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HEREDITARY HEMOCHROMATOSIS: RECENT ADVANCES IN MOLECULAR GENETICS AND CLINICAL MANAGEMENT

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ABSTRACT

Background and Objective. Hereditary hemochromatosis (HC) is an inborn error of iron metabolism leading to increased intestinal iron absorption and progressive iron overload. There have been definite advances in our knowledge of the pathogenesis and management of idiopathic hemochromatosis in recent years, which prompted us to review this subject.

Information sources. The material examined in the present review includes articles and abstracts published in the journals covered by the Science Citation Index® and Medline®. In addition, both authors have been working in this field for several years and have contributed twelve of the papers cited in the references.

State of art and Perspectives. The disease is a late onset autosomic recessive condition, especially frequent in Caucasians. If unrecognized, severe clinical symptoms develop in mid-life related to organ failure. Early diagnosis prevents complications, since an intensive phlebotomy course removes excess iron and offers patients a normal life expectancy. Transferrin saturation is the first examination step, but liver biopsy is still essential for diagnosis and prognosis of HC. The biochemical defect is unknown. Positional cloning of the HC gene has led to the isolation of all the candidate region on the short arm of chromosome 6, telomeric to HLA-A. Recently a putative HC gene has been cloned from this region and found to be mutated in a large proportion of patients. The gene, known as HLA-H, is an atypical MHC class I gene. Although its biological function remains unknown, HLA-H is the first strong HC candidate gene. Molecular screening of patients and carriers is now possible in a significant portion of cases, thereby permitting better control of the disease. If it is unequivocally confirmed that the HLA-H gene is responsible for the disease, understanding of its biological function will provide information on the type and activity of the involved protein, revealing new insights into iron uptake and metabolism in humans.

Key words: hemochromatosis, iron overload, positional cloning

Iron overload is a quantitative increase in total body iron, which usually ranges from 50-60 mg/kg in males and 35-40 mg/kg in females.[1] All conditions associated with iron excess > 5 g may potentially cause tissue damage and relevant pathological findings.[1] Iron overload may be either primary, resulting from a deregulation of intestinal iron absorption as in genetic hemochromatosis (HC), or secondary to other congenital or acquired conditions (Table 1). Among these, iron loading anemias are associated with the most severe degrees of iron overload due to ineffective erythropoiesis and chronic blood transfusions. There is a tendency to use the term *hemochromatosis* to indicate the genetic HLA-associated disorder,[2] whereas *secondary iron overload* is presently used to indicate the non genetic forms of iron loading.[1]

Besides HC, other genetic forms of iron overload have been identified in recent years. The long-recognized sub-Saharan type of iron overload, which develops in individuals who ingest large amounts of home-brewed beer, has been recently shown to have a genetic component which is HLA-unrelated.[3] A newly identified genetic disorder that causes systemic siderosis and neurologic symptoms involves ceruloplasmin,[4,5] a multicopper oxidase involved in iron metabolism due to its ferroxidase activity. This disease has been described recently in Japanese families. Other sporadic reports of non HLA-associated iron overload have not been characterized at the molecular level.[6,7] The recognition of different genetic disorders associated with iron overload reflects the complexity of the mechanisms of iron uptake and transport in humans that are not yet completely understood.

All the genetic and non genetic conditions that may cause significant iron overload are summarized in Table 1. This review will focus on recent advances in genetic, HLA-associated hemochromatosis.

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Table 1. Main conditions associated with significantly increased iron overload.

Condition	*Mechanism of iron overload*
Hemochromatosis	Increased iron absorption
Iron loading anemias	Ineffective erythropoiesis
(β-thalassemia, CDA*, Sideroblastic anemia)	Increased iron absorption Blood Transfusions
Hypoplastic anemias Aplastic anemia, MDS*, PRCA*	Blood transfusions
Chronic hemolytic anemias (spherocytosis, sickle cell anemia, PK* deficiency)	Increased iron absorption
Congenital atransferrinemia	Defective internal iron exchange Increased iron absorption
Neonatal hemochromatosis	?
Juvenile hemochromatosis	Increased iron absorption
Ceruloplasmin deficiency	Decreased ferroxydase activity
Sub-Saharian iron overload	Increased dietary iron Increased iron absorption
Porphyria cutanea tarda	Increased iron absorption
Hepatic disorders	
Chronic viral hepatitis	?
Alcoholic cirrhosis	Increased iron absorption?
Porta-caval shunts	Increased iron absorption

CDA = congenital dyserythropoietic anemias; MDS = myelodysplastic syndromes; PRCA = pure red cell aplasia; PK = pyruvate kinase deficiency

Clinical manifestations of hemochromatosis

HC is a common autosomal recessive disorder in Caucasians, with a prevalence of 1 in 300-500 individuals.[1,2,8,9] If it remains undiagnosed and untreated, severe clinical complications generally occur in the fifth or sixth decade of life. Some of these, such as liver cirrhosis, dilatative cardiomyopathy and diabetes, definitively affect patient survival, while others, such as hypogonadism and arthritis, markedly reduce the quality of life of HC patients.[10-12] Early diagnosis and treatment, however, can completely prevent the development of clinical complications and offer patients a normal life expectancy.[10]

Clinical expression of the disease occurs only in homozygotes. Heterozygotes may have minor abnormalities of those parameters that reflect body iron status,[1,13] but they develop significant iron overload only when other diseases that affect iron metabolism coexist, e.g. heterozygous β-thalassemia, hereditary spherocytosis, or sporadic porphyria cutanea tarda.[14-16] Clinical presentation of HC is variable and depends on two main factors: a) phenotype heterogeneity, and b) time of diagnosis.

The classical clinical picture characterized by melanodermia, liver cirrhosis and diabetes is less and less frequently encountered, whereas the number of patients detected because of the presence of a single symptom or biochemical abnormality is progressively increasing. Unexplained chronic asthenia, arthro-myalgias, hand arthritis, bone demineralization, decrease of libido, amenorrhea, hepatomegaly, arrhythmias, and mild hypertransaminasemia should alert the physician to suspect HC.[12] Moreover, screening studies based on transferrin saturation and/or serum ferritin measurement have allowed diagnosis of asymptomatic patients. For these reasons a new definition of HC, one that requires only homozygosity for the mutant allele, is needed.

All clinical manifestations of HC, excluding arthropathy, are related to the amount of iron overload.[17-20] Liver fibrosis is generally present when liver iron concentration (LIC) or the total amount of iron removed (IR) exceeds 400 μMol/g and 10 g, respectively.[20,21] Iron overload induces liver fibrosis both as a consequence of hepatocellular necrosis and through a direct fibrogenetic effect.[22,23] The coexistence of alcohol abuse or chronic viral hepatitis favors the development of liver cirrhosis at lower amounts of iron overload.[21,24,25] Both insulin resistance, due to iron-dependent hepatocellular dysfunction, and impaired insulin secretion, caused by selective deposition of iron in the β-cells, contribute to carbohydrate intolerance in HC.[18] Hypogonadism depends on hypothalamic dysfunction in the early stage, followed by pituitary failure caused by selective iron accumulation in gonadotropic cells.[18,26] Cardiopathy is characterized by increased thickness of the left ventricular wall, reduced left ventricular compliance and atrial dilatation in the early stage, followed by ventricular dilatation and reduced ejection fraction.[19] Insulin-dependent diabetes, pituitary hypogonadism and dilatative cardiopathy are manifestations of advanced disease and are much more frequent in patients with liver cirrhosis and major iron overload than in those in the prefibrotic stage.[1,20]

Diagnosis of hemochromatosis

In spite of the improvements in recent years, diagnosis of HC is still often missed, mainly because of lack of awareness of the disease and of the appropriate investigations to perform. HC can be suspected on the basis of clinical data, high transferrin saturation and serum ferritin values. An increased serum ferritin value in the absence of high transferrin saturation should be considered with caution. Besides serum ferritin increments due to hepatocellular necrosis or chronic inflammatory disorders, in which the serum ferritin value does not reflect the true amount of iron stores,[27] other hyperferritinemic conditions have recently been described: a) hyperferritinemia and cataract syndrome caused by a single point mutation in the CAGUGU motive that constitutes the loop of the iron regulating element (IRE) of L-ferritin

mRNA.[28,29] In this syndrome, patients present with normal iron stores and early-onset bilateral cataract;[30] b) a syndrome characterized by isolated hyperferritinemia with a mild to moderate liver iron overload, non-HLA-A3 linked, and possibly overweight related.[31]

Liver biopsy is still essential for both diagnosis and prognosis of HC. Both qualitative and quantitative iron measurement should be performed on the biopsy specimen. Iron deposition is mainly hepatocellular, with a typical decreasing gradient from the periportal to the centrolobular zone.[32] The ratio of LIC (μMol/g) to age (hepatic iron index: HII) is the most standardized parameter for defining a patient as homozygous for HC.[21,33] HII equal to or higher than 2 is considered to be diagnostic, but the HII is not as accurate in women as in men due to physiological blood losses (pregnancies, menses). When HII is less than 1.5, homozygosity for HC can be excluded, whereas HII values between 1.5 and 2 should be interpreted with caution.[14,21,33,34] In these cases, only the amount of iron removed by venesection (IR) and family screening can answer the question of their putative genetic status. It is assumed that IR values of 5 g in men and 3 g in women indicate homozygous HC, although a lower IR can be found in earlier stages.[33] IR is the best estimate of total iron burden and we recently observed that the correlation between LIC and IR is not linear and that LIC tends to *plateau* as body iron overload increases.[20,35] Recently, a new grading system based upon the assessment of iron according to its cellular (hepatocytes, sinusoidal cells and portal tract) and lobular distribution in the liver (Rappaport's zones 1, 2 and 3) has been proposed by Deugnier *et al*.[33] This system is quite easy to use, well reproducible, and gives both qualitative and quantitative evaluation of liver iron overload, and can be used as an accurate alternative to HII in the diagnosis of homozygous HC.

Iron overload can be measured non-invasively by a subsequent quantum interference device (SQUID) or magnetic resonance imaging (MRI).[36] SQUID is accurate in measuring tissue iron overload, but it has no other medical applications, is expensive and not readily available. Several investigators have attempted to quantify iron by MRI, but the sensitivity needs further improvement through the use of novel pulse sequences specifically designed to detect and quantitate liver iron.[37,38] With improved diagnostic accuracy and speed, it is likely that MRI will become a widely available, non-invasive and low cost alternative to liver biopsy for the diagnosis and management of HC.

Molecular genetics: a putative gene

Investigation of the gene began 20 years ago, when a fairly accurate genetic localization was established close to the major histocompatibility complex (MHC). This was based both on linkage analysis in families with multiple affected siblings[39] and on linkage disequilibrium of the disease with HLA serotype A3.[40] The MHC maps on the short arm of chromosome 6 (6p) at the cytogenetic band 6p21.3.[41,42] Although these data were extremely important for the aim of cloning the HC gene, progress in further defining the gene localization on the chromosome has been extremely slow. Lack of information about the biochemical defect hampers the possibility of identifying the gene through the *functional cloning* approach, based on the knowledge of the responsible protein. *Positional cloning*, the technique based on finding the gene starting from its position on the chromosome,[43] appeared to be the ideal approach to cloning this gene since its chromosomal localization was approximatively known. However, this approach proved to be both slow and complex in the absence of chromosomal rearrangements and useful recombinants.[44-46] Based on formal genetic studies indicating that the gene was very close to HLA-A,[40,47] a major effort was carried out to hunt for the gene in the HLA-A surrounding region.[48-51] When appropriate markers became available on 6p[52] and HC families were extensively tested, an unusually extended region was found to be in disequilibrium with the disease, encompassing HLA-A and more than 3 megabases (Mb) telomeric (Figure 1).[46,47,53-61] Recently, using linkage disequilibrium combined with haplotype studies in a large number of patients, the HC critical region was narrowed to a 250 Kb region 4 Mb distal to HLA-A. A MHC class I-like gene, HLA-H, isolated from this region has been proposed as the HC gene.[62] The main characteristics of this gene are summarized in Table 2 and its localization is shown in Figure 1. Although its biological function is unknown, HLA-H was mutated in 85% of the American patients studied.[62] A single mutation at position 282 of the gene changes the invariant cysteine to tyrosine (the mutant is indicated as Cys282Tyr or G845A on the basis of amino acid or nucleotide change, respectively). Only 4% of controls possess this mutant and the majority of patients are Cys 282Tyr homozygotes. A second variant, His 63Asp, was found in a low proportion of patients and controls, suggesting the possibility of a polymorphic change. However, part of the patients heterozygous for Cys282Tyr have His63Asp in trans. The role of His63Asp in HC is unclear at present. Similar data have been reported in an independent American study.[63] Preliminary results indicate that the HLA-H gene is also mutated in a considerable proportion of Italian patients.[64] Although the HLA-H gene is a strong candidate for HC, the lack of mutations in HLA-H in a significant number of patients, the absence of causal (deletions, nonsense, frameshift or splicing)

Table 2. Main characteristics of the HLA-H gene.

Localization:	6p22 (4 Mb distal to HLA-A)
Genomic structure:	7 exons (the seventh non-coding) 12 Kb DNA (cDNA: 2.7 Kb)
Predicted protein:	343 amino acids Transmembrane surface and cytoplasmic domains
Homologies:	HLA-A2 HLA-G Fc receptor
Expression:	ubiquitous (low level) 4 Kb transcript

mutations in the gene, and the possibility that Cys282Tyr is a polymorphic change closely associated with another near-by gene leave some degree of uncertainty about the validity of HLA-H.[65,66] Analysis of the HLA-H gene in patients from different populations and functional studies about the gene will clarify this problem in a short time.

The presence of a common mutant in HC was hypothesized from haplotype studies before the detection of the HLA-H gene. Haplotypes associated with the disease can be easily reconstructed by analysis of segregation of polymorphic markers within affected families. Although a variable degree of heterogeneity is observed in different countries, a haplotype characterized by the combination of D6S265-1, HLA-A3, HLA-F2, D6S105-8, CS5-4 alleles was found to be common among patients and very rare in normal subjects in several populations[56-58,67] (Figure 1). As in other genetic disorders in which mutations that have a common origin occur on the same chromosome haplotype background, it was inferred that the HC common haplotype was the ancestral haplotype harboring the ancient HC mutation, which was introduced in different European populations by a *founder* effect. As expected, the Cys282Tyr mutant occurs on the ancestral haplotype. The identification of the ancestral haplotype associated with HC was a major advance in the molecular history of HC. It was used to locate the HC critical region,[62] as in other disorders,[68] but it also provided the opportunity of analyzing genotype/phenotype correlations in patients, even in the absence of the causal mutation.[20,69]

Phenotype heterogeneity

Body iron stores differ in patients with HC. Age, dietary habits, blood donations and blood losses can modify hepatic iron stores and the severity of the disease. Past and recent data showing phenotypic concordance between siblings with homozygous HC indicate that the expression of the disease is strongly influenced by genetic factors[70,71] (Figure 2). Crawford *et al*.[69] found a higher HII in patients homozygous for the ancestral haplotype than in those carrying only one copy or none. In our series we recently found that both homozygous and heterozygous subjects for the ancestral haplotype had a significantly higher amount of total iron removed and IR over age (IR/age) values than patients not carrying the ancestral haplotype.[20] In our study the ancestral haplotype was significantly more frequent in patients with severe phenotype expression, as defined by IR/age greater than 0.33, than in those with a milder one.[20] These data indicated that the gene defect linked to the ancestral haplotype was the result of a single, severe mutation. Accordingly, the majority of our homozygotes for the ancestral haplotype are grouped in the more severe class of phenotype expression,[20] further supporting the hypothesis of a severe mutation linked to the ancestral haplotype. Preliminary data indicate that Italian homozygotes for the Cys282Tyr mutation have a greater iron overload than patients carrying only one mutation or none (unpublished data). In contrast, Feder *et al*.[60] found no obvious clinical-genetic correlation in their patients from the USA.

Biochemical defect

The biochemical defect and the physiopathological mechanism leading to the inappropriately high iron absorption in HC is still unknown. Major hypotheses include a primary defect of the small intestine, reticuloendothelial (RE) system or liver.[72,73] Although much progress has been made in the elucidation of the regulation of cellular iron metabolism, the mechanisms of iron uptake by the intestinal mucosa and its intracellular transport and release to the blood, as well as the release of iron from the liver are still unclear. Several observations have suggested that duodenal epithelial cells in HC behave as in iron deficiency,[74] and that the

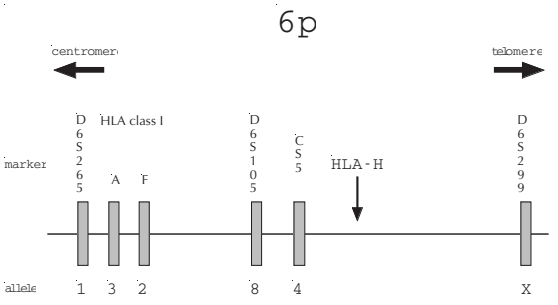

Figure 1. Schematic representation of the HC candidate region on 6p. Relevant markers are shown on the continuous line that indicates the cloned region. The distance from the markers is from Burt *et al*.,[60] Raha Chowdury *et al*.[56] and Malaspina *et al*.[61] The position of the HLA-H gene is indicated. Numbers under the continuous line indicate the alleles of each marker which define the ancestral haplotype (see text for details).

defective control of iron absorption is probably mediated at the level of iron transfer from the cell to the blood.[73]

An increased release of iron from RE cells in the form of ferritin has been observed in HC, but the mechanism by which it occurs has not been clarified.[72,75] To date, studies have failed to reveal any defect in the capacity of these cells to synthesize ferritin or in their ability to take up iron.[73]

Data obtained from organ transplantation indicate that a normal organ (heart or liver) transplanted into a HC recipient does not appear to accumulate iron, and when a hemochromatotic liver was inadvertently transplanted into a normal recipient, iron excess disappeared from the liver.[76] This strongly points to a generalized membrane transport defect or other intracellular defect which might be more evident in the duodenum and liver because of their role in iron metabolism.

The recent discovery of the HLA-H gene led to speculation as to its biological function and how the Cys282Tyr mutation could change it. The HLA-H gene has no metal binding site and thus its product cannot function as an iron transporter. HLA-H protein may behave as a receptor for an iron-binding ligand that limits the iron absorption process. Alternatively, HLA-H protein may sense plasma iron levels and regulate other specific genes or gene products which control cellular iron uptake or release. It was previously suggested that MHC class I molecules may have a more general non-immunologic role.[78] In fact, MHC class I products specifically interact with the insulin and epidermal growth factor receptor systems and regulate the expression of both these receptors.[77,78] Feder *et al*.[62] inferred that tyrosine at position 282 disrupts the binding of the hypothetical class I-like protein codified by HLA-H to β2-microglobulin. Since it has been reported that β2-microglobulin knockout mice accumulate iron in different organs, this rather indirectly supports a function for the gene in iron metabolism.[79]

Screening of hemochromatosis

Its high prevalence, morbidity and mortality, as well as the benefits of early diagnosis and treatment make HC a prime target for screening. HC can be detected before any clinical manifestations occur and even before organ iron loading develops. Recent studies have also demonstrated that large-scale screening for HC is a cost-effective maneuver and have established that transferrin saturation or unsatured iron-binding capacity are sensitive, specific and very inexpensive tests and should be used as the initial screening probe for HC diagnosis.[80,81] Several studies have shown that transferrin saturation of 60% or more accurately predicts the affected homozygous genotype in more than 90 percent of cases in men.[2,82,83] In women, 50% saturation is probably a more appropriate threshold.[82] Serum ferritin is less accurate in assigning the genotype, but it is recommended as a second examination step in those patients with increased transferrin saturation. The finding of a normal serum ferritin concentration does not exclude a diagnosis of homozygous HC, but merely suggests that there is as yet no substantial hepatic iron overload. Serum ferritin concentrations vary with age and sex, and a value more than 2 SD above the appropriate mean is considered abnormal.[81] When both values are elevated, a liver biopsy is strongly recommended to assess the extent of iron overload and to determine the presence of liver fibrosis. When only transferrin saturation is elevated, liver biopsy is probably not indicated since hepatic iron overload has only been reported occasionally in subjects with normal serum ferritin. Figure 3 shows a diagram illustrating a practical approach to the screening, diagnosis and treatment of HC.

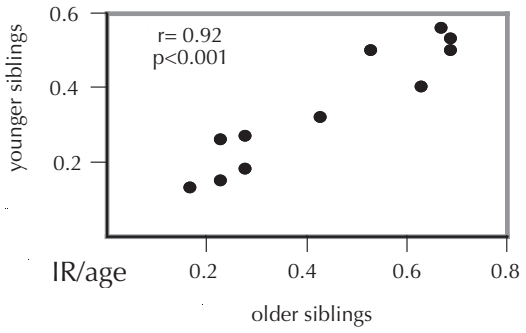

Figure 2. Correlation of the ratio of total amount of iron removed (IR) over age (IR/age) between siblings of the same sex homozygous for HC (personal data).

The optimal age at which individuals should be screened is difficult to determine. Iron overload in homozygotes rarely occurs before the age of 20 years, suggesting that screening would be most effective if applied at that age. The protective effects of pregnancy and menstrual blood losses make it appropriate to rescreen women in the post-menopausal period.

The availability of a molecular test will be extremely useful for defining genotypes at risk in the early stages.

Therapy of hemochromatosis

Phlebotomy is the therapy of choice in HC. It is simple, well tolerated and very efficient. Four to five hundred mL of blood, which correspond to 200-250 mg of iron, are removed every week until the achievement of iron depletion as defined by serum

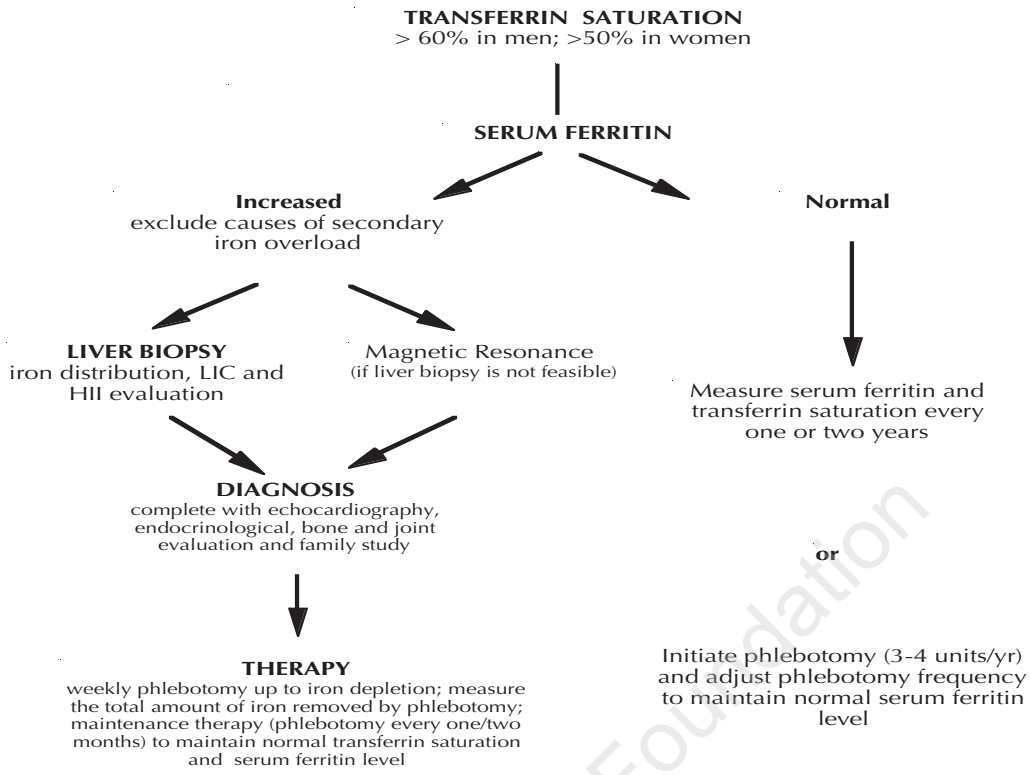

Figure 3. Diagram illustrating a practical approach to screening, diagnosis and treatment of HC.

ferritin below 30 mg/L, transferrin saturation below 20% and a mild anemia that does not promptly recover after cessation of phlebotomies. The frequency of phlebotomies is then adjusted to maintain serum ferritin and transferrin saturation within the normal range. The efficacy of the therapy is strictly related to the time of diagnosis. In the early stage, iron depletion eliminates the risk of developing clinical complications and reverses the initial manifestations of cardiopathy, those related to hypothalamic dysfunction and insulin resistance.[10,11,18,19,26] In contrast, therapy has little effect when pituitary and/or insular β-cell failure have occurred.[18] Although prognosis is severe in the advanced stage, iron depletion can still have beneficial effects, such as reducing the degree of portal hypertension in cirrhotic patients,[84] ameliorating left ventricular function in dilatative cardiomyopathy and improving patient survival.[11,19] However, patients with liver cirrhosis maintain a high risk of developing hepatocellular carcinoma (HCC), even many years after excessive iron has been successfully removed; HCC is the cause of more than 50 percent of all deaths among HC patients with liver cirrhosis.[10,11]

Patients with severe anemia, liver or cardiac failure must be treated with iron chelating agents. This therapy is not as effective as phlebotomies: the amount of iron that can be removed by subcutaneous desferrioxamine (Desferal®) infusion (even performed seven days a week and at a dosage of 40 mg/kg) is about half of that removed by a single phlebotomy of 400 mL of blood (unpublished data). To our knowledge no HC patient has ever been treated with oral deferiprone, whose employment has been restricted until now to patients with transfusion-dependent iron overload who are unable or unwilling to use desferrioxamine.[85]

A low iron diet may a have a protective effect on the expression of the disease by reducing the rate of body iron accumulation, but it has no advantage during phlebotomy treatment.

Patients with HC who require liver transplantation do not fair as well as those with other chronic liver diseases.[86,87] The explanation for these disappointing results lies mainly in the fact that HC patients who are candidates for liver transplantation not only suffer from cirrhosis but usually also present multiorgan involvement.

Future directions

Although the HLA-H gene remains a HC candidate, the high proportion of patients homozygous for the Cys282Tyr mutation and the absence of this

genotype among normal controls[62,63] permit molecular testing in a significant number of patients. Obviously definition of the molecular pathogenesis of HC must await clarification of the molecular defects in cases negative for the Cys282Tyr substitution. Molecular testing will allow earlier diagnosis in patients and screening of carriers. It will also help in dissecting the genetic component of iron loading conditions of uncertain origin.

The recent cloning of the first genes involved in iron uptake in yeast[87-90] has shown an unexpected link between iron and copper metabolism in these organisms.[88,89] Several lines of evidence suggest that this situation has been maintained throughout evolution. They include the role of ceruloplasmin in iron metabolism in mammalians[91] and the discovery of homologues of ceruloplasmin[88] and of Menkes' and Wilson's proteins in yeast.[92] Identification of a defect in the ceruloplasmin gene that causes systemic siderosis is in agreement with this hypothesis.[5] It is possible that increased knowledge of iron-copper metabolism in yeast will shed some light on iron metabolism in humans. No molecular data are available on patients with severe forms of juvenile idiopathic hemochromatosis[93,94] who might have molecular defects different from typical HC.

If HLA-H proves to be the causal gene, functional studies of the involved protein will discover new models of regulation of iron metabolism. At present only hypotheses can be advanced to explain the role of this gene.

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