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 PREVENTION AND CONTROL OF HEMOCHROMATOSIS

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1. INTRODUCTION

Genetic hemochromatosis (GH), also termed hereditary hemochromatosis (HH), is a disease characterized by iron excess especially in the liver. It is genetically transmitted as an autosomal recessive trait and principally determined by a gene located on the sixth chromosome near the A locus of HLA system. This definition, which does not imply substantial iron overload and even less the existence of liver damage, covers all the territory between the unexpressed and the advanced forms of the disease. It does not exclude the influence of possible accessory genetic or environmental factors.

The clinical and pathological entity corresponding to GH was first described by Trousseau (1865) and Troisier (1871) and the term hemochromatosis was later proposed by Von Recklinghausen. Sheldon, in 1935, proposed that GH was due to an inborn error of iron metabolism [102]. This concept was disputed by some authors, especially McDonald, but the discovery in 1975 by M. Simon et al [104] of an association between some HLA antigens and GH followed by the evidence of a genetic linkage between the GH locus and HLA loci ultimately proved the hereditary nature of the disease.

GH is one of the most frequent genetic disease in Caucasians. Indeed, its overall prevalence is greater than 1‰ and reaches 5‰ in some countries. Unlike many other genetic disorders, GH can be easily diagnosed, successfully treated if the patient is diagnosed in time and efficiently prevented among their relatives. Thus, public health strategies must take this disease into account, especially at a time when systematic iron supplementation in the diet is an issue in many countries.

2. GENETICS OF HEMOCHROMATOSIS

2.1 Mode of Inheritance

The debate about a shared environment or heredity as responsible for the familial aggregation of the disease was settled more than 15 years ago through family and population studies which concomitantly provided evidence for an autosomal recessive inheritance [97-98, 106-107]. The following observations all suggested that the presence of two specific homologous genes was necessary for disease to occur:

- (a) in multiplex families
 - (i) the familial segregation of the disease
 - (ii) the horizontal distribution of major iron overload
 - (iii) the high concordance rate for the disease of sib-pairs sharing two HLA haplotypes;
- (b) the genotype frequencies of the HLA-A antigen among unrelated patients.

More recently, statistical methods were used in modelling familial aggregation of the disease [35, 74], and family data from Brittany served to experiment and extend new models for segregation analysis. From recurrent analyses of 147 Breton families ascertained through clinically affected probands [19, 76], it was concluded that the segregation of the disease and of biochemical parameters fitted with the model of a major and recessively-transmitted gene, with a frequency of 0.06 - 0.054 and a sex and age-dependent penetrance. An increased rate of consanguinity has been reported in GH families [37, 97-98]. Although they may have contributed to the high frequency of the gene in certain populations, inbreeding and founder

effect, as well as genetic drift and selection, no longer play a causal part in Breton families. This was shown in a genealogical study comprising 4-7 generation pedigrees of 59 apparently unrelated probands [Yaouanq J, Medicine Thesis, Rennes, 1985]: (i) the average coefficient of inbreeding ($F = 179 \times 10^{-5}$) was similar to that of the Breton population at the same period; (ii) none of the 4 affected inbred subjects was homozygote at the HLA loci, and probably they did not inherit twice the same GH allele from the same common ancestor through both parents; (iii) 4 pairs of probands with a remote relationship did not share any HLA haplotype and, consequently had not inherited a copy of a single GH mutation from their common ancestor.

2.2 Mapping of the HLA-Linked Gene (HFE)

In the absence of a karyotype abnormality which could have facilitated the HFE (hemochromatosis) gene localization, the discovery of a strong association between certain HLA antigens and the GH disease was determinant, pointing to the short arm of chromosome 6 as the putative candidate region. The localization of HFE on 6p 21.3 within the Class I genes region was first suggested by sib-pairs studies (cf above). The close linkage of HFE and the HLA region was confirmed by several genetic linkage analyses [47, 76, 79, 106-107]. The genetic distance predicted between HFE and the Class I genes as a whole is no more than 1 centimorgan, with a cumulative lod score of 60 [47, 76, 79]. Although the order of the HLA-A, HLA-B and HFE genes can theoretically be inferred through multilocus linkage analyses, several problems have hampered a more precise mapping of HFE. Recombinant events are rare, often limited by the availability of family records, and, above all, are difficult to prove in the absence of a specific test for homozygosity. The four recombinant families reported in the world have led to conflicting results in positioning the gene either between HLA-A and B [50] or telomeric to HLA-A [94]. Alternative methods have therefore been sought to refine mapping of the gene relative to HLA-A. Linkage disequilibrium and haplotype studies using HLA markers have mapped the gene closer to HLA-A than to HLA-B and supported a telomeric position of the gene [109]. This approach is currently being pursued using DNA markers during a positional cloning strategy.

Among the likely candidate proteins, an H-ferritin gene located on 6p 21.3 was attractive, but the polymorphisms detected with an H-ferritin CDNA sequence failed to help in the search for HFE. Moreover, the sequence used was later presumed to correspond to a pseudogene which is probably distant from HFE [118]. Positional cloning is a commonly used strategy for cloning and identification of unknown genes [29]. It relies on genetic and physical mapping. Resolution with genetic mapping is rarely finer than 1 centimorgan, and it is difficult to clone a gene in a region corresponding to about 1000 kb. On the other hand, linkage disequilibrium studies using linked DNA markers have proven to be powerful in refining the position of several disease genes. Since the HLA Class I polymorphisms did not prove valuable in detecting polymorphisms associated with the disease, new genetic markers in the HLA Class I region were sought. Several groups embarked on the structural analysis and physical mapping of the region which is cloned in cosmids and YACs [65, 103]. A continuous restriction map has been plotted [53] and seven anonymous genomic probes have been characterized within an interval spanning from 50 kb proximal to HLA-B to 300 kb distal to HLA-A. Using these probes as markers in unrelated patients [21] and then in 66 Breton families, Yaouanq et al (International Conference on Hemochromatosis and Clinical Problems in Iron Metabolism, Jerusalem, Israel, 1993) have established the centromeric boundary of the candidate region at the i97 locus mapping 130 kb proximal to HLA-A. These data suggest that the gene may be located within a 400 kb interval and possibly within a 250 kb region including HLA-A and presenting with a peak of linkage disequilibrium with HFE [127]. Cloned fragments of this narrower region were used to screen cDNA libraries from duodenal mucosa cells, and seven structural genes are currently being sequenced as "hemochromatosis candidate genes" [54].

However one must be aware that the expression of the gene could be specific to another cell type, and that the HFE mutation could reside in non-coding sequences such as introns or regulator sequences.

2.3 Is Hemochromatosis Really a Homogeneous Entity ?

The putative genetic heterogeneity of "non secondary" iron overload is an important question which might, in the near future, hamper the development of presymptomatic testing programmes. In view of the wide phenotypic heterogeneity of the disease, hypotheses involving either more than one gene, or several susceptibility alleles cannot be excluded. Clinical geneticists are familiar with the concept of genetic heterogeneity, and important lessons can be learned from many recessive disorders which are heterogeneous at both the clinical and molecular level, such as thalassaemia, cystic fibrosis, and phenylketonuria.

Is there locus heterogeneity ? - Can similar forms of iron overload be related to genes at different loci? This would be not surprising in view of the number of proteins involved in iron metabolism. However, at present, no linkage and segregation analysis data supported this hypothesis. More recent studies showed no evidence of non HLA-related cases of hemochromatosis [94]. However both dominant and non HLA linked patterns of inheritance were recently described in non Caucasian families [49, 63]. In addition to possible ethnic genetic variations, some methodological pitfalls in the previous studies could explain these apparent discrepancies: (i) selection and ascertainment bias may have operated in favor of genetic homogeneity; (ii) the demonstration of linkage between a disease locus and a marker does not necessarily prove that in a particular family the disease is due to a mutation at the same locus, no matter how high the lod score.

Is there an allelic heterogeneity at the HLA-linked gene ? - The "bigenic" theory of two functionally different but HLA-linked genes was strongly refuted in the past [108]. The absence of a phenotype-genotype correlation is a tempting argument against allelic heterogeneity. Moreover, the presence of HLA-A3 on about 50% of HFE Caucasian chromosomes worldwide had led to the proposal of a unique mutational origin for GH [113]. However, a recent haplotype analysis using DNA markers mapping telomeric to HLA-A favoured at least one additional independent mutational event [127].

One major locus with modifying factors and/or several mutations ? - The effect of additional genes and environmental factors could explain: (i) the intrafamilial variation of the homozygote expression recently reported [4]; (ii) the observation of significantly higher iron overload tests in non carrier (HLA-different) relatives as compared to controls [113]; and, (iii) the debated possibility of partial phenotypic expression in some heterozygotes [20, 94]. On the other hand, Lipinski et al [79] proposed a model of two HLA-linked genes with transactive complementation. Finally, the new molecular definition of a gene allows this last hypothesis to be re-evaluated. If the HFE locus is assumed to correspond to either a functional unit or a transcription unit, different combinations of several mutations affecting the protein-coding function could account for most of the phenotypic variability expression [Yaouanq, J., personal communication, 1993].

3. PHYSIO-PATHOLOGY OF GENETIC HEMOCHROMATOSIS

3.1 Pathology

The major pathological findings in GH relate to the massive amounts of iron found in the parenchymal cells of most organs, particularly liver, pancreas, heart and endocrine glands. The liver is enlarged and nodular in advanced cases. It presents a striking reddish-brown - rusty - colour. On histological examination, iron is found only in the parenchymal cells (= hepatocytes) in early GH [41, 101]. The pattern of iron distribution is peculiar and of relevance to the pathobiology of the disease [41]. Indeed, iron accumulates first within the periportal hepatocytes and in a peri-canalicular location within lysosomes. With time, medio and centro lobular hepatocytes become progressively iron loaded, iron reaches Kupffer, endothelial and biliary cells and portal macrophages and, fibrosis develops from portal tract leading to a "holly-leaf" pattern with large area of preserved parenchyma [41, 92]. In the late stages, cirrhosis occurs with a high risk of developing liver cell cancer [22, 43]. Some particular sublobular nodules consisting of hepatocytes devoid of iron (iron-free foci) have been recently described in the fibrotic or cirrhotic GH liver and are thought to represent pre-neoplastic lesions [44].

The pancreas usually shows heavy deposits of haemosiderin in the acinar cells. Haemosiderin is also found in the heart muscle fibres in nearly all cases of advanced GH but fibrosis is rare. It is also deposited in the conducting fibres of the atrioventricular node and is presumably responsible for the cardiac arrhythmias which may occur in the disease. The pituitary, adrenal, thyroid and parathyroid glands frequently contain extensive haemosiderin deposits, although evidence of functional impairment is usually confined to the pituitary. The epidermis of the skin is atrophic with increased melanin (with or without iron) in the dermis in association with the atrophic epidermis. Increased iron deposition in the skin is very variable and tends to occur around sweat glands [28].

3.2 The Biochemical Defect

One incontrovertible fact is that in GH, iron absorption is inappropriately high in relation to the body iron stores. The basic defect leading to this inappropriately high iron absorption in GH is still unknown. Various hypotheses have been advanced to the basic defect. Theoretically, it could be a defect in the intestinal mucosal cells of the upper small intestine, in the known iron-proteins transferrin or ferritin, or their receptors, in a hitherto unknown transport protein or in the regulation of a transport protein. The defect could be present in the liver, the reticuloendothelial system or could be more generalized.

3.2.1 The Intestinal Mucosal Cell

Several observations have suggested that the intestinal mucosal cell itself functions abnormally in GH and that the cell is behaving as if the body is iron-deficient [73].

Intestinal cell iron-binding proteins

It is a well-known fact that iron absorption is increased soon after stimulation of the bone marrow to increase erythropoiesis, as occurs after haemorrhage. Peters et al [Second International Hemochromatosis Conference, Gold Coast, Australia, 1989] investigated the association of increased iron absorption with increased erythropoiesis in mice with experimentally altered erythropoietic activity and showed enhanced iron uptake *in vitro* in response to hypoxia. *In vivo*, the transfer of iron to

the carcass was markedly reduced in animals with obliterated bone marrow. Nevertheless, such animals did respond to an induced reticulocytosis. These authors also found no response of iron absorption to erythropoietin as it is also seen in nephrectomized patients given this drug. Although these findings suggested iron regulation both at the levels of uptake and of transfer to the body, the mechanism by which increased erythropoiesis results in a rapid increase in iron absorption was not elucidated by these experiments.

Two membrane iron-binding proteins have been described recently, one by Teichmann and Stremmel [119] and one by Conrad and colleagues [30]. The former is a 160 Kd iron-binding protein (a trimer of 54 kD monomers) prepared from solubilized human microvillus membrane proteins. It was localized to brushborder plasma membranes and was present in human intestinal mucosa, liver and heart but not in the oesophagus. An antibody against this protein inhibited Fe^{3+} uptake by more than 50%. These data suggested that the transport of Fe^{3+} across human microvillus membranes represents a facilitated transport mechanism which may be mediated at least in part, by this membrane iron-binding protein [120]. The protein is upregulated in GH and remains so after phlebotomy therapy [116]. The second iron-binding protein described by Conrad et al [30] is a 56 Kd iron-binding protein present in the apical cytoplasm of rat duodenal mucosa cells. These two iron-binding proteins potentially present important sources of the defect, but their biological significance is not yet confirmed.

The role of low molecular weight iron-carriers in mucosal cells

Because of the technical difficulties in studying low-molecular weight iron-carriers, there are few data available and the results to date have been inconclusive. It has been suggested that while in normal subjects, iron-chelates, such as citrate and ascorbate, may act on IRE-binding proteins upregulating the intracellular ferritin synthesis in response to increased cellular iron content, in GH the iron content of these cells is inappropriately low as reflected in the low ferritin content.

Serosal transfer of iron

Although *in vivo* studies of iron absorption in patients with GH have suggested that both net mucosal iron uptake and transfer of mucosal iron to the plasma are increased in this disease [16, 83, 91], at least two *in vivo* studies have provided data which indicate that the defective control of iron absorption in GH is mediated at the level of intestinal cell transfer to the plasma (i.e., serosal transfer as opposed to mucosal uptake). One study used a double labelled radio isotope technique to distinguish between mucosal uptake of iron from the lumen and body iron retention. In a more recent study by McLaren et al [80], mucosal iron kinetics were analyzed using a compartmental model of intestinal iron absorption and systemic ferrokinetics. In subjects with GH, the transfer of mucosal iron to the plasma was inappropriately high, although still inversely related to body iron stores as in normal subjects. This increase in mucosal iron transfer rate appeared to be the major determinant of increased iron absorption.

Transferrin and the transferrin receptor on the mucosal cell

Numerous studies have concluded that both transferrin and its receptor function normally in GH [8, 89]. Recent work supports the hypothesis that the transferrin receptor on the gut cells is concerned more with the transport of iron *from*

the plasma to the mucosal cell, presumably for use within the cell especially during cell growth [6-7]. Moreover, the genes for each of these proteins are on chromosome 3.

It has been suggested that GH relates to a failure to "switch" from neonatal to adult control of iron absorption [114-115]. This hypothesis is attractive but so far unsubstantiated. Anderson et al [7] showed that the intestine of the pre-term rat demonstrated a high level of duodenal transferrin receptor (TfR) along the full length of the crypt-villous axis but soon after birth it was reduced in the area towards the villous tip. There was no correlation between iron absorption and TfR expression in either neonates or adult animals and the crypt receptor density remained high at all ages.

The regulation of ferritin synthesis and TfR expression in GH

There has been a major recent advance in the elucidation of the co-ordinate regulation by iron of both ferritin synthesis and transferrin receptor expression [71-72, 75]. These proteins have now been shown to be coordinately regulated by means of a single binding protein (the IRE-BP) which binds to the iron regulatory elements in the messenger RNA (mRNA) of both transferrin receptor and ferritin. This co-ordinate regulation appears to be intact in GH and the gene for the protein lies on chromosome 9 (and not 6 as for the GH gene). However, in subjects with GH, intestinal mucosal H and L ferritin as well as immunohistochemically detectable ferritin fail to rise in parallel with the serum ferritin levels [59, 122], and it has also been shown that the steady-state mRNA for both H and L is inappropriately low in comparison with the high levels of mRNA for TfR [89]. These results could be interpreted as indicating a primary defect in ferritin transcription in the intestinal mucosal cells. However, it is more reasonable to suppose that these observations merely indicate: (i) that the co-ordinate regulation of the genes for TfR and for ferritin in the gut is still intact; (ii) that the levels of iron in the gut cells are inappropriately low for unknown reasons; and, (iii) that the demonstrated abnormality in intestinal ferritin in GH could be due to a primary abnormality elsewhere in the mucosal cell, to a more remote defect (i.e., in the liver or the monocyte-macrophage system), or to a more generalized cellular or membrane defect. Recent mapping and linkage studies [118] place at least one of the H-ferritin pseudogenes on chromosome 6 centromeric to the GH locus which makes this unlikely to be a candidate gene for the disease. Thus, the available evidence suggests that ferritin synthesis and function in the gut and liver behave normally in GH. Ferritin receptor could be possibly involved in the pathobiology of GH, once further studies will establish its structure and physiological role.

3.2.2 The Liver

In GH, in contrast to secondary iron-overload, the abnormal iron accumulation occurs in the hepatocytes and it is only late in the disease that Kupffer cells, macrophages and biliary epithelial cells of the liver contain stainable iron. In addition, the high percentage saturation of circulating transferrin with iron is observed long before the accumulation of large amounts of iron in the liver occurs. The deposition of iron in hepatocytes and parenchymal cells of other organs could occur by other means. There are at least three mechanisms of iron delivery to hepatocytes in normal animals [32]. In iron-overload condition, iron is also delivered to hepatocytes by non-transferrin-bound iron. The nature of this iron is ill-defined but it probably includes a low-molecular weight form and ferritin iron [15]. Thus, a primary role for hepatocytes in the control of iron accumulation seems unlikely. However, the

production of a circulating regulator of iron absorption has never been excluded. Experiments on rat transplantation have been conducted in attempt to provide an answer [1, 3]. Results would be consistent with a role for the intestinal mucosal cell in regulating iron uptake and body absorption according to its iron content. However, a further role for a humoral factor in regulating iron transfer across the intestinal cell is possible.

Human liver transplantation has also provided some insight into this problem. Available data, which include some 22 subjects with GH successfully transplanted for end-stage liver disease, and a further 4 instances where a liver from a GH subject was inadvertently transplanted into a non-GH recipient, have recently been reviewed by Powell et al [95] who concluded that the combined data could best be explained by a combination of an hepatic and an extrahepatic defect before the disease is fully expressed.

Non-transferrin-bound iron and hepatocyte membrane transport

Hepatic transferrin receptors are reduced in GH [100] suggesting that hepatic iron overload is not due to an increased plasma clearance of transferrin-bound iron. In contrast, recent attention has focused on the low-molecular weight iron complexes collectively referred to as "non-transferrin-bound" iron (NTBI). Although the NTBI accounts normally for less than 1% of the total serum iron, it may account for up to 35% of serum iron in subjects with GH [15]. Hepatic clearance of this form of iron is remarkably efficient [24] and is not down regulated by hepatic iron-loading [123]. Thus, NTBI may be quantitatively much more important than transferrin-bound iron in hepatic iron accumulation in GH. Using the isolated perfused liver model, Wright and colleagues [124] provided evidence that the hepatic uptake of NTBI is mediated by a membrane carrier and occurs by an electrogenic mechanism with a net movement of positive charge into the cell. These authors concluded that, since there is evidence that copper, zinc and manganese share a common carrier with iron [24, 123], hepatic uptake and accumulation of these metal ions may be driven by similar transmembrane gradients. However, uptake of NTBI did not appear to depend on the presence of transmembrane gradients for sodium, chloride or bicarbonate. The early investigators, Sheldon [102] and MacDonald, reported that the concentration of other metal ions, specifically calcium, copper, lead and sulphur (but not zinc or manganese) was also increased in the liver and other organs affected in GH. If this proves true, then other transition metal ions might compete with NTBI for incorporation into a cytosolic binding-site [123, 125]. Against this evidence, the recent demonstration of a membrane transport protein for copper that is defective in a disorder resulting from copper deficiency, Menkes disease, is of particular interest [84].

3.2.3 The Reticulo-Endothelial (RE) System

The observation that Kupffer cells and intestinal macrophages contain little iron in subjects with GH has led to the suggestion that RE cells have a primary defect in iron metabolism leading to reduced iron storage and increased amounts of iron delivered via the plasma to hepatocytes and other parenchymal cells. Kinetic studies involving RE function in GH lend support to this concept. A defect in iron storage, which is common to the two cell types, is certainly compatible with a number of observations in GH including the paucity of iron present in these cells, the increased iron absorption, and early rise in transferrin saturation, etc. To date studies have failed to reveal any defect in the ferritin synthetic capabilities of these cells nor in their ability

to uptake iron. However, increased release of iron from these cells in the form of ferritin has been observed in mononuclear cells of both treated and untreated patients with GH. Fillet et al [57] showed that the early release phase of iron from RE cells was considerably enhanced in patients with GH. The mechanism by which this enhanced release occurs has not been elucidated but may be related to the basic defect present in GH.

3.2.4 A Widespread Parenchymal Cell Defect ?

As discussed above, there is increasing evidence that GH results from a generalized membrane transport or other defect involving multiple organs as occurs, for example, in cystic fibrosis and other disorders. It is possible that one (or more) membrane iron-transport protein(s) could reside on parenchymal cells of a number of organs and, if defective, lead to iron accumulation in those tissues. Alternatively, if confined to the intestine and RE system (i.e., Kupffer cells of the liver), such a protein could be responsible for the rapid removal of iron from these cells enticing them to respond to an apparent iron-deficient state and leading to a relative ferritin deficiency with a parallel increase in TfR concentration. As a consequence, iron absorption is inappropriately increased.

The fact that the iron-loading gene in GH is tightly linked to the HLA loci and displays linkage disequilibrium with HLA-A suggests that abnormalities of a key protein coded at a single locus result in overt hemochromatosis. A number of genes have been considered as potential candidates for the GH gene on the basis of the function of their gene product. However, modern genetic techniques such as genetic linkage analysis, *in situ* hybridization, and somatic cell hybrid deletion mapping panel analysis have demonstrated that most of these genes can be excluded on the basis of incorrect chromosomal location. Despite the evidence cited above, the elucidation of the basic metabolic defect still awaits the cloning and sequencing of the HLA-A linked gene on the short arm of chromosome 6.

4. DIAGNOSTIC CRITERIA FOR GENETIC HEMOCHROMATOSIS

4.1 Clinical Features [25]

The following signs correspond to the full-blown form of the disease. Early diagnosis reduces both their frequency and their intensity. Until the 1960s, GH diagnosis was generally made at an advanced stage when complications occurred, usually in the fifth decade in males and later for females. Indeed, women being relatively protected from iron overload by menstrual bleeding and pregnancies, the prevalence of the overt disease expression in females is about one-fifth that of males. Presently, the face of the disease has considerably changed due to an active diagnostic approach, based on better attention physicians pay to minimal symptoms, and on the wider use of HLA typing in family screening programmes. Thus, GH is recognized earlier, before complications occur, and more often in women.

Liver Involvement

The liver may be considerably increased in overall volume with a predominant left lobe enlargement. It is firm to palpation and its inferior edge is sharp. This hepatomegaly is rarely associated with clinical symptoms of hepatic dysfunction (such as portal hypertension and/or hepatocellular insufficiency). Liver function tests are nearly normal, except for a slight increase

in transaminases (usually less than three times the upper normal limit). The major complication of this liver disease is the development of hepatocellular carcinoma which occurs most often - but not always - in already cirrhotic livers [44].

Cutaneous and Ungual Signs

Excessive skin pigmentation, more often greyish than brown ("bronzed" coloration) is frequently observed, especially in sun-exposed areas, the genital organs and scars. It is attributed to melanin deposition but evolves in tandem with iron depositions in the skin, which are preferentially found around sweat glands. Melanodermy is absent in red-haired people. Other possible signs of the disease are ichthyosis, flattened or even spoon nails and reduced body hair [28].

Cardiac Disease

Electrocardiogram abnormalities consist, by decreasing order of frequency, in T-wave flattening and inversion, low-voltage and rhythm disturbances (atrial tachyarrhythmia and, less frequently, ventricular premature beats and tachycardia). Cardiomyopathy, as assessed by echocardiography, is initially characterized by increased thickness of ventricular walls (reversible alteration) and only later with increasing iron overload by dilated alteration with irreversible cardiac damage. Congestive heart failure is rare but may be fatal, especially in young subjects. Endomyocardial biopsy is a valuable technique to prove the diagnosis. Iron overload within myocardial cells can occur with minimal fibrosis or inflammation. The risk of death related to cardiomyopathy is over 300 times more than the normal population. Autopsy studies have shown that iron deposition is greater in ventricles than in atrias and lowest in conduction tissue.

Endocrine Disorders

The prevalence of diabetes in GH is reported to be about 50-60%. In a series of 163 patients, 55% had overt diabetes and 10% presented with an abnormal glucose tolerance test. The degenerative complications of diabetes are observed in GH with the same frequency as in ordinary diabetes but with less severity. Two main pathogenetic factors contribute to carbohydrate intolerance in GH: (i) impaired insulin secretion caused by selective deposition of iron within the B-cells of the pancreas; (ii) insulin resistance due to iron-related hepatocellular dysfunction.

Hypogonadism is also frequent. In females, it is expressed by early menopause and in males by loss of libido (observed in about 80% of the patients), sexual impotence and testicular atrophy accompanied by a significant decrease in plasma testosterone levels. A decrease in plasma FSH and LH levels with little or no response to clomiphene or LHRH, together with an impaired prolactin response to TRH (thyrotropin-releasing hormone), point to a predominant impairment in gonadotropin secretion.

Bone and Joint Involvement

Bone demineralization is frequent, most often clinically asymptomatic but it may lead to fractures (in particular in the spinal column). In addition to hypogonadism, ascorbic acid deficiency and/or vitamin D deficiency may contribute to bone demineralization. Arthropathy is a common manifestation of GH, often revealing the disease and often misdiagnosed. Clinically, the most characteristic feature is represented by chronic arthritis in the second and third metacarpophalangeal joints responsible for a "painful handshake", a symptom highly suggestive

of the disease. The proximal interphalangeal joints may also be affected as well as the knees, the wrists and the hips. Patients can also experience acute attacks of pyrophosphate arthropathy (pseudogout). Radiologically, the most frequent changes consist of subchondral arthropathy (joint space narrowing, sclerosis and subchondral cyst formation) and chondrocalcinosis (especially in the knees).

General and Miscellaneous Symptoms

Weakness is present in about 80% of patients at the time of diagnosis. A relationship has been suggested between iron overload and the occurrence of infections, particularly by *Yersinia enterocolitica* and by the hepatitis B and C viruses [40, 56]. Susceptibility of GH individuals to infections, such as *Yersinia enterocolitica*, and, in the USA especially to the serious and often fatal septicemia caused by the virulent *vibrio vulnificus*. The bacteria reside in over 50% of shell-fish, especially raw oysters, as well as in shrimp, crab, clams and mussels found in coastal and sea waters. Abdominal pain is a classical symptom of GH. Its frequency in the early stage of the disease is however diversely appreciated. Vitamin deficiencies, especially C and A, have been reported.

4.2 Biological Tests [23, 25-26]

Serum Iron

Normal serum iron levels are about 20 $\mu\text{mol/l}$, slightly higher in males than in females. In fact, the range of normal values is large; furthermore serum iron concentrations exhibit important circadian fluctuations (the levels are maximal in the morning, minimal in the afternoon) as well as marked day-to-day variations (of 30% or more). In patent GH, values greater than 36 $\mu\text{mol/l}$ are the rule.

Transferrin Saturation

Normally, transferrin, the protein devoted to iron transport, is on average 30% saturated, slightly more in men than in women (0.32 and 0.26 respectively-personal data). In full-blown GH, transferrin is often totally saturated.

Serum Ferritin

Normal ferritin levels, which are less unstable than serum iron, are usually less than 200 $\mu\text{g/l}$ in women and than 300 $\mu\text{g/l}$ in men. Ferritin increase is proportional to the degree of iron excess. In fully expressed GH, ferritin values greater than 1 000 are common.

Overall Interpretation of these Parameters

In GH, transferrin saturation is the best screening test in homozygotes. Indeed, in a series of 537 subjects belonging to 18 GH families, a transferrin saturation superior to 0.62 diagnosed 92% of the homozygous siblings. However, its "ceiling" (1.00) is reached for relatively moderate iron overload, which accounts for its failure to predict the degree of iron excess (the same holds true for serum iron). Ferritinaemia is not subject to this limitation and is valuable for quantitating iron overload. It should be noted, however, that all three parameters can underestimate massive iron overload due the development of ascorbic acid deficiency. Liver dysfunction, either acute or chronic, and whatever its cause (but particularly when alcoholic) can lead to an increase of these serum parameters and therefore to overestimation of iron load.

4.3 Imaging Methods

Computed Tomography (CT)

CT provides a non-invasive assessment of hepatic iron by detecting a rather specific increase in X-ray density [= increased liver attenuation (LA)]. A good correlation between LA 1-and liver iron overload has been reported in case of major iron overload. However, single energy CT lacks sensitivity (LA values may be in the normal range for LIC up to 150 $\mu\text{mol/g}$ dry weight), which limits considerably its clinical usefulness [84]. Dual-energy unfortunately does not permit to improve sensitivity of the method [66].

Magnetic Susceptibility Measurement (MSM)

Based upon the paramagnetic properties of ferritin and haemosiderin, MSM provides a rapid, safe, reliable and quantitative measurement of liver iron load. However, the instrumentation for this technique is scarcely available.

Magnetic Resonance Imaging (MRI)

It is based on the ability of stored intracellular iron (ferritin, haemosiderin) to become strongly magnetized when placed into a magnetic field, with subsequent shortening of the relaxation time T2. MRI appears a promising noninvasive technique to assess tissue iron levels [17, 60-61, 67].

4.4 Liver Biopsy [41-44, 101]

Histological Examination

Using Perls or Tirmann-Schmelzer's techniques, liver biopsy ascertains iron overload, precises its predominant periportal and hepatocytic distribution [41], provides a semi-quantitative evaluation of iron excess thanks to various grading systems [42, 101], may enable the quantitation of hepatic iron through computerized measurement of stainable iron [38, 88], and informs on the degree of tissue injury (especially existence and degree of fibrosis) and associated lesions (i.e., alcoholism) [41].

Liver Iron Concentration

Liver biopsy permits the determination of liver iron concentration (LIC) which correlates closely with the measurement of iron stores by depletive therapy and has become a key method for quantitating iron overload [23]. The upper normal value of LIC is about 36 $\mu\text{mol/g}$ dry liver weight. Iron overload can be considered as moderate under 70, important between 70 and 160 and major above 160 $\mu\text{mol/g}$ dry liver weight. Furthermore, LIC determination permits the calculation of the hepatic iron index (i.e., the LIC to age ratio) which enables to distinguish homozygous hemochromatosis (in which hepatic iron index is superior to 1.9) from heterozygous subjects [2, 11-14, 94, 99, 117].

4.5 Genetic Markers [110, 112]

Diagnostic Value of HLA Typing

If the theoretical importance of a linkage GH-HLA is evident, the practical diagnostic value of HLA typing is quite dependent on the clinical situations: (i) within a family where an

affected individual has been diagnosed (= proband), HLA typing permits to identify subjects at risk (especially the HLA-identical siblings, i.e., those sharing two haplotypes with the proband); (ii) in a given individual: when iron overload is diagnosed, the presence of HLA-A3 represents an argument in favour of GH but does not permit to affirm GH since about a quarter of normal subjects are "A3-carriers". On the other hand, the absence of A3 should not be used as a criterion to exclude the genetic nature of iron overload, due to the fact that approximately a quarter of authentic GH are "devoid" of A3. It should also be pointed out that: (i) the haplotypes A3-B7 or A3-B14 are more suggestive of the disease than A3 alone; and, (ii) B7 or B14 without A3 are of no diagnostic value.

Direct Analysis of Genomic DNA, which would represent the ideal means to identify affected subjects, is not yet feasible.

4.6 Quantitative Phlebotomy [23]

It provides an accurate determination of the degree of storage iron and serves as a reference for the other methods, provided that the venesections are carried out according to a strict protocol (400-500 ml/week). With good record keeping the degree of storage iron is easily obtained by totalling up the number of phlebotomies (knowing that 500 ml are equivalent to 250 mg of iron) and correcting the data for the alimentary iron input (i.e., by adding about 2 mg of iron per day during the venesection programme).

4.7 Diagnosis Strategy

In practice, whatever the clinical and biological expression of the disease, GH diagnosis relies on:

4.7.1 Affirmation of a Liver Iron Overload Compatible with GH:

Liver biopsy is necessary to ascertain liver iron overload, to precise its type (digestive hyperabsorption), and to calculate the biochemical and/or the histological hepatic iron index which allows differentiation between putative homozygotes and heterozygotes [41, 43].

Other cases of iron overload within the family of a putative homozygote add an ultimate argument in favour of GH, if recessivity and linkage to HLA are demonstrated. However, this proof is often lacking, due to the family structure, and the inconstant recurrence of a recessive disease.

4.7.2 Exclusion of Other Causes of Iron Overload:

Iron-Loading Anaemias

A variety of haematological disorders are involved. Some of them are congenital diseases such as thalassaemias major and, to a lesser extent, intermedia, sickle-cell disease, red cell dysplasia or congenital sideroblastic anaemia. Other diseases are acquired, such as refractory sideroblastic anaemia, and hypoplastic or myelodysplastic disorders. Under these conditions, iron accumulation is related: (i) mainly to transfusions (e.g., aplastic anaemias); (ii) to increased iron absorption due to increased erythroid activity (e.g., thalassaemia intermedia); or, (iii) to both mechanisms (e.g. thalassaemia major or sideroblastic anaemias). The iron absorbed in excess is mainly deposited in parenchymal sites (i.e., hepatocytes but also pancreas and

heart) in a similar fashion to GH. Transfused iron is initially deposited within macrophages in the mononuclear phagocytic system but subsequently iron redistribution occurs, leading to parenchymal deposition and damage so that, finally, the pattern of organ involvement resembles that encountered in GH. In the case of thalassaemia major, most patients require 200-300 ml/kg/year of blood transfusion which corresponds to 0.25-0.40 mg of iron/kg/day. It has been claimed that iron levels below 400 mg/kg are safe, levels of 750 mg/kg are toxic (growth failure, endocrinopathies) and levels of 1000 mg/kg are lethal. A relationship between liver fibrosis and liver iron concentration has been demonstrated.

Chronic Alcoholic Liver Disease

Iron overload is found in about 30% of chronic alcoholics and is usually mild to moderate. Body iron is increased to only 25-50 mg/kg and LIC is less than 100 $\mu\text{mol/g}$ - i.e., inferior to three times the upper normal limit. The mechanism(s) of this slight LIC increase remain(s) under discussion: (i) a systematic involvement of heterozygotism for GH has been ruled out; (ii) a direct favouring effect of alcohol on iron absorption has not been conclusively shown; (iii) indirect effects of alcohol are possible through: (a) the iron content of alcohol beverage, particularly red wines; (b) folic acid deficiency which may increase iron absorption; (c) transferrin desialylation which could account for an increase in parenchymal cell iron; (d) impaired release of iron from the mononuclear phagocytic system; (e) porta-caval shunt (either spontaneous or surgical) could be an additional factor. Clinically, the picture may involve some degree of melanodermy associated with hypogonadism, glucose intolerance, marked increase in serum iron load parameters (iron, transferrin saturation, ferritin), so that the diagnosis of "hemochromatosis" can be evoked. It is then of utmost importance to resort to direct iron load parameters especially liver biopsy (if necessary through transjugular route) in order to visualize and quantify hepatic iron. In case of alcoholic siderosis, iron deposition is mild and found primarily within Kupffer cells rather than hepatocytes. Furthermore, the LIC to age ratio is below 2, which helps to separate alcoholic siderosis from iron overload in young GH homozygotes. The differentiation between alcoholic siderosis and GH heterozygotism is extremely difficult in the absence of familial arguments for GH. On the other hand, when, in an alcoholic patient, major hepatic iron excess is found, homozygous GH associated with alcoholism can be asserted, the concept of hemochromatosis secondary to iron overload being no more valid.

Porphyria Cutanea Tarda (PCT)

PCT is a chronic hepatic porphyria due to deficient activity of uroporphyrinogen decarboxylase (Uro-D). Two main forms of the disease are described: in sporadic PCT, uro-D deficiency is restricted to the liver (erythrocyte Uro-D activity is normal), whereas in familial PCT the genetic deficiency is found in all tissues (particularly the red cells), and is transmitted in the autosomal dominant mode. The excessive amount of porphyrins in blood and skin accounts for the characteristic cutaneous photosensitivity (with skin fragility and bullae). Clinical expression of both forms of the disease usually require exogenous factors such as alcohol, oestrogen intake, liver disease and iron. Iron overload is frequently observed in PCT but remains of moderate intensity (it does not exceed four times the normal values); its origin is controversial but its favouring effect on the disease expression is certain. Oral or parenteral iron administration is followed by relapse of PCT whereas phlebotomies lead to clinical and biochemical remission of the disease with normalization of hepatic Uro-

D activity. The mechanisms whereby iron interferes with PCT expression remain poorly understood. *In vitro*, iron might inhibit Uro-D activity by a dual effect: (i) direct interaction of iron with the sulphhydryl groups of the enzyme and; (ii) indirect action through the generation of free radicals altering the enzyme. *In vivo*, however, Uro-D activity is decreased neither in rat experimental iron overload nor in human genetic iron overload. Moreover, phlebotomy treatment is efficient even in the absence of iron overload indicating that iron removal may not be the sole factor accounting for the beneficial effects of venesections. Clinically, serum ferritin determination is a useful tool for the evaluation of iron stores despite the associated liver damage.

Chronic Haemodialysis

Iron excess, sometimes massive, is due for overcompensation of blood losses (estimated to about 3 mg per day), by parenteral (mainly transfusional) iron rather than oral iron. It is responsible for visceral - especially liver and spleen - involvement. Serum ferritin levels are considered reliable indicators of iron overload, more convenient and more accurate than bone marrow iron estimation. Liver iron evaluation remains, however, the reference method. Classical treatment consists of: (i) discontinuance of iron supplementation and; (ii) chelation therapy using desferrioxamine during dialysis. Promising therapeutic approaches are represented by oral chelation (possibly using hydroxypyridinones) and treatment of anaemia by recombinant erythropoietin.

Iron Overload in Non-HLA-Linked Hereditary Disorders

Neonatal Hemochromatosis (NH)

This is a rare disease, usually fatal (within a few days to weeks), characterized by massive hepatocytic iron deposition with cirrhosis. Iron overload is present, to a lesser extent, in parenchymal cells of endocrine organs, the heart and kidney. Little iron is found in the mononuclear phagocytic system. The overall pattern of iron excess recalls that of GH. NH pathogenesis is unknown but since NH tends to group within families a genetic component is likely. The metabolic defect accounting for this disease is unknown. NH and GH do not seem to be genetically related.

Cerebrohepatorenal Syndrome (Zellweger's Syndrome)

It is a fatal autosomal recessive disorder, clinically characterized by hypotonia, abnormal facies and polycystic kidneys. In some cases, increased parenchymal iron is found in the liver (with fibrosis), spleen, kidneys and lungs.

Hereditary Tyrosinaemia

Hepatic iron overload is inconstant and moderate in this disease dominated by a peculiar "fishy" odour, cirrhosis and renal abnormalities.

Congenital Atransferrinaemia

Due to the absence of transferrin, (the plasma iron transport protein) this exceptional disorder is characterized by microcytic hypochromic anaemia contrasting

with iron overload in the liver (within the hepatocytes) but also in the heart, kidneys, thyroid and pancreas. Anaemia is improved by transferrin infusion.

African Iron Overload

It concerns sub-Saharan Africa and results from excess dietary iron related to traditional maize beverage fermented in iron pots at a low pH which favours intestinal iron absorption. Iron deposition is found in the mononuclear phagocytic system throughout the body as well as in parenchymal cells. African iron overload may present clinical manifestations similar to GH. It differs, however, from GH by its good correlation between bone marrow and total body iron stores. In addition, the existence of a gene distinct from any HLA linked gene has been suggested on the basis of family studies.

5. TREATMENT OF HEMOCHROMATOSIS

5.1 Symptomatic Therapy of Visceral and Metabolic Complications [33]

Against asthenia, vitamin C should be avoided since it is potentially deleterious especially towards the heart. For the liver, alcohol consumption should be suppressed. For heart failure digitalis should be used with caution since it may precipitate rhythm disturbances. Diabetes requires adequate diet and, when overt, most often insulin. Joint inflammation responds to salicylates and non steroidal anti-inflammatory drugs but corticosteroid should not be given due to the risk of diabetes.

5.2 Elimination of Iron Overload

Methods

Davis and Arrowsmith [36] demonstrated that weekly venesections allowed tissue iron to be mobilised and the subject's iron stores to normalize. Although there have never been specific controlled trials, subsequent uncontrolled studies have indicated that venesection therapy prolongs life span, and that the tissue damage is at least partly reversible. Venesection therapy still represents the best means to eliminate iron overload, according to a two-phase protocol:

The "attack" treatment consists of 400-500 ml venesection per week (corresponding to the removal of 200-250 mg of iron). Heavily iron-loaded patients can be treated by two weekly venesections of 500 ml each. The follow-up is based: for tolerance, essentially on haemoglobin values, and for efficacy, on serum ferritin levels. The duration of the attack phase depends on the degree of iron overload and is predictable from the LIC basal value obtained before starting the phlebotomies. Attack treatment is stopped as soon as serum iron load parameters reach the low normal range ($\approx 50 \mu\text{g/l}$ for ferritin, $\approx 10 \mu\text{mol/l}$ for iron, ≈ 0.20 for transferrin saturation and ≈ 0 for NTBI).

The maintenance therapy consists of 400-500 ml venesections every one to three months, theoretically for lifetime. The goal of maintenance therapy is to keep serum iron parameters (iron, transferrin saturation and ferritin) within the normal range.

Results

They are usually excellent for general health, skin pigmentation, liver and heart symptoms and moderate for diabetes. Insulin dependence is not reversible but insulin dose can be reduced in about one-third of the patients and in the patients with glucose intolerance, carbohydrate metabolism is improved in about half of the cases. Unfortunately, results are poor for joint symptoms (which may even worsen despite phlebotomies) and for gonadal insufficiency. Moreover, regarding the liver, when cirrhosis is present at the beginning of phlebotomy therapy, the risk of development of hepatocellular carcinoma remains despite an adequate depletive therapy. On the whole, curative treatment of GH results in a significant improvement in life expectancy which may even be normal in patients who present without cirrhosis or diabetes at the time of diagnosis, which underlines the importance of an early diagnosis.

5.3 Questions Under Discussion

At Which Point Should Weekly Venesections be Halted ?

This question is still controversial. All would agree that it is important not to render the patient symptomatic. However, in patients with cirrhosis or fibrosis, substantial quantities of iron remain in macrophages and Kupffer cells even when the conventional indices of iron storage are in the low normal range, i.e., serum ferritin $< 20\mu\text{g/l}$ and transferrin saturation $< 10\%$. In such subjects, it would seem prudent to render the subject mildly iron deficient for a short while to ensure that all excess storage iron is removed. In this respect it is of interest that the very earliest studies [69] advocated that venesections should continue until the peripheral blood haemoglobin concentration reached 10.5 g / dl and remained at that level even when venesection is discontinued. At this point all excess storage iron is usually removed. In subjects diagnosed in this pre-cirrhotic and pre-fibrotic stage of the disease, less intensive venesection therapy is required, although again all excess iron should be removed.

Is Follow-up Liver Biopsy Required After Venesection ?

An important practical point relates to whether follow-up biopsy is required after the course of venesection is complete. Hepatic cirrhosis is not always reversible and patients who are cirrhotic at diagnosis do not need to be subjected to repeated biopsy. Likewise, subjects who have normal liver architecture without fibrosis at diagnosis do not need to be subjected to repeated biopsy since cirrhosis will not develop in these subjects. Patients with fibrosis and some disturbance of hepatic architecture at diagnosis require special consideration and possible follow-up with repeated liver biopsy, especially if the degree of fibrosis on the initial biopsy suggested that sampling errors may have resulted in failure to detect established cirrhosis, or if there is an associated cause of liver damage.

Are There Alternative or Complementary Treatments to Venesections ?

Low Iron Diet

The common view is that low iron intake is useless since a one-year diet is equivalent to only two or three venesections. However, generalization of iron supplementation to more and more numerous components of the diet in Western countries could modify the opinion as a free diet is indicated in GH.

Chelation Therapy

When venesection is not feasible, for example in patients with hepatocellular insufficiency, chronic anaemia, or general disease (i.e., arteriosclerosis, malignancy), therapy with chelating agents should be used. For example desferrioxamine can be given intravenously in glucose saline following each transfusion (1 g eight-hourly) and up to 18 μmol (1 g) of iron may be excreted over 2-3 days during such therapy. Long-term therapy can be instituted with subcutaneous infusions overnight two-three times a week. However, desferrioxamine therapy is not satisfactory in GH because the amount of iron chelated, 270 - 360 μmol / day (15 - 20 mg / day), is not comparable with that removed by venesections. Treatment by oral iron chelating agents is eagerly awaited. However, except in therapeutic trials, such treatment is not yet available at the present time. The ultimate treatment of course will be gene replacement therapy based on the eventual identification of the gene. However whether gene therapy will be cost effective in a disease that can now be diagnosed in its early stages and treated effectively, will be controversial.

6. PROGNOSIS IN GENETIC HEMOCHROMATOSIS

From 1959 to 1992, 251 subjects have been diagnosed at the University of Düsseldorf as suffering from GH. These patients have been extensively followed in order to assess prognosis criteria in GH [87].

6.1 Clinical Presentation at Diagnosis

In the total group of 251 GH patients, abnormalities in liver function tests (75%), weakness and lethargy (74%), skin hyperpigmentation (70%), diabetes mellitus (48%), arthralgia (44%), impotence (45% in males), and ECG abnormalities (31%) were the most frequent findings and symptoms at diagnosis. In recent years, about 50% of patients were detected without having liver cirrhosis and 20% of patients did not have any symptoms and pathology except iron overload.

Iron removal in general ameliorated liver disease - if previously compensated -, weakness and cardiac abnormalities. Endocrine alterations due to iron overload did only partially respond to iron removal which, at least, prevented the progression of these alterations. Therapy, however, did not influence arthropathy which even worsened in several patients. Iron removal also failed to reverse insulin-dependent diabetes. This was probably related to irreversible destruction of islet cells caused by iron deposition. Mild abnormalities of glucose metabolism, such as abnormal glucose tolerance or non-insulin-dependent diabetes which may be due to liver disease, could be ameliorated by iron depletion in some patients probably reflecting the improvement of liver disease. Signs of liver failure or portal hypertension, such as jaundice, ascites, or splenomegaly were infrequent findings at the time of diagnosis. However, if present at the time of diagnosis, they usually did not regress after iron removal. In non-cirrhotic patients hepatomegaly, as well as histological and laboratory signs of liver disease, often completely disappeared after removal of iron. In almost 50% of patients who showed fibrosis on the initial liver biopsy, the fibrotic alterations were markedly improved after iron removal. However, complete regression of cirrhosis was not observed in any of the patients who had complete cirrhosis on the initial biopsy.

6.2 Life Expectancy

The total group had a significantly reduced life expectancy compared to the normal population.

Cirrhosis and Diabetes

Survival in the non-cirrhotic patients was virtually identical to the survival expected in a sex- and age-matched normal population. This was also true for the subgroup of patients who did not have diabetes at the time of diagnosis. Thus, in the absence of cirrhosis and diabetes, iron removal by phlebotomy therapy prevents further tissue damage and guarantees a normal life expectancy. Cirrhotic patients had a significantly worse prognosis compared to the group of non-cirrhotic patients when survival between the two subgroups was compared by a log rank test. Similarly, non-diabetic patients had a markedly better prognosis compared to patients who already had developed diabetes at the time of diagnosis. Sex and presence of arthropathy did not predict prognosis.

Amount of Iron Excess

The prognosis of GH appears to depend directly on the amount (and probably also on the duration) of iron excess. Patients who could be de-ironed during the initial 18 months of venesection therapy had a significantly better survival than patients who were not depleted during this period. In addition, patients who died during the period of follow-up had significantly more mobilizable iron compared to patients who were living at the end of the study. When the amount of iron excess was evaluated by the number of phlebotomies necessary to achieve iron removal, this hypothesis was supported: again, patients with massive and long-lasting iron overload had a worse prognosis than patients with less severe iron excess.

6.3 Causes of Mortality

During a mean follow-up of 13.4 years, 69 deaths occurred in the 251 patients.

Liver Cancer

In 19 patients, death was due to liver cancer (27.5%) including 16 hepatocellular carcinomas (HCC) and 3 intrahepatic bile duct carcinomas. Thus, the risk of a patient with GH to die from a liver neoplasm was calculated as more than 200 times that of a matched normal person. In many patients who died from liver cancer, tumor developed up to 22 years after the diagnosis had been made and despite excessive iron had completely be removed. In this series, all liver cancers developed in cirrhotic livers. However, it is of note that some cases of liver cancer arising in fibrotic but non-cirrhotic GH livers have been recently reported. The development of liver cancer could also depend directly on the amount and duration of iron overload since patients who died from liver cancer showed significantly greater iron stores compared to patients who died from other causes. Hepatitis surface antigen (HBsAg) was detected in none of the patients who developed liver cancer.

Liver Cirrhosis

In 14 patients, death was caused by liver cirrhosis. Thus, liver cirrhosis as a cause of death was more frequent in GH compared to the normal population. Patients who presented with major complications of liver cirrhosis, such as ascites, oesophageal varices, splenomegaly,

and markedly decreased liver function had the worst early prognosis and often died before iron depletion could be achieved.

Cardiac Disease and Diabetes

Five patients died from cardiomyopathy and three from diabetes mellitus.

Other Causes

The frequency of other causes of death did not differ from the frequency expected for a matched normal population including extrahepatic malignancies.

Survival in the present series was better when compared to previous studies. This improvement in survival of GH patients is in part due to the fact that, in recent years, an increasing number of patients have been identified at an early stage of the disease. This underlines the interest and the need of a screening policy for GH in order to diagnose the disease before the onset of complications, such as liver cirrhosis and diabetes mellitus.

7. EPIDEMIOLOGY OF GENETIC HEMOCHROMATOSIS

The availability of the HLA system as a linked marker for GH has made it possible to define the distinction between homozygotes and heterozygotes among the relatives of patients with fully developed GH, and to detect this genetic disease prior to the development of clinical signs and symptoms. Thus, the pattern of clinical manifestations among many "affected" subjects is often considerably less severe than was suggested earlier. It is now known that many homozygous individuals are asymptomatic, and the classic triad of hepatomegaly, skin pigmentation, and diabetes mellitus is uncommon. These facts explain, at least in part, the discrepancy between the high frequencies of abnormal homozygotes revealed by genetic surveys and the lower frequencies of GH in clinical and autopsy studies. More and more cases are now being detected by finding abnormal tests of iron status in patients with non-specific complaints such as weakness, arthropathy and abdominal pain or with isolated elevation of serum transaminase levels in standard multiple biochemical analysis.

7.1 Prevalence of Genetic Hemochromatosis in Countries with Established Registers

Prevalence of GH in Australia

Early estimates suggested that GH occurred in the homozygous state in about 1 in 400 persons of European extraction with a carrier rate of about 1:10. To determine the prevalence of the disease in Australia, a population survey of employees of a large Australian Banking Corporation and of a large Australian insurance company was recently conducted [77]. The prevalence of iron overload due to homozygous GH in an asymptomatic Australian (predominantly Caucasian) population was determined by surveying 1968 employees of the two large corporations. Subjects were screened by measurement of transferrin saturation and serum ferritin concentration and, in all subjects with elevation of both indices, percutaneous liver biopsy was performed to establish whether significant iron overload was present. The prevalence of iron overload due to GH in this population was 0.36%. The prevalence rate was not significantly different between males and females. The positive predictive value of a transferrin saturation consistently > 45% together with an elevated serum ferritin concentration was 64%. From this study, it was concluded that the prevalence in Australia of significant iron overload due to homozygous GH warranting treatment is approximately 1: 300 and that

transferrin saturation should be included in existing adult health screening programmes. One can believe that this is a conservative estimate for a number of reasons. Firstly, some of the subjects donated blood, thus potentially masking disease expression. Secondly, one subject with iron indices suggestive of GH declined liver biopsy and family screening and has not been included as a homozygote. Thirdly, stringent diagnostic criteria, demanding clear evidence of phenotypic expression of the disease in the form of hepatic iron overload have been applied. In the absence of a genetic marker which can directly identify the GH gene, liver biopsy findings, including increased hepatic iron concentration and an hepatic iron index greater than 2, are widely regarded as the definitive tests on which the diagnosis is based. Family studies employing HLA typing, although useful, rely on the existence of homozygous relatives. Liver biopsy was not performed on subjects with elevated transferrin saturation alone since liver iron content is very unlikely to be elevated when serum ferritin is normal. Thus, it is possible, indeed likely, that some individuals with the homozygous genotype and as yet incomplete or absent phenotypic GH expression have not been diagnosed, especially since the study was biased more towards younger subjects than the general population. It is noteworthy that there was no statistically significant difference in prevalence rates in males or females, which suggests that this autosomal recessive disorder is expressed equally in females, given an adequate dietary iron supply.

Prevalence of GH in the USA

A study was conducted in 1988 by Edwards et al [51] in order to determine the prevalence of GH in the American Red Cross blood donors in Utah. Healthy blood donors (5840 men and 5225 women) were screened using the fasting transferrin saturation method (TS). A diagnostic procedure - including liver biopsy and family study - was performed in participants with a TS of 62% or more. The estimation of the frequency of homozygosity for the GH gene was based on the data in men because the threshold value of 62% for the TS identified only half as many female homozygotes suspected. The frequency of homozygosity was 4.5‰, corresponding to a gene frequency of 6.7%.

Prevalence of GH in France

In 1976, the prevalence of clinically patent GH in the Breton population, France, was estimated as 1.1‰ [Alexandre, J.L., Medicine Thesis, Rennes, France, 1976]. More recently, data obtained through extensive family studies and an ongoing population study conducted in "Ille et Vilaine" (one of the four "counties" of Brittany) has led to an estimated homozygote prevalence as high as 3.6‰ in Brittany. In addition, analysis of death certificate data leads to the suggestion that GH may be about 3 times less frequent in France as a whole than in Brittany.

Prevalence of GH in Italy

By analyzing the geographical origin of Italian patients referred to Milan for GH, it was possible to define some areas of North Italy where GH is more common. Interestingly, these areas correspond to known settlements of the ancient Celts, supporting Simon's hypothesis of a Celtic origin of the disease [110]. In addition, several patients who came separately originated from the same village. For the above reasons, an epidemiological survey of GH by analyzing different areas of North Italy was undertaken. This study is still ongoing and some of the results are preliminary.

In collaboration with transfusion centres selected on the basis of their geographical location, the prevalence of donors with serum ferritin (SF) above normal values in the various

areas was first assessed. According to the study protocol, subjects with increased ferritin were recalled and invited to stop, if ascertained, alcohol intake. After two months, a second examination with SF and transferrin saturation (TS) measurements was performed. At the time of this second examination, the patients were tested also for gammaglutamyltranspeptidase as a marker of alcohol intake, to verify alcohol withdrawal, because of the known interaction between alcohol and ferritin. If iron overload was confirmed, a medical examination was carried out to rule out the presence of inflammatory processes or coexistent diseases. Subjects with evidence of iron overload were asked to undergo percutaneous liver biopsy.

The prevalence of donors with increased SF in the various transfusional centres are shown in Table 1.

- Transfusion Centre of Garbagnate Hospital: 1301 subjects, 976 men and 325 women, aged 18-65 years, who came to donate blood over one year were investigated for iron overload. For 83 of them it was the first donation. Garbagnate Hospital is the only centre where SF and TS are routinely performed in all donors. At first screening, ten men but no woman showed increased iron values (increased TS and/or SF). At second testing, five male donors presented persistent raised values of TS and/or SF. Liver biopsy performed in 3/5 subjects showed the presence of homozygous GH in two, giving a prevalence of GH of 2/970 (0.2%) in the male donors .
- Transfusion Centre of Brescia Hospital: 3200 donors, 2400 men and 800 women were investigated, aged 25-61 years. One hundred were donating blood for the first time and the others were regular donors. Fifteen had increased SF values. At second testing 12/15 had a persistent increase of SF and/or TS.
- Bergamo Hospital and Transfusion Centres around Bergamo: SF was measured in 10523 regular blood donors, 7787 men and 2736 women of mean age 39 years. One hundred and eighty-five men and 23 women had increased SF. Analysis of the geographical origin of the patients with increased SF showed that the incidence of subjects with raised values varied in the different villages, ranging between 0 and 7.93%. This analysis considered only villages with more than 50 donors. Interestingly, the higher incidences were observed in the villages from which several GH patients originated. Liver biopsy will be proposed to patients with persistent increases of SF.
- Transfusion Centre of Domodossola Hospital: 2830 donors, 1844 men and 976 women were studied. SF, at the time of the first donation, was increased in 151 men and in 8 women. After regular donations, 30 subjects had a persistent increase of SF and/or TS. Seven of these 30 potential GH subjects underwent liver biopsy which revealed GH in four. On the basis of this subgroup, it is possible to estimate the prevalence of GH in the Domodossola area as at least 0.2%, but it is probable that at the end of the study the prevalence will be higher.

From these results, of which only those of Garbagnate are complete, it appears that the prevalence of GH in Italy is at least 0.2%, with a gene frequency of 4.5 % and a heterozygote frequency of 9%. Since studies on epidemiology of GH in blood donors may underestimate the real prevalence of GH, mainly in women and young men, these values could be higher. In addition, the prevalence of GH in Italy may vary in different regions and within individual areas in the different regions. In fact, on the basis of the geographical origin of the GH patients, it is possible to conclude that GH is very likely more frequent in north than in south Italy and also that in the north it is possible to identify areas with a particularly high prevalence of GH. For example, the areas of Domodossola and around Bergamo, both areas corresponding to ancient Celtic settlements, appear to have a higher prevalence of GH. Liver biopsy of potential GH

patients will confirm the diagnosis and will provide more consistent data on the epidemiology of GH in these areas.

7.2 French Experience of the Assessment of the Prevalence of GH

Assessment of the GH Gene Prevalence Through Family Studies

One family study was reported by Marcel Simon [Iron Club Meeting, Amersfoort, The Netherlands, 1985]. From a total of 916 independent (each counted only once) haplotypes identified in 191 Breton families, 382 bearing the hemochromatosis gene (HFE) were present in probands and were used to assess the HFE genotype of the other haplotypes within the family members. Each haplotype was defined as carrying either the HFE or the normal allele only if it was present in an obligate carrier (on haplotype in common with a proband). Therefore, 61 haplotypes remained undefined since they were present only in HLA different relatives. Among the remaining 473 haplotypes present in relatives sharing one haplotype with a proband, 27 were defined as *certainly* carrying HFE since present in subjects with confirmed iron overload, and 13 as *possibly* carrying HFE in women or young obligate carriers whose genotype (either heterozygous or homozygous) was uncertain. Based on the overall data the gene frequency calculated in the population studied was 0.057 (27 / 473) to 0.085 (40 / 473), and based on the male data it was 0.064 (18 / 283) to 0.099 (28 / 283). The minimal gene frequency of 0.057 corresponded to a heterozygote frequency of 0.11 and a homozygote frequency of 3.25‰, assuming a Hardy Weinberg equilibrium. In addition, the segregation analyses of genetic hemochromatosis with serum iron [76] and serum ferritin [18] concentrations within 147 pedigrees from Brittany, favoured a recessive inheritance transmission with a gene prevalence of 0.060 and 0.054 respectively.

Assessment of the Prevalence of GH Through Population Study

A population study is in progress in the Ille et Vilaine County with the collaboration of the "Mutualité Sociale Agricole" (MSA) which is a part of the French National Health Insurance and specifically covers farmers, agricultural workers and ancillaries. Farmers and their families can benefit from a free MSA check up every five years, and are evaluated according to the date of birth of the main insurance beneficiary. The check up consists of: (i) venous blood drawn in fasting subjects between 8 and 10 am to determine serum glucose, triglyceride, cholesterol, GGT and haematological parameters; and, (ii) a questionnaire concerning general health, and dietary and alcohol habits. In case of abnormal results, subjects are asked by their general practitioner to benefit from further investigation and follow-up. In 1992, screening tests for iron overload were added to the check-up. Then, blood donations and gynaecological history were added to the questionnaire. The initial screening consisted of serum iron and transferrin saturation (TS) measurements. If TS was greater than 59%, additional measurements of serum ferritin (SF), AST and ALT were made. The older subjects who had TS > 59% and SF greater than the upper normal laboratory values (200 ng/ml in women and 300 ng/ml in men) were recalled by the MSA nurses for a second test. Those who were alcohol abusers were asked to stop their alcohol intake between the two screening tests. Between 01/03/92 and 10/31/92, 5479 subjects (i.e., 62%) replied favourably. There were 2741 men (mean age \pm SD: 55 \pm 11 y; min-max: 17 y - 67 y) and 2,738 women (mean age \pm SD: 56 \pm 8 y; min-max: 19 y - 67 y). Six subjects were known to already have GH. Diagnosis was confirmed early after the first screening in a woman. Thus, the prevalence of GH in this population is at least 1.27‰ (7 / 5479). This prevalence is in agreement with the previous assumption that the homozygous state in Caucasian populations is never less than 1‰ [113]. The definitive results are not known for the entire population studied. If the individuals who have not yet been

investigated are included, a maximum prevalence can be calculated, given that about 1/3 of the subjects who undergo a liver biopsy are confirmed as having GH according to classical criteria.

Then:

- 14 subjects (8 men, 6 women) needed liver biopsy after the first screening, and 13 (8 men, 5 women) after the second. Assuming 9 GH patients among these 27, the total patient group should reach 16, which gives a prevalence of about 2.92‰ (16 / 5479).

- 21 subjects had a discordant elevation of TS and SF at one of the two tests. Nine were chronic alcoholic males over 55 years; 12 were females or young males (9 and 3 respectively) who could be homozygotes in the early stage of iron overload. Assuming that a long term follow-up of these last 12 subjects would possibly detect GH in 1/3, the prevalence should finally reach 3.65‰ (20 / 5479). Interestingly, it must be noted that the homozygote prevalence of 3.65‰ corresponds exactly to the gene frequency of 0.06 calculated within families. M. Simon himself pointed out that family studies might have overestimated the true frequency of homozygosity in the general Breton population. The concordance between the two approaches was thus unexpected. Although the results in the present population study are extrapolated, the estimate of 1/3 seems real and even if the estimate was 1/5, the expected prevalence would be as high as 2.73‰ (15/5479), i.e., far greater than that of cystic fibrosis which is currently considered as the most frequent recessive disease in Caucasian populations.

Approach of the Prevalence in France Through Mortality Data

The only epidemiological data on hemochromatosis currently available for all of France are mortality data recorded by the National Institute of Health and Medical Research (INSERM) from death certificates. Since 1979, GH is coded "275.0" according to the International Classification of Diseases, Injuries and Causes of Death (9th revision, ICD 9). A French population census was carried out in 1982 by the National Institute for Statistics and Economic Studies (INSEE). A total of 393 deaths from GH (primary cause of death) was recorded in France over the period 1979-1983. This comprised 313 men and 80 women aged from 25 to 90. From these, 49 were recorded in Brittany (43 men aged from 35 to 79 and 6 women aged from 35 to 90). The number of deaths was too small to allow accurate calculations of age and sex-standardized mortality rates. Therefore the study was focused on the expected number of deaths in men in France adjusted to the mortality rate in Brittany for the same age group aged 35-79. In France, the observed number of male deaths was 297 and the expected number according to the data in Brittany was 874 (observed/expected ratio = 0.34). This study does not directly assess the prevalence of GH in France, but can be considered as an interesting comparative approach. It suggests that the disease may be 3 times more frequent in Brittany than in France. The probable underestimation of GH-related deaths on death certificates does not invalidate the interpretation in terms of a ratio, insofar as the proportion of undeclared cases does not vary from one region to another. Whether the influence of M. Bourel and M. Simon during the period considered has led to a better ascertainment in Brittany than in France is open to question. General practitioners in Brittany can be expected to be more aware of the disease, which would explain more frequent and earlier diagnosis than in other French regions. However, it can be assumed that men who died from GH as a primary cause were those with unambiguous and major clinical presentation of the disease, and consequently the diagnosis would have been made whatever the region. Thus, the difference observed between Brittany and France in the data studied is probably not due to a bias in ascertainment.

It has been pointed out that studies initiated in regions where there was an *a priori* assumption of high prevalence might overestimate the global prevalence of the disease [113].

The French mortality data suggest a lower frequency in the country as a whole than in Brittany, and there is no reason to believe that a systematic ascertainment bias has inflated the ratio in favour of Brittany. Together with the data from the autopsy and population studies in Sweden [68, 78], and from the N-HANES II in USA [55], this finding suggests that the average prevalence of GH is about 1‰ in Caucasian populations. On the other hand, there are undoubtedly regions of unknown size and number where the prevalence is considerably higher. In addition, regional differences within a country have already been reported in Sweden [68, 78] and Portugal [90]. Such striking geographic variations of prevalence among populations of European Caucasian ancestry as well as ethnic variations are well known in many other recessive diseases. These differences become important when discussing genetic risks, public health intervention and prevention strategies at the level of a country.

8. APPROACH FOR FAMILY SCREENING IN GENETIC HEMOCHROMATOSIS

8.1 General Approach

Today, in the absence of a specific test, homozygosity for HFE is only clinically postulated, once the phenotypic expression has occurred. The indirect genetic tests currently available, i.e., HLA serotyping and/or DNA genotyping for linked specific or anonymous markers [94, 126], are of no diagnostic value but are used to assess the genetic status of each relative of a proband. Then, once a GH patient has been identified, a screening programme must be offered to his family. In addition to periodic phenotypic testing for iron overload which can be performed by a general practitioner, genetic testing can be proposed to healthy relatives. This requires interpretation and the following must be included in genetic counselling: (a) accurate diagnosis; (b) education and information about the disease and genetics; (c) careful interpretation of the results in close collaboration with laboratories that perform the genetic tests; (d) awareness of the genetic risks and the options to avoid the disease. Assiduous phenotypic follow-up to confirm the predicted outcome is also important. The genotype of unaffected relatives can be predicted as homozygote normal (no haplotype in common with the proband), obligate carrier (one haplotype in common with the proband), or homozygote for HFE (both haplotypes). The risk of homozygosity for obligate carriers depends on the frequency (p) of the gene within the population, and on the subsequent frequency of heterozygote-homozygote and double homozygote marriages (0.4‰ and 0.01‰ for $p = 0.06$). In practice, genetic tests are valuable in assessing the genetic status of siblings, but they are of poor value in the proband's children except in some particular areas where the risk of carrying the mutation for a given haplotype within the population subgroup studied is already known (this risk has wide regional variations).

Simple practical problems may limit the predictive accuracy of the tests: unavailability of crucial subjects (proband and/or parents), incomplete family records and, no or partial informativity of the family [46]. Discordances between the clinical outcome and the predicted status are well documented [46, 93], and require re-interpretation of the family as a whole. A major unsolved problem is the unclear etiology of atypical and borderline forms of iron-overload that do not fulfil the classical criteria, and may represent other genetic or acquired conditions [Yaouanq, J., personal communication, 1993].

The new genetic bio-technology offers great hope for the improved care and prevention of the disease. The future strategies of genetic testing and population screening will depend on the mutational profile of GH, i.e., the number and distribution of the mutation(s) in the population [81, 85]. Once the HFE gene is isolated, and assuming little if any heterogeneity, direct diagnosis and screening for HFE homozygosity independently of family history could

become a reality, and should be considered in certain high risk populations. On the other hand, mass screening for heterozygosity would be inappropriate for GH since neither an individual benefit, nor a reduction of the incidence at the population level can be expected for a recessive disease with delayed age of clinical expression, preventable and compatible with a normal life expectancy.

In the event of marked molecular heterogeneity at the HFE locus, many affected subjects could be in fact compound heterozygotes. Therefore, direct DNA-based testing might be of limited value for accurate diagnosis, and genetic testing for families with a history of HFE could still require the use of linked markers. Pilot programmes should be initiated in order to assess the feasibility of genetic screening and to define the appropriate target populations. In the meantime, extensive educational efforts should be made to advise on the technical limits of this kind of preventive medicine. On the other hand, the molecular description of the different mutations should provide important insight into the pathophysiology of the disease.

8.2 Genetic Basis [110].

Disease Transmission and Expression

It is now well admitted that GH is transmitted according to an autosomal recessive transmission. Therefore, only patients bearing two GH genes (= homozygotes = HH) express fully the disease. However, due to the variability of penetrance and the effects of environmental factors, clinical expression varies widely from one homozygote to another. Patients bearing one GH gene only (= heterozygotes = HN) do not express clinical GH symptoms, but may present with mild abnormalities in serum iron parameters, and slight and steady liver iron overload in 25% of cases [108]. Marriages between two heterozygotes produce on average 1/4 homozygotes, 1/2 heterozygotes and 1/4 GH free subjects in the offspring (Fig. 1). Mating between one heterozygote and one homozygote is less frequent leading statistically to 1/2 homozygotes and 1/2 heterozygotes in descendants (Fig. 2). Mating between two homozygotes is exceptional in which case all children are homozygote.

Relationship Between GH and the HLA system

The GH Gene and the HLA-A Locus

The GH gene has not yet been fully identified but has been located however on chromosome 6, very close to the HLA-A locus. Due to their proximity, the GH gene and the HLA-A allele are transmitted together. Therefore, HLA-A does not contain the GH gene itself, but is a close indirect marker for the gene.

The GH Gene and HLA-A Antigens

Among the antigens encoded by the HLA-A gene, the A3 antigen is the most frequently associated with the GH gene [105]. 55-100% (average = 73%) of GH patients are positive for HLA-A3 as compared to 19 to 32% observed in the general population. The HLA-A3 antigen can be therefore considered as a special marker for the GH gene but not as an exclusive and specific marker since 27% of GH patients are negative for HLA-A3 and up to 32% of controls are positive for HLA-A3. Hence, HLA-A typing has no diagnostic value in a given patient.

The GH Gene and HLA Haplotypes

By way of the HLA-A antigens, the GH gene is indirectly associated with certain HLA-B locus antigens since the HLA-B antigen is transmitted along with the HLA-A antigen. As a result, two haplotypes have been found to be preferentially associated with the GH gene: A3-B14 and A3-B7 [109]. However, (i) for a given subject, the demonstration, for example, of A3, A11, B7 and B35 antigens does not necessarily imply A3-B7 and A11-B35 haplotype matching. The study of HLA antigen transmission in the subject's family is hence necessary in order to identify family haplotypes (which, in this example, could be A3-B7 and A11-B35 as well as A3-B35 and A11-B7, the former two being statistically more frequent than the latter); (ii) what has been shown for HLA-A3 antigen is valid for haplotypes: in a given patient, the presence of A3 and B7 or B14 alone does not permit the diagnosis of GH. Finally, the linkage between the GH gene and HLA provides crucial information in the siblings of a homozygous GH subject (= proband). It allows (i) the identification of the proband haplotypes as markers of the GH gene within his family, and (ii) the prediction of homozygosity in siblings who have two haplotypes in common with the proband.

8.3 Initial Hypothesis

The validity of a family screening relies on the hypothesis that the proband is homozygote for the GH gene. Therefore, it is absolutely necessary to validate the following steps before starting family screening:

Presence and Quantification of Iron Overload

Iron overload must be confirmed and quantified on liver biopsy: (i) Clinical and biological data often lead to overdiagnosis of GH, especially in alcoholic patients, and to underdiagnosis in early GH; (ii) Imaging procedures are not as sensitive and specific as previously claimed: iron-related increase in CT liver density is inconstant in early GH (due to its lack of sensitivity) as well as in advanced disease (due to possible false negatives related to associated steatosis and fibrosis) [66]; magnetic resonance imaging is more specific but its sensitivity is still low [17, 67]. However, the development of new MRI sequences could improve its efficiency in the near future [61]; (iii) Histological iron scoring [23, 41-42] and determination of liver iron concentration [23] remain, therefore, the best prospective means for quantifying liver iron content and total body iron.

Determination of Iron Overload Mechanism

The major mechanism(s) of liver iron deposition in GH is iron hyperabsorption [25-26]. This explains why hepatocytic iron deposits always predominate in peri-portal and peri-lobular areas with a decreasing gradient from zone 1 (peri-portal) to zone 3 (centro-lobular). In advanced GH, when hepatocyte iron storage capacity is overpassed leading to the development of sideronecrosis, iron deposits attain macrophages, endothelium walls and biliary duct cells [41]. But even in advanced stages, iron overload predominates within hepatocytes in peri-portal and peri-lobular areas [41]. Histological examination permits to rule out iron overload related to abnormalities in iron turn-over (such as inflammatory syndrome, hepatocellular necrosis, haemolytic syndromes without dysmyelopoiesis...), or to parenteral iron administration which are characterized by iron deposits in macrophages without zonal distribution [101].

Assessment of the Genetic Origin of Iron Overload

Due to the lack of a specific marker, this step relies upon the elimination of other causes of iron hyperabsorption such as sideroblastic anaemia and chronic oral iron intake, and on the existence of GH in the patient's family medical history, especially in siblings.

Validation of Proband Homozygosity

This last step remains the most difficult one since there exists no specific marker able to differentiate homozygotes in their early stage, from heterozygotes who express mildly iron overload. Homozygosity can be putatively affirmed, however, on iron quantification data, and in particular the hepatic iron index (HII), defined as the liver iron amount to age ratio [13]. The HII can be determined by using a liver iron concentration assay [10] which provides a biochemical hepatic iron index (BHII) [11], or by histological examination which defines a histological hepatic iron index (HHII) [42]. Based on results from family screening data, one can conclude upon homozygosity when the BHII is greater than 2 [2, 94, 99, 117], and/or when the HHII is greater than 0.2 [42]. These two indexes have been shown to be well correlated [42]. These diagnostic tools are still insufficient however as (i) they have been validated only in GH families and not in the general population, and (ii) they do not always correctly classify putative probands, especially when homozygosity is under-expressed due to weak penetrance, blood donation or blood loss.

8.4 Screening Methodology

Subjects to be Screened

Screening must be proposed to all proband's siblings. Whenever possible, the proband's parents, spouse, and children over 10 years old should be included in the screening. The screening of children of homozygote-heterozygote parents by the age of five is encouraged in order to prepare the family for a possible plebotomy which may become necessary due to significant iron overload.

Data to be Collected

Phenotypic Data

One should screen for clinical and biological signs of iron overload and for possible causes of over-estimation (contraceptive pill intake, chronic alcoholism, liver disease, haemolysis...), or under-estimation (blood loss, blood donation, pregnancies, intra-uterine device, inflammatory syndrome) of serum iron parameters. This implies careful clinical examination and several simple biological tests such as transferrin saturation, serum iron, ferritin, alanine amino transferase (ALT), asparto amino transferase (AST), and gamma glutamyl transferase (GGT) serum levels, sedimentation rate and red cell count.

Genotypic Data

Complete HLA typing (i.e., testing all known HLA-A and HLA-B antigens) must be carried out. Testing only for HLA-A3, HLA-B7 and HLA-B14 antigens is useless and meaningless.

Data Interpretation

The first step consists in reconstituting HLA antigen transmission in the family studied. It then becomes possible to define the haplotypes which in the family studied are transmitted together with the GH gene. By definition, both proband haplotypes are markers for the GH gene.

The second step consists in comparing genotypic and phenotypic data.

In siblings (and parents)

Irrespective of parental mating (HN-HN, HH-HN, HH-HH), siblings who are HLA identical to the proband (two haplotypes in common with the proband) are considered as being homozygote for the GH gene (Figs. 3 and 4). Interpretation of HLA semi-identical and HLA different siblings of the proband (i.e., one or no haplotype in common with him, respectively) may be more difficult, due to the problem of a possible heterozygote-homozygote parental mating, in areas of high frequency of the gene.

- If parents have been screened, it is easy to define their genetic status due to their advanced age. Then:
 - If both parents do not express any clinical or biochemical abnormality (negativity of phenotypic screening), it can be concluded that they are both heterozygote. Consequently (i) their offspring HLA semi-identical to the proband are heterozygote and, (ii) those HLA different to the proband are free of the GH gene (Fig. 3). This is the most frequent case.
 - A parent suspect of iron overload may possibly be homozygote for the GH gene. It then becomes necessary to validate homozygosity as previously explained. If homozygosity is indeed confirmed, this implies the existence of 3 GH genes (and hence, 3 haplotypes marking for the GH gene) in the family. As a result, (i) half of their offspring semi-identical to the proband are heterozygotes and half are homozygotes, and (ii) all their offspring HLA different are heterozygote for the GH gene (Fig. 4).
 - If both parents are validated as homozygotes, their four HLA haplotypes are markers for the GH gene and, then, all their children are homozygote irrespective of their HLA typing. This situation is quite exceptional.
- If parents have not been screened, the conclusions drawn depend on phenotypic data:
 - For semi-identical subjects: when phenotypic expression lacks, these subjects are likely heterozygotes born from HN-HN parents. However, attention must be paid to young subjects, specially women, who could be homozygotes free of phenotypic expression. In cases of phenotypic expression, overestimation of serum iron parameters due to expression of a heterozygous status or to chronic alcoholism, oral contraceptive or iron intake, non-hemochromatotic liver disease or haemolysis have to be considered before concluding in favor of a possible HH-HN parental mating. These considerations require careful clinical and biological follow-up and, on some occasions, liver biopsy.

- For HLA different subjects: these subjects are most likely to be either free of the gene (HN-HN parents) or heterozygote (HH-HN parents). Hence, before suspecting an unlikely homozygous status in these subjects, overexpression of serum parameters must be excluded in the former, and expression of a heterozygous state must be considered in the latter.

In children (and spouse)

All children born from a homozygote subject are, by definition, heterozygote. However, they have a risk to be homozygote since their definitive genetic status depends on that of the proband's spouse.

- Phenotypic data of proband's children are difficult to interpret as these subjects are usually young. It may be impossible to differentiate heterozygous from homozygous expression in such subjects. Phenotypic data from the proband's spouse can help if positive, but they are most often negative and are not conclusive as to genetic status (since a healthy subject may be GH free or heterozygote).
- Genotypic data allow for a statistical estimation of the risk of homozygosity in an obligatory heterozygote, according to the probability for the haplotype inherited from the proband's spouse to carry a GH gene. As an example, the risks reported in Table 2 have been established in the Breton population. However, as discussed above (see point 8.2), they cannot be used in other subgroups of the Caucasian population.

8.5 Therapeutic Consequences

The ensuing therapy depends upon the reliability of the genotypic assignment.

In cases of certainty:

Homozygotes: except for young homozygotes free of any clinical or biological signs of iron overload, they should benefit from a liver biopsy in order to confirm homozygosity before starting the screening of their own family (spouse and children), and determine the extent of liver damage (in terms of fibrosis and associated lesions), and define treatment modalities (duration of venesection programme).

Heterozygotes are advised to be venesected (400 ml two to four times a year) in order to treat or prevent a putative slight iron overload.

Subjects free of the GH gene do not require any particular follow-up.

In cases of uncertainty between:

Free status and heterozygosity: no follow-up is necessary but these subjects are encouraged to be venesected two to four times a year;

Heterozygosity and homozygosity: these patients should be directed to a venesection programme if they express phenotypic abnormalities; if not, they should undergo a serum iron parameter follow-up every year. If serum ferritin steadily increases, liver biopsy is indicated.

8.6 Costs and Benefits

If diagnosed before the onset of complications such as liver cirrhosis, diabetes and cardiomyopathy, and if correctly treated, GH does not decrease life expectancy as compared to the general population [56, 87]. In view of the fact that GH patients detected by family screening go uncomplicated in 95% of cases, there is no doubt that such a programme is profitable in terms of individual and public health. In addition, by way of GH family screening other frequent conditions such as chronic alcoholism and diabetes are detected. The mean cost of screening in Brittany, France, including physical examination, blood tests (serum iron parameters, ALT, AST, GGT, blood count and HLA typing) and running charges of the screening centre, can be evaluated at about US\$ 250 per subject. Based upon the last 179 family screening procedures performed in 1990 and 1991 in the Breton Family Screening Centre for Hemochromatosis, the cost per homozygote detected is estimated at about US\$ 1 500. Given the medical and social cost of a fully expressed disease complicated by diabetes, arthropathy, cardiomyopathy, impotence and cirrhosis during a mean duration of 10 years and by hepatocellular carcinoma in the late course of the disease, the cost of family screening is by comparison quite acceptable. In order to gain substantial savings, some authors have proposed not to include HLA typing in family screening procedures [5]. Such a proposal should be considered with caution at a time when heightened awareness of physicians and general public information are leading to early diagnosis of probands and, hence, the screening of younger and younger GH patients. On the other hand, assessment of the genetic status in young males and in females before menopause, is unreliable when based only on clinical and biochemical data. Then, the risks are: (i) to over-diagnose homozygote GH in subjects with increase in serum iron parameters due to heterozygote status, or to non hemochromatosis-related causes, leading to useless follow-up (recurrence of blood tests), inadequate diagnosis procedures (wrong indication for liver biopsy), useless venesection programmes and worthless descendance screening which could cost much more than HLA typing of the whole family; or (ii) to under-diagnose homozygote GH in subjects with normal serum iron parameters such as in young women who are wrongly considered as free of GH or classified as of uncertain genetic status. The former are regularly followed-up, but the cost of such useless follow-up exceeds the cost of initial HLA typing. The latter are not accurately followed-up and develop clinical symptoms insidiously.

9. CONCLUSIONS

Family screening for genetic hemochromatosis implies a methodical procedure which relies upon (i) a proper understanding of its mode of inheritance and of the linkage between the GH gene and HLA system, (ii) the scrupulous validation of proband homozygosity, (iii) the collection of phenotypic and genotypic (HLA typing) data in the proband's parents, siblings, spouse and descendants, and (iv) the analysis of its mode of transmission in the family studied. Until new and reliable DNA markers can be used in routine tests [126], HLA typing remains necessary. Such a policy is justified since it allows for highly effective prevention of complications from a frequent and potentially fatal genetic disease.

Once the gene is identified, any decision concerning the systematic integration of genetic screening for GH into primary health care should be taken in the light of the strengths and weaknesses of previous screening programmes [62, 86, 96]. Apart from economic evaluation to define the most appropriate public health policy, efforts should be made to inform the public and the practitioners in order to avoid negative psychological effects of genetic screening in a healthy population [52, 58, 82].

10. LIST OF PARTICIPANTS

- Dr Pierre Brissot, Liver Unit, Centre Hospitalier Régional et Universitaire de Rennes, Pontchaillou, F-35033 Rennes, France
- Dr Yves Deugnier, Liver Unit, Centre Hospitalier Régional et Universitaire de Rennes, Pontchaillou, F-35033 Rennes, France (**Chairman/Rapporteur**)
- Dr C.Q. Edwards, LDS Hospital House Staff Clinic, College of Medicine, University of Utah, 325 8th Avenue, Salt Lake City, Utah 84143, USA
- Dr Silvia Fargion, Istituto di Medicina Interna e Fisiopatologia Medica, Pad.Litta Ospedale Maggiore, Università degli Studi di Milano, 35 via F. Sforza, I-20122 Milan, Italy
- Dr June Halliday, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland 4028, Australia
- Dr C. Hershko, Department of Medicine, Shaare Zedek Medical Centre, P.O. Box 3235, Jerusalem, Israel
- Dr Lawrie Powell, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland 4028, Australia
- Dr Georg Stromeyer, GI Unit, Department of Medicine, Heinrich-Heine-University, Moorenstr. 5, D-4000 Düsseldorf, Germany
- Dr Jacqueline Yaouanq, Department of Public Health, Centre Hospitalier Régional et Universitaire de Rennes, Pontchaillou, F-35033 Rennes, France.

HEMOCHROMATOSIS FOUNDATION SECRETARIAT

- Mrs M. Krikker, President, Hemochromatosis Foundation Inc., P.O. Box 8569, Albany, NY 12208, USA
- Mrs M. Warder, President, Canadian Hemochromatosis Society, P.O. Box 94303, Richmond, B.C., Canada V6Y 2A6
- Mrs J. Fernau, Hemochromatosis Society, Hollybush House, Hadley Green, Barnet, Herts EN5 5PR, UK

FRENCH HEMOCHROMATOSIS ASSOCIATION SECRETARIAT

- Mr P.-M. Morel, President, French Hemochromatosis Association, B.P. 7777, F-30912 Nîmes, France
- Dr P. Delon, 8 chemin Maurice Ravel, CH-1290 Versoix, Switzerland (unable to attend)

WHO SECRETARIAT

Dr Victor Boulyjenkov, Responsible Officer, Hereditary Diseases Programme, Division of Noncommunicable Diseases, World Health Organization, CH-1211 Geneva 27, Switzerland (Secretary)

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Table 1

**PREVALENCE OF INCREASED FERRITIN IN DONORS
FROM DIFFERENT TRANSFUSIONAL CENTRES OF NORTH ITALY**

CENTRES	TOTAL	MALES	INCREASED FERRITIN				
			SCREENING		FEMALES	SCREENING	
			#1	#2		#1	#2
			%	%		%	%
Garbagnate	1301	976	1	0.5	321	0	0
Brescia	3200	2400	0.5	0.4	800	0.4	0.1
Domodossola	2830	1844	8.1*	1.5	976	0.8*	0.2
Bergamo	10523	7787	2.1	**	2736	0.8*	

* Prevalence estimated at the time of the first donation.

** Done in 35 patients: ferritin was elevated in 20.

Table 2

**RISK OF HOMOZYGOSITY IN AN OBLIGATORY HETEROZYGOTE SUBJECT OF
THE BRETON POPULATION ACCORDING TO HLA HAPLOTYPES [111]**

FIRST HLA HAPLOTYPE (= marker for the GH gene)	SECOND HLA HAPLOTYPE (= unknown GH status)	RISK OF HOMOZYGOSITY (percentage)
Aa - Bb	+ A3 - B14	57
	+ A3 - B7	21
	+ A3 - By	19.5
	+ A11 - By	13
	+ A3 - B non 7 non 14	10.5
	+ Ax - By (random)	6
	+ A non 3 - By	4
	+ A non 3 non 11 - By	3.5

Fig. 1

**TRANSMISSION OF THE GH GENE (BLACK SQUARE)
AMONG OFFSPRING OF HETEROZYGOTE PARENTS**

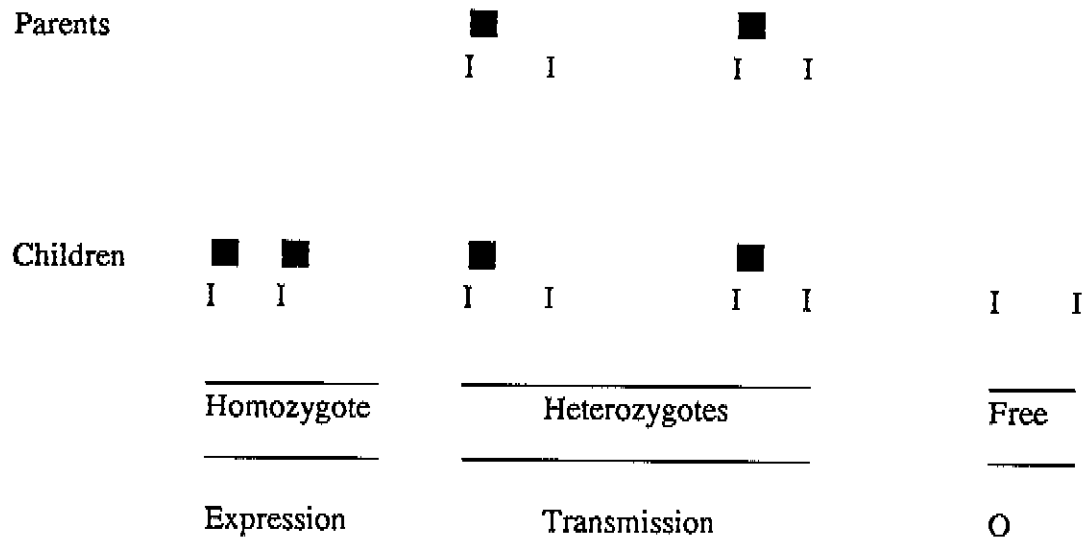


Fig. 2

**TRANSMISSION OF THE GH GENE (BLACK SQUARE)
AMONG OFFSPRING OF HETEROZYGOTE AND HOMOZYGOTE
(PSEUDO DOMINANT TRANSMISSION) PARENTS**

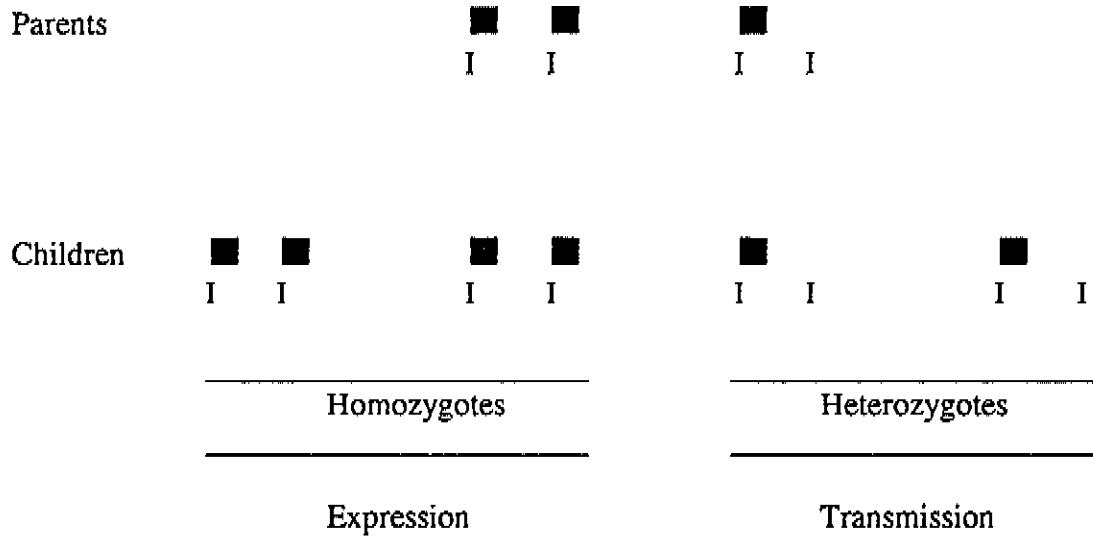


Fig. 3.

**HAPLOTYPIC RECONSTITUTION AND TRANSMISSION OF THE GH GENE
(BLACK SQUARE) IN SIBLINGS BORN FROM HETEROZYGOTE PARENTS**
id = identical, ≠ = different

Parents	■		■					
	I	I	I	I				
	A2	A9	A3	A1				
	I	I	I	I				
	B12	B5	B7	B8				
	■	■	■		■			
	I	I	I	I	I	I		
	A2	A3	A2	A1	A9	A3	I	
	I	I	I	I	I	I	I	
	B12	B7	B12	B8	B5	B7	B5	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■		■	
	I		I		I		I	
	A2		A3		A9		A1	
	I		I		I		I	
	B12		B7		B5		B8	
	■		■		■			

Fig. 4

**HAPLOTYPIC RECONSTITUTION AND TRANSMISSION OF THE GH GENE
(BLACK SQUARE) IN SIBLINGS BORN FROM
HETEROZYGOTE AND HOMOZYGOTE PARENTS**
id = identical, ≠ = different

Parents

■	■	■	
I	I	I	I
A2	A9	A3	A1
I	I	I	I
B12	B5	B7	B8

■	■
I	I
A2	A3
I	I
B12	B7
Proband	

■	■
I	I
A2	A3
I	I
B12	B7
HLA id	

■	■
I	I
A9	A3
I	I
B5	B7
HLA semi id	

■	
I	I
A2	A1
I	I
B12	B8
HLA Semi id	

■	
I	I
A9	A1
I	I
B5	B8
HLA ≠	

Homozygotes

Heterozygotes

Expression

Transmission