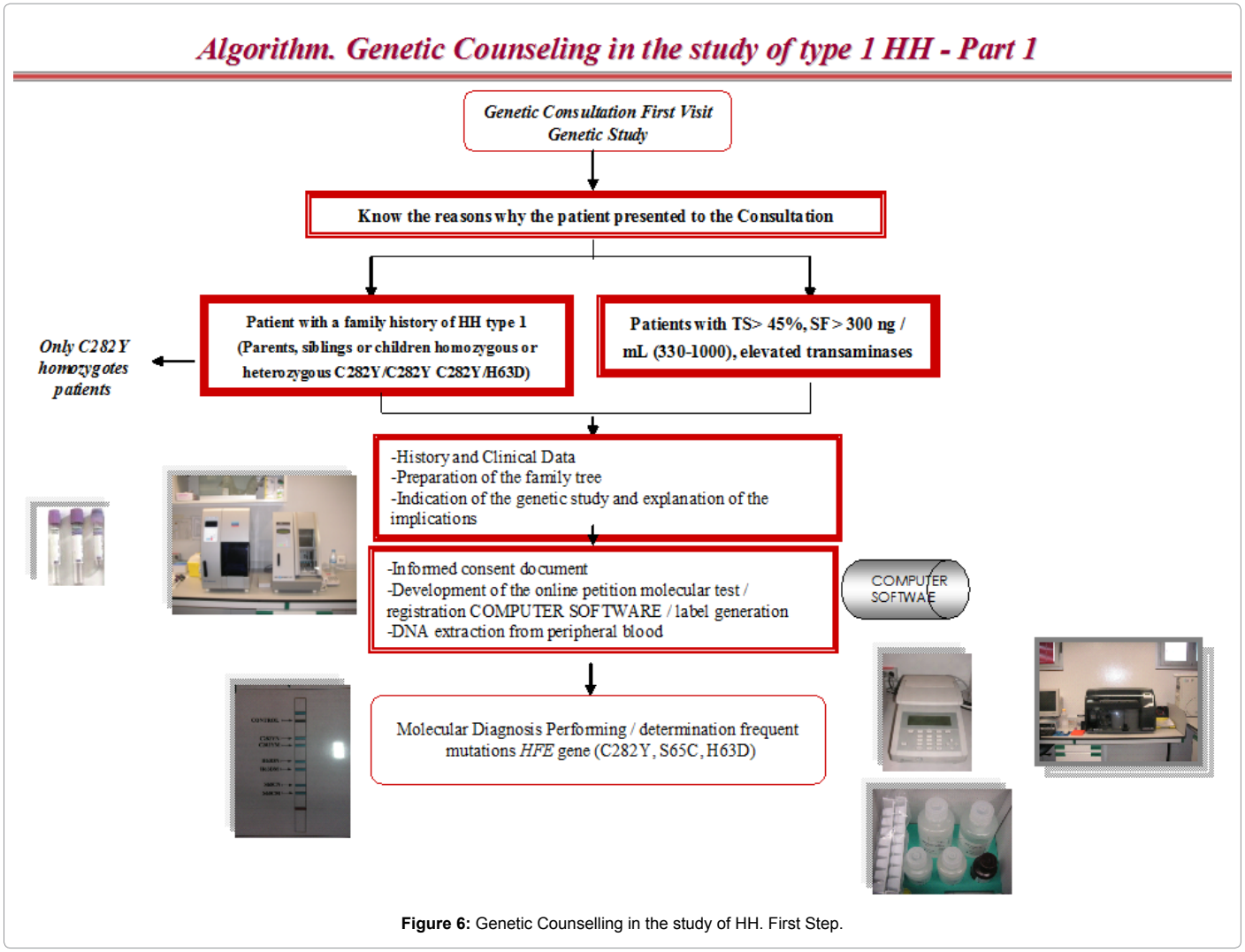

Figure 6: Genetic Counselling in the study of HH. First Step.

with 13% exhibiting other symptoms. Iron overload is present in 39% of females under 40 years with no additional symptoms manifesting [5]. In all cases, the penetrance of clinical hemochromatosis is much lower that the penetrance of iron overloads [4].

Predictive referral (i.e. individual currently unaffected) and C282Y/ H63D compoundheterozygous: Five percent of individual with HH have this genotype but so do 2% of the general population and some individuals with this genotype have iron overload but to a lesser degree than patients with C282Y mutation in homozygous state. The individual may be at risk of developing iron overload/HH and it is recommended that the indices of iron overload (TS, ferritin) be regularly monitored. Suggested frequency for biochemical monitoring of individuals with this genotype is every three years. Additional comments could suggest a referral to a specialist. The report may refer to implications of the results for other family members and suggest that genetic counselling be considered (Figure 6,7). It is not accurate to state that the individual has HH or will develop HH [4].

Predictive referral (i.e. individual currently unaffected) and C282Y heterozygous: The individual is at less than the population risk of developing HFE related HH. The presence of additional risk factors cannot be excluded. Suggested frequency for biochemical monitoring of individuals with this genotype is every five years. The report may refer to implications of the result for other family members and suggest that genetic counselling should be considered [4] (Figure 6, 7).

Predictive referral (i.e. individual currently unaffected) and H63D homozygous: It has been suggested that H63D homozygous have a slight risk of iron overload, but regular monitoring of the biochemical indices should be suggested. The report should suggest that genetic counselling be considered (Figure 6,7). It is not accurate to state that the individual has HH or will develop HH [4].

Predictive referral (i.e. individual currently unaffected) and H63D heterozygous: The individual is at no increased risk of iron overload. The report may refer to implications of the result for other family members and should suggest that genetic counselling be considered [4] (Figure 6, 7).

Predictive referral (i.e. individual currently unaffected) and normal genotype: The individual is unlikely to develop HH.

Carrier status referral and C282Y heterozygous: The individual is a carrier of the C282Y mutation. The report may refer to implications of the result for other family members and suggest that genetic counselling be considered (Figure 6, 7). The report may suggest carrier testing for any partner [4].

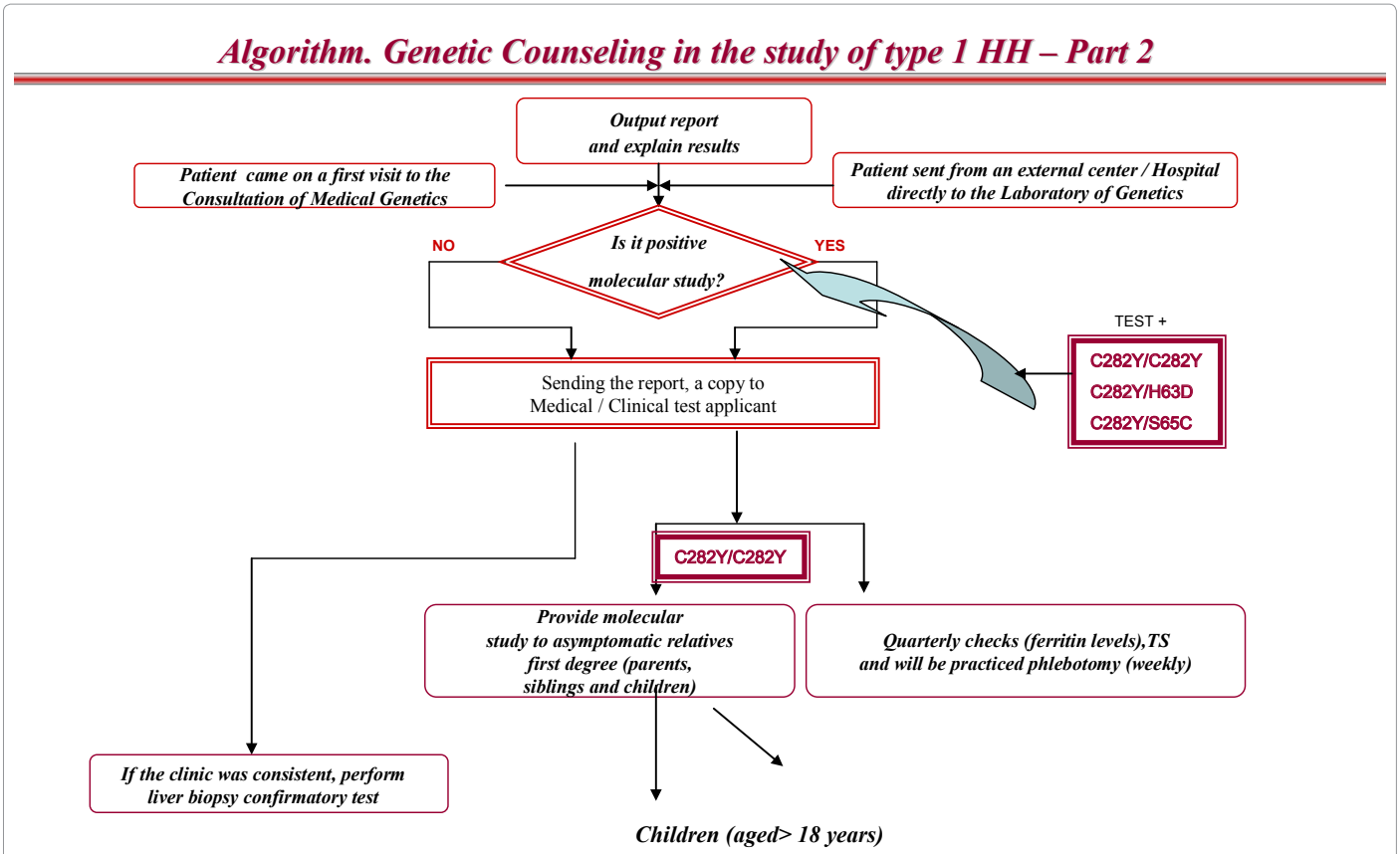

Figure 7: Genetic Counselling in the study of HH. Second Step.

Carrier status referral and H63D heterozygous: The individual is a carrier of the H63D mutation. The report may suggest carrier testing for any partner.

Carrier status referral and normal genotype: The individual is not carrier of the C282Y or H63D mutations.

Information points on reports

Reports should contain information on the method/genetic test used to generate the result, the sensitivity and specificity of the method if known and figures relating to hemochromatosis in the population being reported in, complete with references. These information points are best kept peripheral and not in the main body of the report.

Guidelines from Clinical Genetics Society and the American College of Medical Genetics recommend not testing minors (under 16 years of age) for carrier status for late onset disorders. It is possible to test parents and from this information, determine the appropriate risk to each child of inheriting a genotype predisposing to the development of HH [4].

H63D testing: It is necessary?

Some clinicians think that seeing families where only H63D is segregating may not be the most appropriate use of health care resources. Other specialties such as hepatology feel that the risk of iron overload in H63D homozygous is evident in their clinical experience. When there is a family history of HH, both mutations should be tested for, even if the index case is homozygous for C282Y and especially if extensive screening has not been performed in the family. The reason is that, due to the high carrier frequency of H63D (25% in the general population), it is possible for both C282Y and H63D to be present in the same family and therefore for a family member to be a compound heterozygote at risk of iron overload [4].

It is interesting to note that the phenotypic expression of H63D homozygote, C282Y and H63D heterozygote genotypes appear significantly only in women, suggesting HFE-gender interactions. Elevations in TS are more evident in men with HFE compound polymorphism involving H63D variant than elevations in SF. Organ damage associated with hemochromatosis occurs only when both TS and SF are significantly elevated, the risk of which appears to be somewhat higher in these cases [4, 24].

Genetic family screening

Screening is recommended for all first degree relatives of an index case (Figure 8). The absence of heterozygous state excludes inheritance of *HFE* mutations that confer increased risk of iron overload (i.e. p.CysC282Tyr/p.Cys282Tyr or p.Cys282Tyr/His63Asp) in the offspring. There are still no definitive guidelines regarding when to screen for these mutations in the general population. Given the generally progressive nature of iron overload, clinicians could usually defer testing young subjects until 18 to 20 years unless there was history precocious iron overload [8,23,25,26].

Genetic testing strategy for a proband

- Adults with transferring-iron saturation higher than 45% warrant targeted mutation analysis (Figure 8). Individuals homozygous for the C282Y mutation or compound

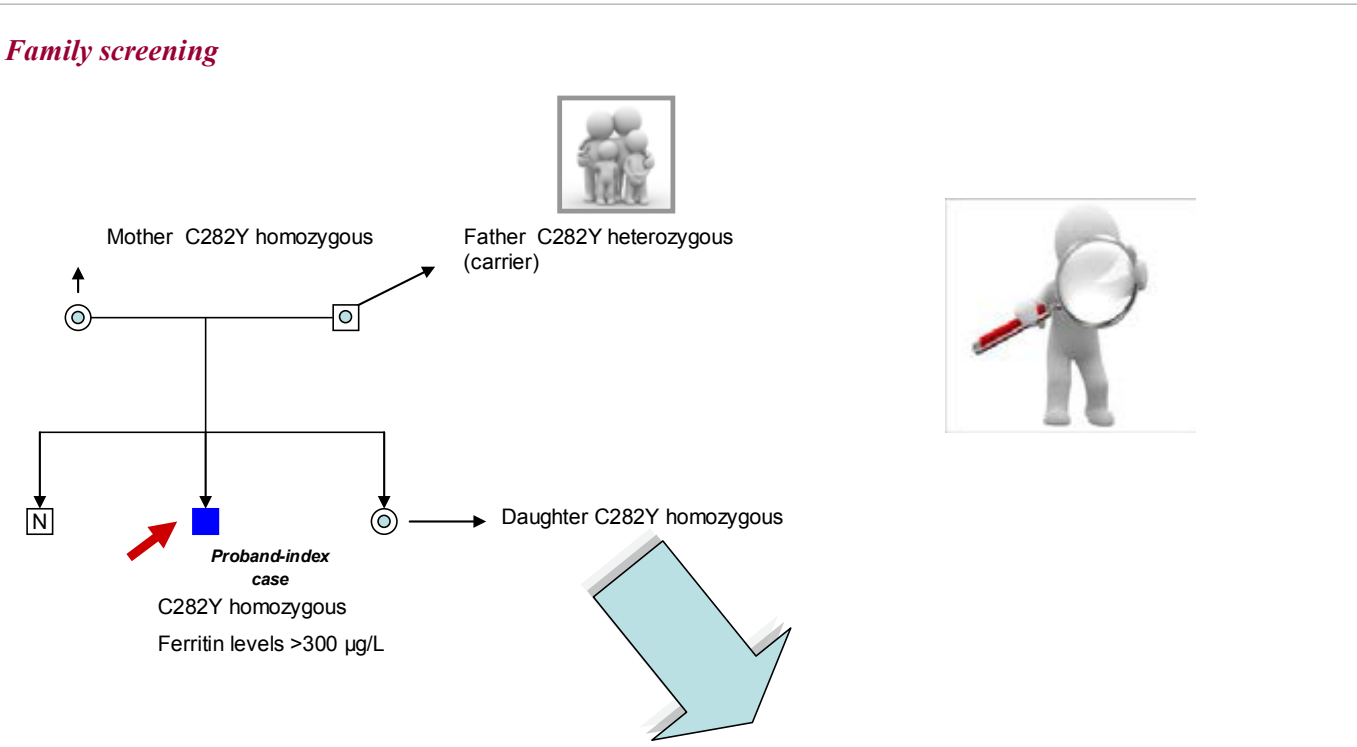

Figure 8: Pedigree, affected members and carriers.

heterozygous for the C282Y and H63D mutations can be diagnosed as having the genetic make-up to develop HFE-HH.

- Individuals who are not C282Y homozygotes generally represent a heterogeneous group who may suffer from liver disease unrelated to HFE or have other metabolic syndromes. These individuals should undergo liver biopsy with assessment of histology and measurement of hepatic iron concentration as a next diagnostic step [7] (Figure 9).

If the patient has not C282Y homozygous or C282Y/H63D compound heterozygous genotype, and there is clinical symptoms of HH, it should be study no HFE genes [2] (Figure 10).

Prevalence of HFE C282Y homozygosity

The frequencies of each HFE genotype are different in racial/ethnic groups. The estimated prevalence of C282Y homozygotes in non-Hispanic Cucasians was higher than in Native Americans, Hispanics, African Americans, Pacific Islanders, or Asians [17,27,28].

Who should be tested for genetic predisposition to HH?

- Family members of subjects with hemochromatosis
- Family members of subjects shown to have an altered HFE gene
- Patients with symptoms which may be early manifestations of hemochromatosis
- Tiredness
- Arthralgia
- Loss of libido
- Upper abdominal discomfort
- Patients with liver disease of unknown cause, including patients with suspected alcoholic liver disease
- Patients with conditions which could be complications of hemochromatosis
- Arthritis especially involving metacarpophalangeal (MCP) joints, but also wrist, shoulders, knees and feet
- Cardiomyopathy
- Impotence
- Diabetes mellitus in association with liver disease [9].

Diagnostic and monitoring strategies in HH

Unfortunately the early clinical signs and symptoms of iron overload are both common and non-specific. At the same time, the clinical penetrance of *HFE* mutations is quite low, making population screening unjustified. A high index of clinical suspicion is, therefore,

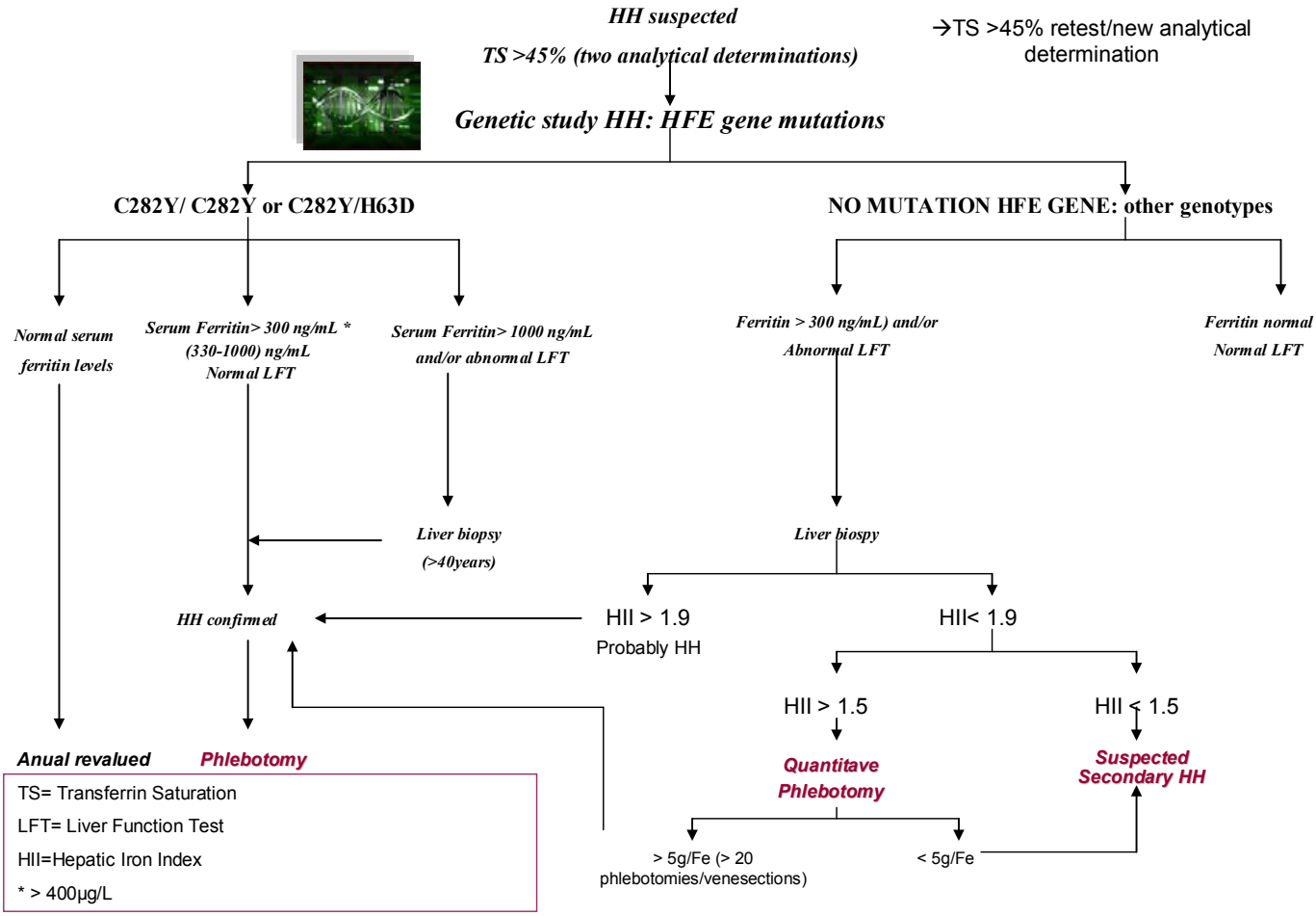

Figure 9: Strategy for diagnosis of Iron Overload Status/Algorithm.

essential for early diagnosis. In recently developed evidence-based guidelines physicians are recommended to consider testing for the disease in patients who have had unexplained symptoms consistent with iron toxicity for several months. The combined measurement of serum iron concentration, transferrin or TIBC (and calculation of TS) and ferritin concentration provide simple a reliable first assessment of body iron levels. *HFE*-gene testing is needed only in those cases with increased TS and ferritin and when acquired causes of hyperferritinemia have been excluded [15,29,30,31].

If the patient is homocygous for *HFE* C282Y, the diagnosis of *HFE*hemochromatosis can be established. If serum iron parameters are elevated but the pathognomonic *HFE* genotype (C282Y homozygous) is absent, determination of hepatic iron concentration is warranted. Magnetic resonance imaging (MRI) is increasingly recognized as a useful non-invasive approach for this measurement, provided that proper software and calibration techniques are used, which is often problematic. In the selected cases with documented hepatic iron overload, a search for the non-classical genetic defects is then indicated (Table 5) [15,16].

Family history of hemochromatosis: strategies of study

For first and second degree relatives of an index case, HFE gene testing should be undertaken to screen for disease. Iron studies should be also ordered if there are any signs or symptoms suggestive of HH. If the individual is not C282Y homozygous HH is highly unlikely to occur. Patients should be counselled that rarely HH can occur from other genetic mutations.

Once diagnosedthe individual (C282Y homozygous), family screeningis requiredand is recommended forfirst-degree family members (Figure 11). It should be studied inall brothers and sisters of the probandIfthe probandhasmore than one child it must be analized the spouse ofindex case.Spouse has a probability about 33 percent of being a carrierofC282Ymutation.If genetic study is negativechildrenwill only beheterozygous carriers.Ifgeneticstudyis positive it must be studied children.Ifspouse hasH63Dmutation(low penetrance); notcontinue the studyon children.Ifthe probandhas only one son/daughter it shouldbe done the genetic study.

When both parents are C282Y heterozygous, siblings of a proband at the time of conception have a 25% risk of having the 2 alleles mutated HFE, a 50% risk of having a mutated HFE allele and 25% of inheriting the alleles normal HFE. When a parent is heterozygous and one homozygous, siblings of a proband at the time of conception have a 50% risk of having the 2 alleles mutated HFE and 50% of inheriting a mutated allele [25].

In some studies it has been seen that morbidity among first-

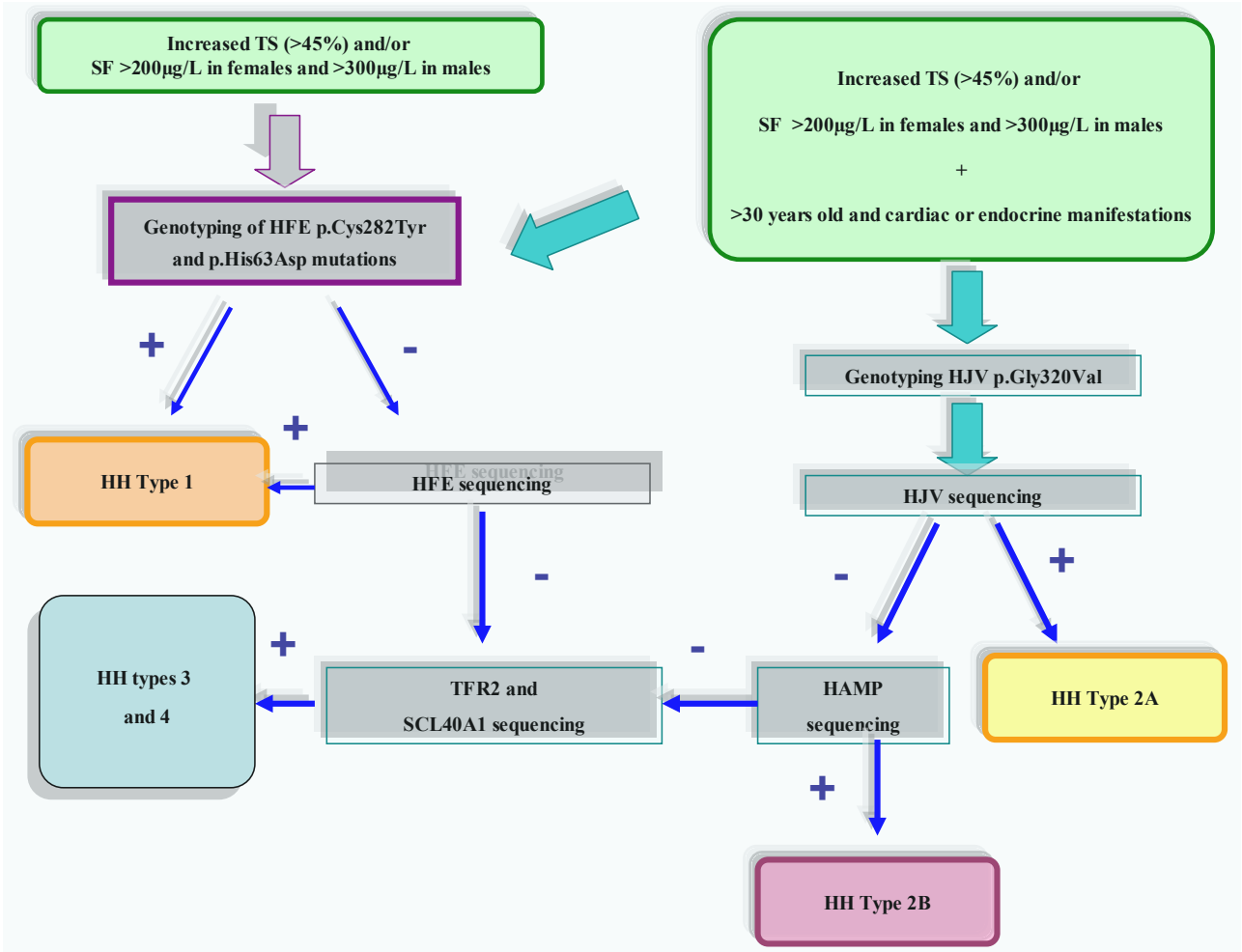

Figure 10: Algorithm of genetic diagnostic strategy for patients suspected HH. TS: transferrin saturation; SF: serum ferritin; JH: juvenile hemochromatosis; +: means positive result; -: means negative results [2].

STEP	ACTION UNDERTAKEN
1	***Basic biology and clinical examination*** O *Hyperferritinemia in an adult (male >300µg/L, female >200µg/L)* O *High transferring saturation (male >60%, female > 50%)* O *Exclude acquired causes: hematologic disorder, alcohol, cell necrosis*
2	***Basic genetic testing*** o Test *HFE* (C282Y) ⇒ C282Y homozygote: *HFE* hemochromatosis stop (unless very severe iron overload, ***follow step 4-2***) ⇒ C282Y heterozygote ▪ Test *HFE* (H63D) • C282Y/H63D compound heterozygote + mild iron overload: ***stop*** • C282Y/H63D compound heterozygote + high iron parameters: ***follow step 3*** • No H63D: ***follow step 3*** ⇒ Other HFE genotypes: ***follow step 3***
3	***Quantification of hepatic iron (magnetic resonance Imaging or liver biopsy)*** ***1. HII ≤1.9 µmol/g/year:STOP*** ***2. HII >1.9 µmol/g/year:follow step 4***
4	***Specialized genetic testing*** o Sequence the *HFE* gene: *1. Presence of a private HFE* mutation (can explain iron overload) 2. No additional *HFE* mutation: sequence iron-related genes (HAMP, HFE2/HJV, TFR2, SLC40A1)

Table 5: Step strategy for the diagnosis of rare HFE mutations associated with C282Y in a compound heterozygous state [14].

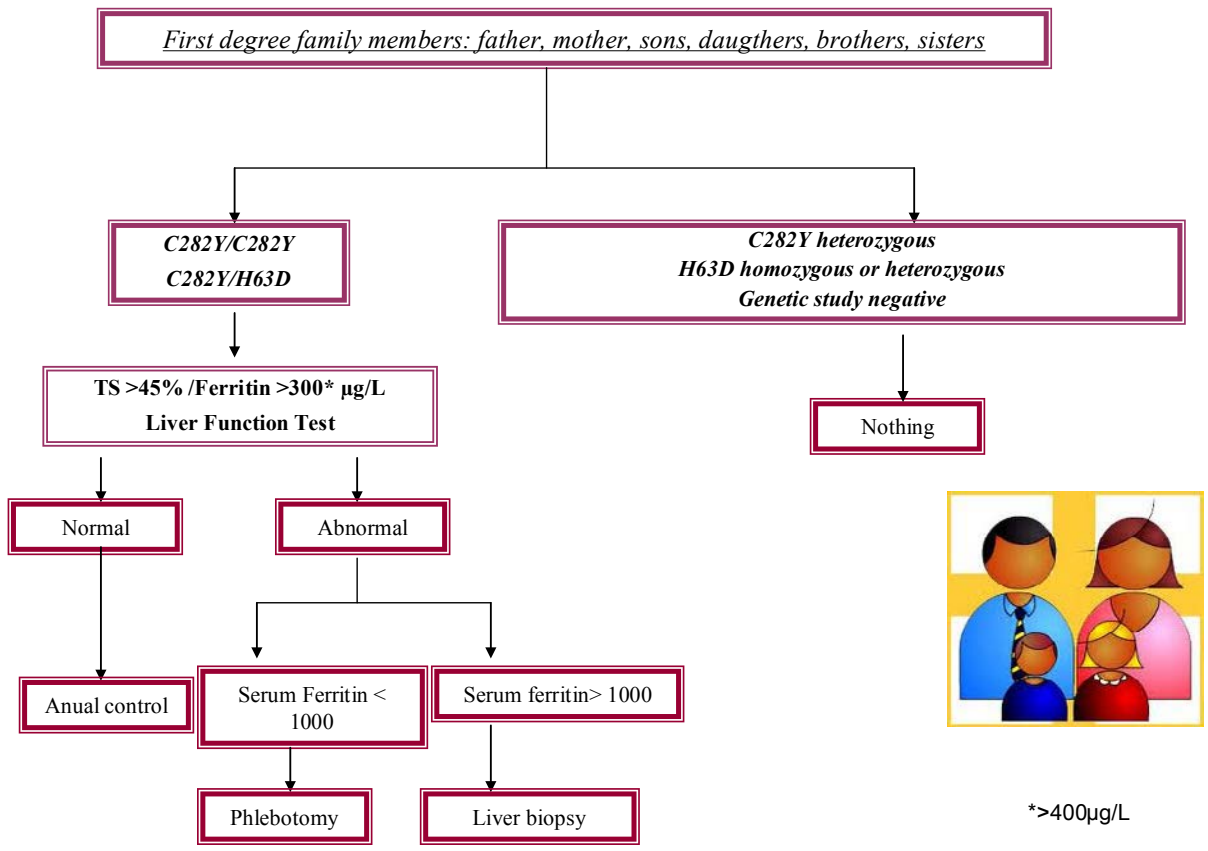

Figure 11: Algoritm family screening.

degree family members of C282Y-homozygous probands previously diagnosed with clinically proven HH is higher than that in an age-and gender-matched normal population [9,26,32].

Algorithms for guidelines for diagnosis and therapeutic strategy for patients with suspected HH (HFE and no HFE)

It has been established a variety of algorithms and flow charts for diagnostic strategy of HH (HFE and non HFE) by scientific associations (Figure 12,13).

Liver Biopsy: The liver is the most readily accessible tissue with which to formally assess the degree of iron overload by measurement of the Hepatic Iron Content (HIC). However, liver biopsy no longer has a primary role in the diagnosis of hemochromatosis in patient with C282Y mutation in homozygous state. The usefulness of liver biopsy in these patients is as a prognostic indicator to establish or exclude the presence of cirrhosis [8,18].

Liver biopsy is usually recommended as a tool to determine severity of liver disease, particularly when other causes (such as alcohol or viral hepatitis) could be implicated and when *HFE*gene mutation testing is negative. Those subjects with C282Y mutation in homozygous state for whom a liver biopsy has been recommended include those aged > 40 years, serum ferritin >1000 µg/L or those with clinical evidence of liver disease, including elevated transaminase levels [8] (Figure 12,13).

Liver biopsy no longer has a primary role in the diagnosis of HH. In patients with HH, liver biopsy is nowadays performed for three main reasons:

1) To determine prognosis of the disease, calculating the fibrosis grade in the liver sample; a SF>1000 µg/L, raised ALT and platelet count <200 000 /µL are considered markers of advanced liver disease, and liver biopsy has been recommended for these patients;

2) To diagnose the presence of other diseases which produce iron overload, such as alcoholic liver disease and Non-Alcoholic Speatohepatitis (NASH), and to determine their severity. When there is a coexistent pathology, liver biopsy may help to clarify the main cause of liver disease, and

3) To identify preneoplastic lesions, including iron-free foci and dysplastic nodules [33].

An assessment of the quantity (HIC) and distribution of hepatic iron should be made when liver biopsy is performed. Perls´s Prussian blue staining is used to demonstrate the extent and distribution in peri-portal hepatocytes which contrasts with non-HFE forms of HH where the pattern of hemosiderin accumulation is in the sinusoidal lining cells without acinar zone predilection. In liver biopsy the Prussian Blue staining reveals abundant deposit fine granular material corresponding to Fe in the interior of hepatocytes.

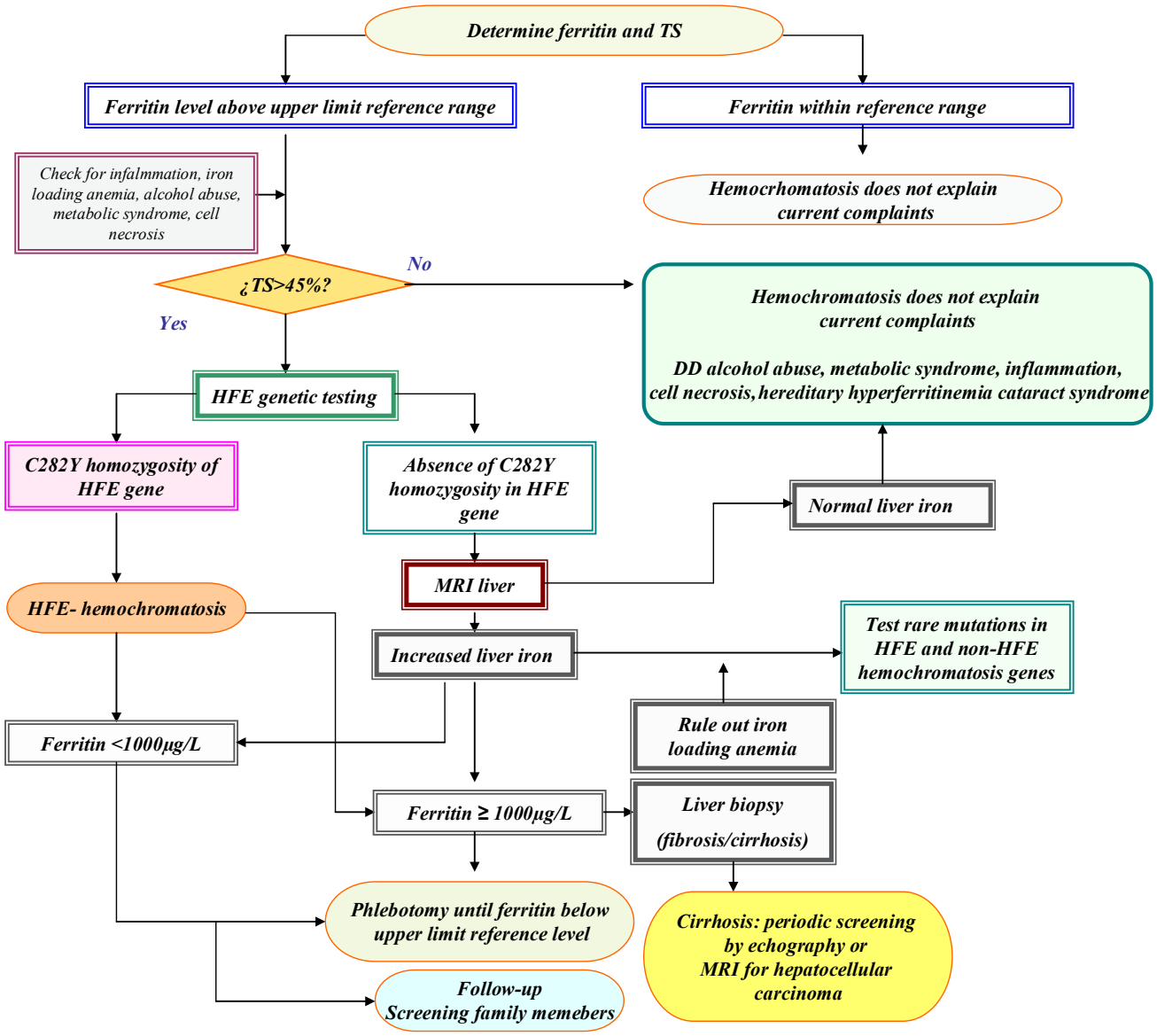

Figure 12: Diagnostic and therapeutic algorithm for patients with suspected HH. TS: transferring saturation; MRI: Magnetic Resonance Image; Figure adapted from van Bokhoven et al, and Swinkels DW and Fleming RE [15].

Non-invasive Hepatic Iron Quantification

Liver biopsy remains the definitive test for the quantification of HIC. However, there is increasing evidence regarding the use of MRI techniques to accurately estimate HIC. Cardiac MRI can be useful to estimate the degree of iron deposition in the heart [8,33].

Management of Hemochromatosis

The primary management goal in HH is the removal of excess iron (de-ironing) through venesection. Secondary goals involve management of liver and non-liver complications which requires:

a) the recognition of cirrhosis and its complications;

b) the identification of other contributing causes to liver disease;

c) the clarification of the nature of any genetic cause;

d) the identification and management of iron deposition in other tissues.

These factors influence therapy and have prognostic implications. Finally, consideration should be given to screening of first degree relatives, particularly to detect early disease.

Venesection is usually commenced once serum ferritin increases above 200 µg/L in premenopausal women and 300 µg/L in postmenopausal women and men. The mainstay of de-ironing is regular phlebotomy to achieve a SF level of less than 50 µg/L without anemia or iron deficiency. One venesection typically removes 250 mg of iron and in the classical presentation with iron overload, removal of 8 to 25 units of blood is usually sufficient to normalise serum ferritin which should be assessed after each 1-2 g of iron removed. Once ferritin levels have normalised, venesection frequency can be individualised and may require once every three to four months [8,9,29].

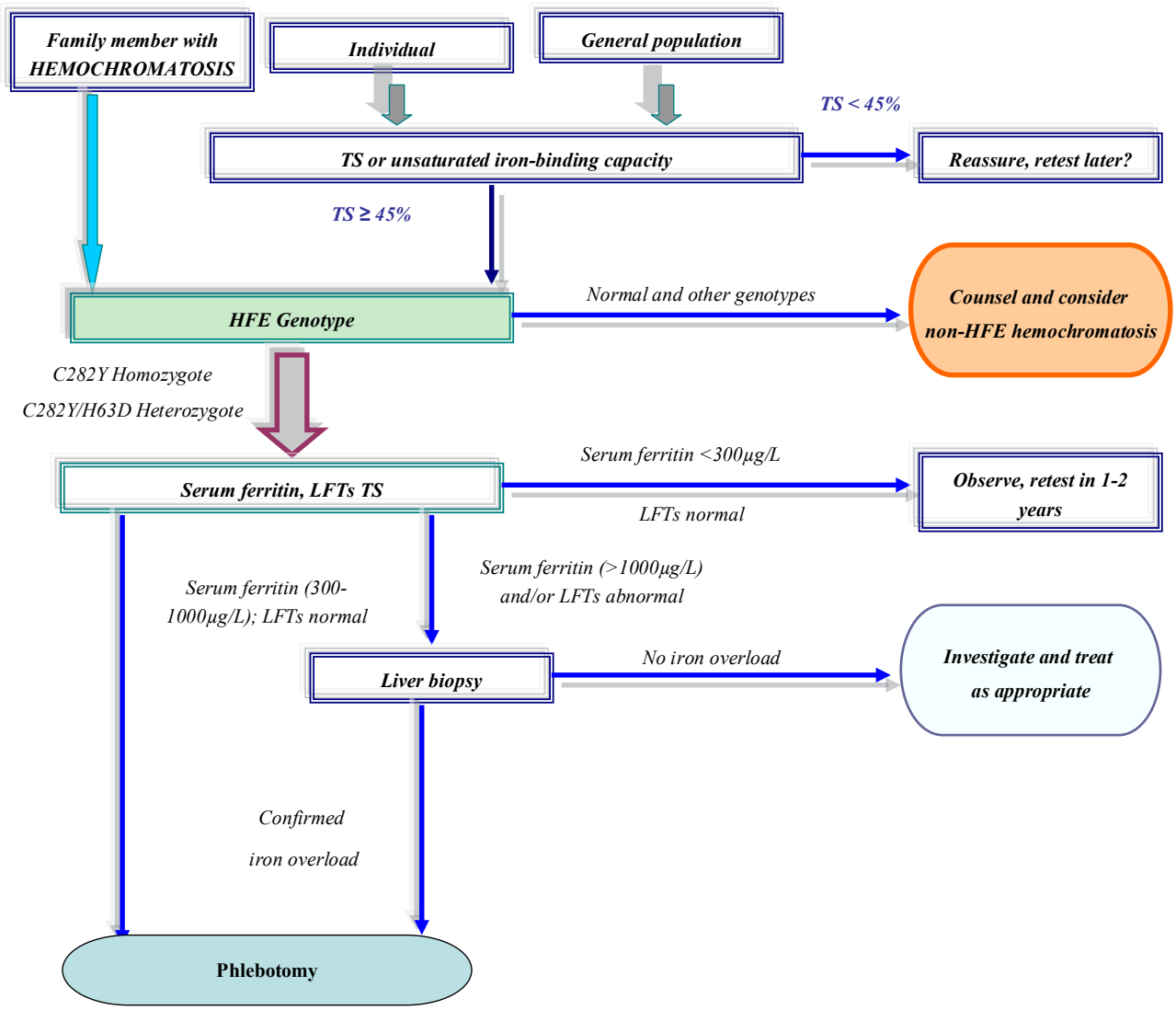

Figure 13: Algorithm for screening for HFE-associated hemochromatosis. LFT: Liver Function Test; TS: Transferrin Saturation. Figure adapted from Clark P, Britton LJ, Powell LW. ClinBiochem Rev Vol 31 February 2010 and published in The Canadian Journal of Gastroenterology 2000; 14(2): 121-5 [8].

Desferrioxamine is a subcutaneous chelating agent but is rarely required except in those patients with HH who are intolerant of venesection due to hypotension, anemia or cardiac failure. An effective oral iron chelating agent has been keenly awaited. Desferasirox (EXJADE®) is effective and available for patients with secondary iron overload, e.g. thalassemia, but its role in HH is still under investigation [8].

It is critically important to determine whether a patient with HH has cirrhosis. Traditionally liver biopsy has been performed to determine HIC as a guide to diagnosis and management of HH. Testing for the *HFE* mutation has now provided an effective substitute test for the majority of patients. The reduction in biopsy however has also reduced valuable histologicalinformation about fibrosis and cirrhosis and also diagnostic clarification in non-HFE iron overload. Certain patient profiles have been found to be correlated to the absence of fibrosis (age <40 years, SF < 1000 µg/L, absence of hepatomegaly and normal liver enzymes). The role of non-invasive tests for fibrosis in HH is evolving [8,34,35,36].

Patients without cirrhosis are not considered to be at increased risk of liver or non-liver related mortality. While cirrhosis is irreversible, iron depletion improves portal hypertension as assessed with abdominal ultrasound and upper gastrointestinal endoscopy [8].

Non-cirrhotic patients with HH do not appear to have increased risk for Hepatocellular Carcinoma (HCC) and therefore do not need to undergo screening.

Cirrhotic patients with HH increased risk of HCC and should undergo appropriate screening. Subjects with cofactors for liver injury such as viral hepatitis, non-alcoholic steatohepatitis and/or alcohol excess have significantly increased risk of more aggressive liver disease and developing HCC. Steatosis has been identified as a cofactor in liver injury in subjects with hemochromatosis [8].

The effect of HH and iron overload on survival post liver transplantation remains contentious. Poorer outcomes for patients undergoing transplantation with hemochromatosis and /or iron overload were suggested by early studies. Results of more recent

work with ascertainment of patients that includes the HFE gene mutations have found contrasting results independent of the degree of iron overload-the prognostic implications of which remain debated. Successful liver transplantation appears to correct the underlying metabolic defect in the disease [8].

Iron overload classically may lead to a number of extrahepatic complications. Cardiomyopathy and conduction disturbances are the most common cause of sudden death in HH however they are generally reversible with venesection. Impaired cardiac function could necessitate a less aggressive venesection program or the use of chelating agents. Patients should be assessed for cardiac symptoms on history and baseline electrocardiograph should be performed with a low threshold for echocardiography [8].

There are numerous endocrine manifestations of iron overload which may present with insidious symptoms of lethargy, weakness, fatigue, loss of libido and impotence. Hypogonadotrophic hypogonadism can be assessed by testing serum testosterone in men and follicle stimulating hormone and luteinising hormone levels in women. Osteoporosis may complicate hypogonadism and should be assessed with plain radiographs and bone mineral densitometry. Fasting blood glucose and thyroid function tests should be performed to assess for diabetic mellitus and thyroid dysfunction. Arthralgias could be assessed with radiographs and referral for rheumatologic opinion as appropriate. Established hypogonadism, diabetes and arthritis do not reverse with venesection but some improvement may occur [8].

What is the prognosis for patients with HH?

Non cirrhotic patients diagnosed and treated early have a normal life expectancy provided they continue treatment. The response to venesection treatment depends on the presenting symptoms and the stage of disease at the time of diagnosis (Figure 14). Those with SF < 1000 µg/L are at low risk of cirrhosis and do not require a liver biopsy. A normal lifespan can be expected if venesection to normalise iron levels is implemented [9,29].

Early symptoms and signs are completely reversible with normalisation of iron indices, including elevated aminotranferases and liver fibrosis. However, cirrhosis rarely regresses to normal despite venesection therapy, nor does it develop if the patient is non-cirrhotic at diagnosis and is an adequately treated. Patients with cirrhosis have a risk of primary liver cancer even when complete iron depletion is achieved. These patients should be screened every six months with hepatic ultrasound and serum alpha-fetoprotein levels. It is not yet known whether HH associated arthritis is reversible with venesection, and it is the only HH associated condition for which prophylactic venesection may not prevent disease progression [9,37].

Hereditary hyperferritinemia-cataract syndrome (HHCS)

The combination of congenital cataracts and hyperferritinemia without evidence of iron overload or inflammation was suggestive of HHCS. HHCS is a rare differential diagnosis of HH. It should be suspected in patients with raised ferritin concentration, but no evidence of iron overload, and in the absence of mutations in the *HFE* gene. Awareness of this condition prevents unnecessary liver biopsies and allows accurate genetic counselling since HHCS is an autosomal dominant disorder. The danger of treating these patients by phlebotomy in the same manner as those with HH is highlighted [38,39,40,41].

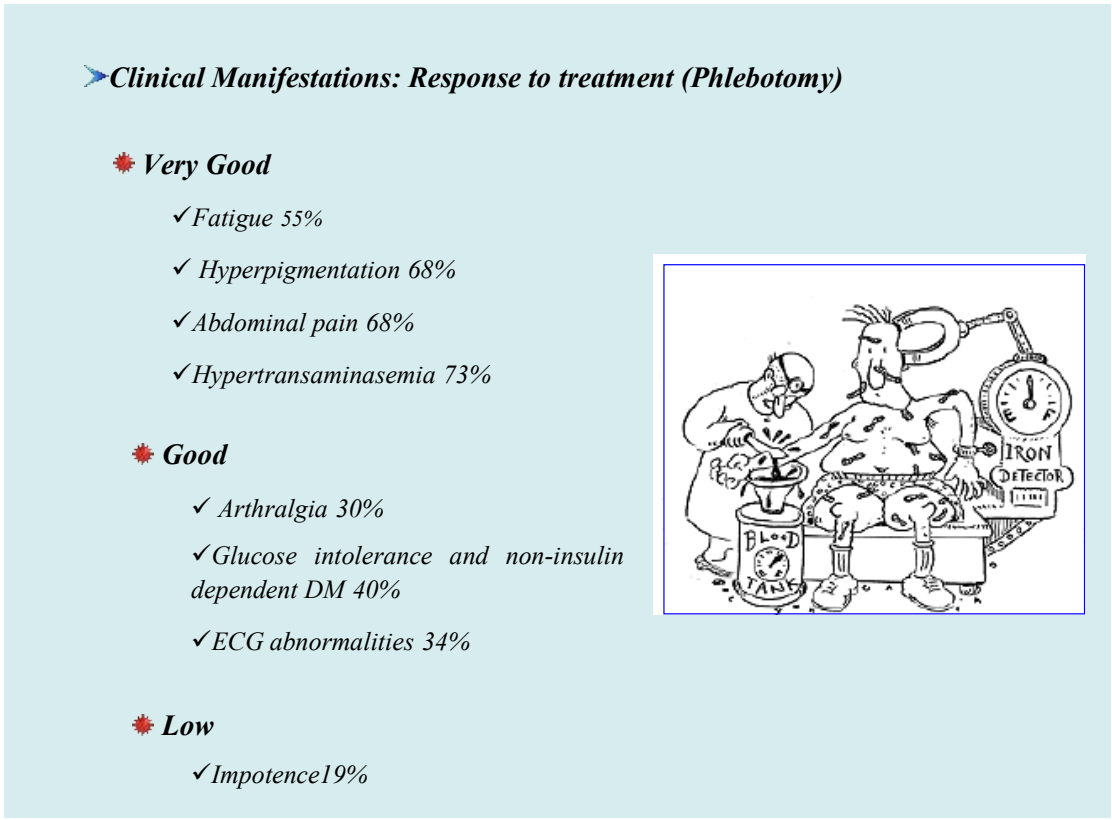

Figure 14: Response to treatment in patients with HH.

HHCS is a rare autosomal dominat genetic disease, which was first described in 1995 independently by the groups of Bonneau and of Girelli [38,39]. They reported two families in whom elevated serum L-ferritin concentration without iron overload, presenting with juvenile bilateral cataracts, was inherited as an autosomal dominant trait. Cataracts comprise crystalline deposits of L-ferritin. The underlying molecular defect in both the early reports of HHCS was identified as point mutations in the 5´Untranslated Region (5´UTR) of the L-Ferritin gene (FTL), in the region corresponding to the Iron-Responsive Element (IRE) of L-Ferritin messenger Ribonucleic Acid (mRNA).These mutations lead to loss of suppression of L-ferritin mRNA translation by the iron-dependent Iron Regulatory Protein (IRP) leading to dysregulated expression of the L-ferritin protein. Since these early reports, a series of other point mutations and short deletions of L-ferritin IRE associated with HHCS have been reported.

The bilateral nuclear cataracts are caused by a direct effect of L-ferritin accumulation in the lens and may develop at a young age rather than being congenital. Apart from visual impairment there are no other symptoms and no treatment is required except for symptomatic cataract removal. Treatment as for HH with phlebotomy is contraindicated as it can rapidly precipitate severe iron deficiency anemia.

The three different subunits composing the proteinous shell of human ferritin, L, H and G arrange to form different isoferritins. The intracellular ferritin contains mostly L and H subunits. Serum ferritin consists of L and G subunits. Ferritin synthesis is regulated by the availability of iron. An interaction between the IRP and the IRE of the FTL gene controls the translation of the L-ferritin gene. The IRE is a non-coding stem loop sequence located on the 5´UTR of the L-ferritin mRNA.

In the presence of abundant cellular iron there is a structural change in the IRP, that prevents the IRP from binding to the IRE, and ferritin synthesis will proceed. When there is a shortage of cellular iron, there is no relevant structural change and IRP binds to IRE and ferritin translation is inhibited.

In 1995, Bonneau et al. speculated that the reason for the accumulation of L-ferritin in HHCS is a mutation on the IRE coding region of L-ferritin. In 1995, two groups in Italy and France simultaneously described the first two point mutations in the IRE of L- ferritin gene. These mutations all change the structure of the IRE in a way which reduces or abolishes binding to the IRP. This leads to unregulated translation of the L-ferritin gene and consequently elevated levels of circulating L-ferritin.

Direct DNA sequencing was initially used to identify mutations in FTL and most of the know mutations are still detected by direct DNA sequencing. Another, faster method is double–gradient denaturing gradient gel electrophoresis, which is able to detect the mutations in a single run.

A distinguishing feature of HHCS is bilateral juvenile cataracts, which have an unusual morphology. They are described as “sunflower-type” morphology or “bread-crumb-like”. The opacities consist of abundant L-ferritin protein. The precise mechanism by which this occurs is unclear. Less opacities might be caused by a yet occurs is unclear. Lens opacities might be caused by a yet unknown interaction between L-ferritin and the lens proteins, or by a disturbed metabolism of L-ferritin within the lens. The high protein concentration in the lens, the slow turnover of mature lens fibers may also be involved in the interaction. No involvement of organs other than the eye has been reported in patients so far. Ferritin levels in HHCS can exceed values over 6000 μg/L without any correlation to the severity of the affected lens [40,41].

The prevalence of HHCS is different populations is unknown. It has been describe more than 50 mutations in FTL gene in relationship with HHCS [42,43].

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