CHAPTER 24

Diagnosis and treatment of non-HFE-haemochromatosis

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1. Introduction

Non-HFE-haemochromatosis is a clinical term that encompasses all forms of genetic haemochromatosis unrelated to HFE mutations. At variance with HFE-haemochromatosis, which is common in individuals of Northern European ancestry and is generally due to a specific (C282Y) mutation, all other forms are rare and genetically heterogeneous (1-2). Transferrin receptor 2 (TfR2)-associated haemochromatosis was the second form of haemochromatosis characterised at the genetic level. It is also called "type 3 haemochromatosis" and is similar to HFE-related disease in terms of abnormalities of iron parameters, clinical complications and type of liver iron storage (3) but may present early in life. Juvenile haemochromatosis or "type 2 haemochromatosis" is characterised by early parenchymal iron accumulation and a severe clinical course. Presentation with hypogonadism is common and cardiac complications are relatively frequent (4). Juvenile haemochromatosis is by itself genetically heterogeneous: type 2A is due to mutations of HJV or HFE2, encoding haemojuvelin (5), type 2B is due to mutations of HAMP, the hepcidin encoding gene (6). TfR2 and juvenile haemochromatosis share with HFE-haemochromatosis autosomal recessive inheritance, increased duodenal iron absorption, increased macrophage iron release, increased transferrin saturation and prevalent hepatocyte iron deposition (7-8).

Ferroportin disease (improperly called "type 4 haemochromatosis") is due to mutations of SLC40A1 that encodes the iron exporter ferroportin, is inherited in a dominant fashion and is phenotypically heterogeneous. Two main forms have been defined: the more frequent is distinct from haemochromatosis in that patients show normal/reduced transferrin saturation and preferential iron accumulation in macrophages (these cases indeed represent the true "ferroportin disease"). A rarer form has clinical and pathological features of classic haemochromatosis (9). The molecular pathogenesis of the different phenotypes has been clarified: ferroportin disease is due to loss of function mutations that reduce ferroportin on the plasma membrane; the haemochromatosis-resembling phenotype is produced by ferroportin mutations that gain the plasma membrane but are resistant to the hepcidin-mediated degradation (10).

The haemochromatosis disorders characterised at the genetic level are listed in Table 1.

2. How to make a diagnosis of non-HFE-haemochromatosis

The diagnosis of non-HFE-haemochromatosis requires sequential steps. Clinical evaluation, biochemical tests, assessment of total body iron and molecular tests concur to reach the correct diagnosis.

Table 1: **Classification of the genetic forms of haemochromatosis**

Disease	Type	Gene	Protein	Chromosome	Inheritance	Phenotype	OMIM* number
Haemochromatosis	1	*HFE*	HFE	6p	AR	classic	+235200
Juvenile haemochromatosis	2a	*HJV*	Haemojuvelin	1q	AR	juvenile	#602390
Juvenile haemochromatosis	2b	*HAMP*	Hepcidin	19q	AR	juvenile	#602390
Haemochromatosis	3	*TfR2*	TfR2	7q	AR	classic	#604720
Ferroportin disease	4	*SLC40A1*	Ferroportin	2q	AD	atypical (classic)	#606069

OMIM = On line Mendelian Inheritance in Man at http://www.ncbi.cnlm.nih.gov*

Step 1: suspecting non-HFE-haemochromatosis

The first diagnostic step is to have the suspect that primary iron loading is present, as in HFE-related haemochromatosis. This is frequently based on the findings of abnormal transferrin saturation (TS), usually >45% in both sexes and/or increased serum ferritin (SF) (>200 µg/L in adult females and >300 µg/L in adult males). More rarely the suspicion derives from clinical symptoms related to iron-induced organ failure or from complications in the presence of abnormally high iron parameters. This occurs especially in the severe juvenile form, which frequently presents symptoms and signs of organ damage at the time of diagnosis. As in HFE-disease the diagnosis of non-HFE-haemochromatosis requires the exclusion of other conditions which can cause secondary iron overload, especially haematological disorders, like iron-loading anaemias or transfusional siderosis (9).

Step 2: diagnosis of a non-HFE type of genetic haemochromatosis

The following step is to exclude mutations in the HFE gene. In theory, this would require the sequencing of all gene exons and intron-exon boundaries. In clinical practice the HFE genotype is often considered "wild type" when the presence of the two common (C282Y and H63D) mutations has been excluded. Other mutations in HFE have been reported, but they are extremely rare, private and not recurrent (11), and often present in trans to a C282Y allele (12). The possibility of a familial disorder (either recessive or dominant) should always be investigated. Unexplained cardiac deaths in young subjects are sometimes reported in juvenile haemochromatosis families (personal observation). A dominant disorder suggests ferroportin disease, although exceptions exist with recessive forms in the presence of a high degree of inbreeding (13). The finding of the same iron overload phenotype in the presence of a discordant HFE genotype (e.g. one sibling homozygous and the other heterozygous for H63D mutation) strongly suggests the presence of causal mutations in genes other than HFE.

Step 3: evidence of increased tissue iron

If the diagnosis of HFE-haemochromatosis is not confirmed by finding a susceptible HFE genotype, it is advisable to demonstrate increased total body iron, before starting expensive and time-consuming search for mutations in other genes. Liver iron concentration (LIC) is considered a measure of total body iron and liver biopsy remains the gold standard for defining LIC (14). A dried fragment of the specimen obtained is used for atomic measurement of iron and the results are expressed as mMoles iron/g dried weight (dw) (normal values 3-33 mMol/g dw). Non-invasive techniques for measuring LIC based on magnetic resonance imaging are rapidly replacing liver biopsy. However, in the diagnosis of non-HFE-haemochromatosis, a liver biopsy is valuable for several reasons. First, it is a mean of assessing the stage of the disease by demonstrating iron-related histological damage (fibrosis/cirrhosis), which is especially important in the severe juvenile form. Second, it shows the distribution of iron, a feature that can point to a more precise diagnosis. As an example if iron is found mainly in the Kupffer cells, it is unlikely that the disease is due to HJV or HAMP mutations: in this case ferroportin is the most likely candidate and should be studied first.

Step 4: the precise molecular diagnosis

The precise molecular diagnosis requires demonstration of the nucleotide change at the DNA level. Since there are at least four genes to be sequenced and their mutations are non-recurrent and often novel, this approach is expensive and time-consuming. However, finding a causal (nonsense or frameshift mutation) is a definitive proof of the non-HFE (and of the genetic) character of the disease. Due to their rarity, all HAMP (2) and most TfR2 (15, 16) mutations are present in the homozygous state. This is not the case for HJV mutations: approximately 1/3 of the subjects are compound heterozygotes for 2 different molecular defects (17). Mutations identified in TfR2 are rare and spread all over the coding sequences (8). HAMP mutations are either nonsense or affect the invariable cysteines required for the correct protein folding (1). More than 30 different mutations affecting HJV have been described, the most common being G320V (1). Both HAMP and HJV genes are rather small and can be easily studied by direct sequencing. TfR2 is a large gene and usually requires mutation screening techniques. HJV mutations may lead to different phenotypes in different racial contexts. In two Japanese families three (two related) patients homozygous for novel (D249H and Q312X) HJV mutations were not diagnosed until middle-age (18). It is unlikely that this is due to a mild effect of these mutations themselves, since the Q312X produces a truncated protein, as occurs in several juvenile haemochromatosis-associated mutations. The effect of possible genetic modifiers remains to be established.

Heterozygous mutations of ferroportin are present in different populations: usually mutations are private but a single amino acid deletion (Val162del) has been reported in different populations (1). The inheritance is dominant, but "de novo" mutations can occur (19).

For a complete discussion of mutations in non-HFE-haemochromatosis the reader is referred to the gene test web site at http://www.genetests.org.

Rare patients with a single C282Y allele carry heterozygous mutations in HAMP (20). In a single family, mutations of HFE and TfR2 resulted in a juvenile phenotype (21). These cases are true examples of digenic inheritance, a mechanism expected in a disorder in which several proteins cooperate along the same pathway. However, digenic inheritance is rare in haemochromatosis, as demonstrated by the results of screening for mutations in HAMP and HJV genes in HFE-patients, which led to only a few positive results of simultaneous mutations in different genes (22-24).

Commercial tests have been developed for simultaneous diagnosis of multiple mutations (25). Unfortunately many patients have their own private mutations that require intensive gene sequencing. Molecular tests for this type of diagnosis are available only in a few centres, often on a research basis. Denaturing high-pressure liquid chromatography (DHPLC) proved to be a convenient technique for mutation screening, reducing the need of intensive sequencing (22-24). However, new approaches simultaneously sequencing multiple genes have been developed (26). As a molecular diagnosis is expensive, time-consuming and in some cases unable to provide a clear diagnosis its utility is sometime questioned. Considering that non-HFE-haemochromatosis is rare and acquired iron overload is common, careful selection of patients for molecular tests is recommended.

A positive family history, an especially motivated patient, and the possibility of reassuring siblings when the mutation characterised in the proband is excluded are important reasons for performing molecular diagnosis. Another important reason is the possibility of a conclusive diagnosis of a genetic disorder that may in some cases lead to individualised treatment (see below). However, the decision to proceed to molecular diagnosis should only be taken after extensive discussion with the patient. During genetic counselling it should be clearly explained to both the patient and the family that in some cases, despite complex and repeated investigations, no conclusive results are achieved. Several reasons may account for this failure: either the mutation lies in gene areas not explored by the screening technique (e.g. promoters or introns) or it affects yet unidentified genes. One of the most common reasons for failure, however, is the non-genetic nature of the iron overload.

3. Specific features of the different disorders

3.1 Inheritance

It is important to remember that haemochromatosis is a genetic disease and thus inheritance and family history are important. All disorders are autosomal recessive, with the exception of ferroportin disease, which is autosomal dominant. In a recessive disease it is also important to investigate possible consanguinity within the family. Most of the rare forms have been identified at the homozygous state in families with consanguineous parents (3, 5, 8, 17).

3.2 Clinical features

The age at presentation, although not an absolute criterion, may point towards the diagnosis. Juvenile haemochromatosis presents clinically before 30 years, with few exceptions (27). However, as more cases are reported it is clear that TfR2-related disease may also present early in life: in some cases liver iron overload has been documented in the second and even the first decade of life (8, 28).

Most patients with juvenile haemochromatosis have complications at the time of diagnosis: commonly hypogonadism, less frequently cardiomyopathy, diabetes and cirrhosis (27). In contrast, patients with typical ferroportin disease are often symptomless, even if diagnosed in advanced age. They may have disproportionately high levels of ferritin associated with a benign clinical phenotype (29) and may be asymptomatic despite ferritin levels that are always associated with complications in other forms of haemochromatosis. This is because iron is deposited in macrophages rather than in parenchymal cells. Serum ferritin levels and tissue iron loading increase with age, and the level of transferrin saturation may also increase, but it rarely reaches 100% as it does in HFE-haemochromatosis. However, in rare cases ferroportin mutations may lead to hepcidin resistance, and hence to iron deposition in the hepatocytes and to a phenotype of true haemochromatosis (9).

3.3 Genotype/phenotype correlation

The correlation of genotype with phenotype in haemochromatosis is rather weak. The penetrance of HFE-disease is a matter of controversy (30-31), but it is accepted that the clinical expression is generally low (32). Experience is obviously limited in the other forms of haemochromatosis because of the limited number of families reported. We encountered variable penetrance in a few families with specific TfR2-disease, but never in juvenile haemochromatosis. The latter is usually fully penetrant in the second-third decade of life, in agreement with direct (HAMP) or indirect (HJV

mutations) inactivation of the key regulator of iron metabolism hepcidin (5, 6). Indeed a high penetrance is expected in severe diseases, a low penetrance in less severe disorders.

4. Treatment

4.1 Phlebotomy

There is limited experience with treatment of non-HFE-haemochromatosis, but an approach similar to that developed for HFE-haemochromatosis appears reasonable (33). Phlebotomy remains the most effective treatment for juvenile and TfR2-haemochromatosis (2). Due to the different pathogenesis and variable phlebotomy tolerance the treatment of patients with ferroportin mutations should be individualised. Patients with the haemochromatosis-like phenotype should follow the same treatment as classic haemochromatosis, whereas the treatment of typical ferroportin disease should probably be less aggressive to avoid anaemia. In some patients with ferroportin disease, a weekly phlebotomy program is not tolerated and mild anaemia rapidly occurs despite elevated serum ferritin levels.

There is no evidence that treatment should be started in all cases with TfR2 or ferroportin disease, because some patients will not progress to overt disease. In practice patients are treated according to a retrospective study in HFE-haemochromatosis which shows that patients treated before the development of complications have an improved survival (34).

The recommended protocol is to remove one (rarely two) 400-500 mL blood units weekly, until ferritin levels drop to < 50 µg/L. Each blood unit contains approximately 200-250 mg iron. A variable time from few months to years, depending on the iron burden, is required to reach iron depletion. Phlebotomy induces iron mobilisation from the liver in all types of haemochromatosis, as shown by repeated liver biopsies after depletion in selected cases. In all disease types, once iron depletion is reached, lifelong therapy with maintenance phlebotomy is required, the interval between phlebotomies being shorter in juvenile cases (at least one per month) than in the classic HFE type, because of the greater dietary iron absorption from the gut in the former condition.

As in HFE-haemochromatosis, phlebotomy is able to prevent organ damage and improve survival, if started in the pre-cirrhotic phase of the disease, because mild fibrosis is reversible. The cut off for fibrosis risk (ferritin >1000 µg/L) is inferred from experience in C282Y homozygous patients (35). Hypogonadism and insulin-dependent diabetes are irreversible and require lifelong treatment. Arthropathy is iron-independent and often does not improve following phlebotomy.

4.2 Iron chelation

At present the only alternative to phlebotomy is iron chelation by subcutaneous deferoxamine (DFO), which is used in anaemic patients or those with severe cardiac disease (36). Protocols are similar to those established for secondary iron overload: DFO is given subcutaneously at the dose of 20-40 mg/kg with a portable pump for 8-12 hours/day. Serum ferritin and urinary iron excretion are used to monitor the treatment efficacy.

Oral iron chelators have been developed for transfusional iron overload. If deferiprone (L1) is established to be superior to DFO in removing iron from the heart (37), this chelator might prove of utility in juvenile haemochromatosis patients with heart disease. The oral iron chelator deferasirox has proved to be as efficacious as DFO in clinical trials in anaemic patients with secondary iron overload (38) and is approved for the treatment of transfusional iron overload. In theory this drug might have a role also in selected patients with primary iron overload.

Erythropoietin has been used in selected cases of non-HFE-haemochromatosis, to increase haemoglobin levels and to allow concomitant phlebotomy (39). However the cost of the drug prevents its extensive use.

In ferroportin disease, treatment experience is limited and in some cases with moderate ferritin increase, careful monitoring alone will probably be of benefit. In any case it is reasonable to apply a phlebotomy protocol less stringent than in haemochromatosis, because macrophage iron is less prone to cause tissue damage than hepatocyte iron and because there are some reported cases of impaired phlebotomy tolerance.

5. Future perspectives for diagnosis and treatment

Hepcidin is the most important regulator of intestinal iron absorption and macrophage iron release. Hepcidin has been measured for research purposes by quantitative Real Time-PCR of HAMP RNA from liver biopsy (40), immunoblot (41), mass spectroscopy of urine or serum samples (42, 43) and more recently by ELISA (44). Hepcidin is normal/reduced in patients with C282Y haemochromatosis (45), and almost absent in juvenile haemochromatosis with both HJV (5) and HAMP mutations (46). Patients with TfR2-disease also have very low hepcidin levels (47). These data are in keeping with the iron accumulation in the hepatocytes and low iron in macrophages that characterises all these conditions, though with different degrees of severity. In ferroportin disease hepcidin is not reduced, at least as indicated by the present limited experience (19, 48). Will hepcidin assay become a test for diagnosis of all forms of haemochromatosis? Although in selected cases hepcidin assay might be of help in diagnosis, e.g. the extremely low levels in HJV

juvenile forms (42), we must take into account that hepcidin may also be drastically reduced in acquired conditions, including anaemia or hypoxia from different causes (48, 49), whereas it is increased in inflammatory disorders (41).

Once the molecular mechanisms of intestinal iron absorption and of hepcidin function are fully clarified it is not unrealistic to expect that hepcidin agonist molecules (or molecules that modulate its activator HJV) will become available as a supportive treatment in iron overload, to modulate iron absorption/release. Hepcidin agonists can be expected to mimic the effect of hepcidin on ferroportin degradation, decreasing iron export. It is presently unclear if they may be of benefit in established iron overload or may be only useful as a maintenance therapy after iron depletion. An alternative to hepcidin agonists could be the development of inhibitors of cellular iron transport - as divalent metal transporter 1 (DMT1) - which could reduce iron import from the lumen to the enterocyte. This approach might be useful in all type of iron overload independently of their genetic or acquired origin, provided that intestinal iron absorption is increased.

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Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. Juvenile haemochromatosis is:
a) A type of HFE-haemochromatosis with severe clinical manifestations at young age ☐
b) A genetic disease due to mutations of either haemojuvelin or hepcidin with early onset of iron overload ☐
c) A secondary iron overload that occurs before 30 years of age ☐
d) An autosomal dominant form of genetic haemochromatosis ☐

2. Ferroportin disease is:
a) A rare type of autosomal dominant haemochromatosis ☐
b) A rare type of autosomal recessive haemochromatosis with mutations of ferroportin ☐
c) A polygenic form of haemochromatosis ☐
d) A secondary iron overload with macrophage iron storage ☐

3. Transferrin receptor 2 is:
a) A member of the TF receptor family involved in hepcidin control ☐
b) A receptor involved in transferrin bound iron uptake in the liver ☐
c) A cellular iron exporter ☐
d) The receptor which binds hepcidin on macrophages and duodenal cells ☐

4. Treatmen of juvenile haemochromatosis without cardiac complications is based on:

a) Intensive iron chelation by parenteral route ☐
b) Intensive phlebotomy treatment ☐
c) A combination treatment (phlebotomy and iron chelation) ☐
d) Intensive chelation using oral iron chelators ☐

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5. Ferroportin disease is characterised by:

a) High transferrin saturation and high serum ferritin ☐
b) High serum ferritin and high macrophage iron ☐
c) Low serum ferritin and high hepatocyte iron ☐
d) High serum ferritin without iron overload ☐

NOTES