

Hypogonadism and Reduced Bone Mineral Density in Heterozygous H63D Mutation in the HFE Gene: An Unusual Presentation of Hereditary Hemochromatosis

Case Report

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Hereditary hemochromatosis (HH) is the most common inherited metabolic disease in the white population. It is a disorder of iron regulation that brings about an excess absorption of dietary iron, which gradually accumulates in the parenchymal cells of liver, pancreas, heart, anterior pituitary, and gonads. Human iron homeostasis depends on the coordinated functions of numerous genes, the precise roles of which are in many cases still obscure.

Classic HH is an autosomal recessive disorder associated with a mutation of the HFE gene, which was discovered in 1996 and is located on chromosome 6. The most common form of HH is related to homozygosity for the C282Y mutation of the HFE gene, resulting in a change of tyrosine for cysteine at amino acid 282 (Feder et al, 1996) This mutation leads to a chain of events that may culminate in severe damage to multiple organs. The C282Y mutation is largely confined to whites of north European origin, and the frequency of homozygosity decreases from the north to the south of Europe, being lowest in Italy and the Mediterranean countries (Carella et al, 1997), where hemochromatosis is genetically heterogeneous (Piperno et al, 1998). Another mutation of the HFE gene, termed

H63D mutation, results in the substitution of aspartate for histidine at amino acid 63 and does not appear to have clinical effects (Gochee et al, 2002). The clinical significance of compound heterozygosity for C282Y and H63D is still controversial (Rochette et al, 1999). It is possible that several genes, other than HFE, may play a role in the disease in a few patients, presenting similar forms of iron overload which act as modifiers of the phenotype, as seen in HFE knockout mice (Bensaid et al, 2004). Therefore, it is difficult to predict whether and to what extent such a mutation will be phenotypically expressed.

The Online Mendelian Inheritance in Man (OMIM) database currently lists 5 types of HH (Bomform, 2002), each caused by mutations involving a different gene, but cases of iron overload have been diagnosed in those genes, known to be associated with this disease, that have a normal sequence, indicating that there are still other genes to identify.

In the most extreme forms of HH, the disease manifests itself as cirrhosis, hepatocellular cancer, destructive arthritis, cardiomyopathy, and endocrine problems such as diabetes mellitus and sexual dysfunction due to hypogonadotropic hypogonadism. Gonadal problems in hemochromatosis generally result from the destructive action of hemosiderin in the anterior pituitary (MacDonald and Mallory, 1959). A review by Pedersen-Bjergaard et al. on the pituitary function in hemochromatosis highlighted an insufficiency of pituitary gonadotropic secretion with clinical hypogonadism in 46% of the patients, a subclinical insufficiency of the growth hormone axis in 15%, of the lactotropic axis in 8%, of the thyroid axis in 4%, and of the adrenocortical axis in 1.5% of the patients. Moreover, the same authors underlined that lactotropic, thyroid, or adrenocortical insufficiency was usually associated with hypogonadism or growth hormone insufficiency (Pedersen-Bjergaard et al, 1996). Hypogonadotropic hypogonadism appears to be unusual in patients with lesser degree of hepatic siderosis at diagnosis (McDermott and Walsh, 2005). Therefore, partial hypopituitarism alone, in the absence of any important damage to other main parenchymal

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Dynamic responses and basal hormone concentrations

Tests	Hormone	Baseline	30'	60'
Hypoglycemia insulin test (0.1 U/kg IV bolus)	GH (ng/mL)	0.21		0.19
	ACTH (pg/mL)	13.00		14.00
	Cortisol (mcg/dL)	20.80		21.10
CRH test (100 mcg IV bolus)	ACTH (pg/mL)	19.00		17.00
	Cortisol (mcg/dL)	26.30		20.2
GnRH test (100 mcg IV bolus)	LH (mIU/mL)	1.50	1.80	
	FSH (mIU/mL)	1.80		1.80
TRH test (200 mcg IV bolus)	TSH (mcU/mL)	0.66	4.07	

organs, notably the liver, associated with reduced bone mineral density (BMD), is an unusual presentation of the disease.

We describe a male patient with secondary hypogonadism and reduced bone mass as a presenting feature of hereditary hemochromatosis.

Case Report

The proband, a 45-year-old patient, father of one 11-year-old child, was referred to our department for the evaluation of a decreased libido associated with impotence and profound asthenia, which had begun some months back.

He had no previous medical history. Physical examination revealed bronze skin (only a mild generalized hyperpigmentation) also, from family history, present in his father, nicknamed the "Moor," who died when he was 50 years old because of cardiac problems.

Parameters related to iron metabolism and basal hormonal evaluation, verified with analogous results on 2 consecutive occasions, showed the absence of anemia, normal serum iron concentration, and transferrin percent saturation during fasting ($\leq 42\%$); a marked increase of ferritin levels (633 ng/dL with reference range 16–220 ng/mL); normal 24-hour urinary uroporphyrin and coproporphyrin concentration; reduced levels of total and free testosterone (1.2 ng/dL and 5 pg/mL with normal range 2.7–11 ng/dL and 9–47 pg/mL, respectively), associated with low levels of luteinizing and follicle stimulating hormone (1.4 mIU/mL and 1.5 mIU/mL with normal range 1.3–9 mIU/mL and 1.4–16 mIU/mL, respectively) and normal levels of prolactin. Except for mild elevation of fasting glycemia and impaired glucose tolerance at 2 hours following oral glucose-tolerance test, fasting routine laboratory tests (hemochrome, aspartate aminotransferase, alanine aminotransferase, electrolytes, and HbA_{1c}) gave normal results as well as hemoglobin electrophoresis, glucose 6 phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, haptoglobin, and the reticulocyte count, evaluated to rule out thalassemia or hemolytic conditions. TSH, free fractions of T₄ and T₃, IGF-I, plasma

cortisol, and urinary free cortisol were also normal, as well as plasma aldosterone (PA), plasma renin activity (PRA), and PA/PRA ratios in the basal state and in the upright position.

Decreased response of serum gonadotropins to GnRH stimulation (100 μ g IV bolus) revealed a secondary hypogonadism. An insulin-induced hypoglycemia test, with a reduction of blood glucose less than 40 mg/dL from the starting level, highlighted a basal GH deficiency and the lack of response of plasma ACTH and cortisol, which was also confirmed by AM CRH test (100 μ g IV bolus). On the contrary, a stimulation test with thyrotropin-releasing hormone (TRH) (200 μ g IV bolus) gave normal results (Table).

In view of partial hypopituitarism, the brain MRI scan, carried out to exclude the presence of pituitary abnormalities or signs of hemosiderin deposition, was normal. MRI images of the liver were suggestive of iron deposition and did not highlight liver and spleen enlargement.

According to the patient's wishes, we did not perform a liver biopsy. Moreover, it has been demonstrated that the measure of liver iron concentration loses most of its diagnostic significance after cloning the HFE gene (Camaschella, 2005) and that subjects with normal biochemical liver function tests, without hepatomegaly and with serum ferritin levels less than 1000 μ g/L, rarely have significant fibrosis (Qaseem et al, 2005; Schmitt et al, 2005; Yen et al, 2006). Nevertheless, liver biopsy can still be useful in some patients to provide additional information on histology (presence of fibrosis, cirrhosis) as well as on the distribution of iron (hepatocytes vs Kupffer cells) (Camaschella, 2005).

Considering the association of hemochromatosis and osteoporosis (Diamond et al, 1989) and the well-known deleterious effects of hypogonadism on bone, we evaluated the BMD using dual x-ray absorptiometry densitometer. The patient showed reduced BMD at lumbar spine (L₂₋₄: 0.956 gr/cm²; T-score: -2.59 DS) and at nondominant proximal femur neck (0.831 gr/cm²; T-score: 1.83 DS). In light of these results, in order to study the bone metabolism, fasting blood samples

were taken for the measurement of serum calcium, phosphorus, intact parathyroid hormone, bone-specific alkaline phosphatase, C-terminal cross-linking telopeptide of type I collagen (CTX), and 25-hydroxyvitamin D. All these laboratory tests were within normal range with the exception of CTX, which was slightly increased (data not shown).

The proband did not report any previous blood transfusion, iron-containing medications, or daily consumption of alcohol.

Although a normal value (<45%) of serum transferrin saturation normally rules out HH, we considered it reasonable, on account of raised ferritin levels together with the familial and personal history of the patient, to perform the genetic testing for HFE mutations with the patient's consent. Heterozygosity for the H63D mutation of the HFE gene confirmed the diagnosis for HH.

In relation to these results the proband began an androgenic and corticosteroid replacement therapy.

The response to therapeutic phlebotomy highlighted the decrease of serum ferritin in parallel with transferrin saturation, in the absence of anemia, and an increase of the free interval of androgenic replacement therapy (intramuscular).

Materials and Methods

Biochemical Evaluation—We measured: after overnight fasting, serum iron using the ferrozine method (ADVIA 1650; Bayer, Leverkusen, Germany), serum transferrin using the nephelometric method (BN II Instrument, DAE Behring, Deerfield, Ill), and serum calcium and phosphorus by automated routine procedures (intra- and interassay coefficients of variation $\pm 2.0\%$); by chemiluminescent immunometric assays: serum ferritin, cortisol, estradiol, total testosterone, FSH, LH, prolactin, free fractions of T_4 and T_3 , and TSH (ADVIA Centaur; Bayer), IGF-1, 25-hydroxyvitamin D and PTH (Dia Sorin, Saluggia, Italy), GH (Medical Systems, Genoa, Italy), CTX (beta-CrossLaps; Roche, Basel, Switzerland); ACTH, fasting insulin and dehydroepiandrosteron sulfate (Immulite; Diagnostic Products Corp, Los Angeles, Calif); by radioimmunoassays: androstenedione (DSL), free testosterone (Biosource, Nivelles, Belgium), and bALP (Tandem-R Ostase; Beckman Coulter, Inc, Fullerton, Calif); by chromatographic purification using high-performance liquid chromatography (HPLC): 24-hour urinary free cortisol (CLU) and porphyrins. The intra- and interassay coefficients of variation of chemiluminescent immunometric assays and radioimmunoassays were $\pm 6.0\%$, except for bALP ($\pm 10.0\%$).

Bone Densitometry—Spinal (L_2 – L_4) and femoral BMD at 4 femoral sites were measured by dual x-ray

absorptiometry densitometer (Prodigy Lunar, Madison, Wis): neck (FN), Ward's triangle (WT), great trochanter (TR), and total hip.

Genetic Testing

DNA Isolation and Amplification—Genomic DNA was prepared from blood, using the isolate 2 DNA extraction kit. DNA fragments were amplified by PCR, using the primers described (5'-ACATGGT-TAAGGCCTGTTGC-3' and 5'-GCCACATCTGGC-TTGAAATT-3' for the C187G mutation at codon 63; 5'-TGGCAAGGGTAAACAGATCC-3' and 5'-CTC-AGGCACTCCTCTCAACC-3' for the G845A mutation at codon 282 of the HFE gene) (Feder et al, 1996). A "hot start" PCR at 96°C was followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 56°C for 60 seconds, and extension at 72°C for 60 seconds. PCR products were desalted using Ultrafree R-MC (30 000 NMWL) filter units (Millipore Corp, Billerica, Mass).

Restriction Fragment Length Analysis—Mutations were detected by restriction fragment length analysis. The C-to-G transversion at nucleotide 187 abolishes the DNA sequence recognition site for endonuclease Bcl (New England Biolabs, Ipswich, Mass). Restriction fragments were separated on 2% NuSieve GTG agarose and 1% agarose MP (Boehringer Mannheim, Basel, Switzerland) gels containing ethidium bromide and visualized by ultraviolet transillumination.

Discussion

Hereditary hemochromatosis is an iron overload disorder and the most common recessive disease in Caucasians. About 80% of hemochromatosis patients are homozygous for the C282Y mutation in the HFE gene (Njajou et al, 2004), but in Italy, typical HFE mutations account for only 64% of cases of overt HH (Carella et al, 1997), and the estimated prevalence of C282Y homozygosity in the general Italian population is 1 in 3900 (Cassanelli et al, 2001). Moreover, screening data from both northern (Barosi et al, 2002) and southern Italy (De Marco et al, 2004) revealed a high prevalence of HFE-unrelated iron overload.

In HH, transferrin saturation values are of great importance because iron accumulates first in the transferrin pool, resulting in an increase of serum transferrin saturation, and subsequently in tissue stores, especially the hepatic parenchyma, with a progressive increase in concentrations of serum ferritin. Therefore, morning fasting transferrin saturation is generally regarded as the best single screening test for HH, even if there is no agreed-on cutoff for the optimal detection of disease (EASL, 2000; Pietrangelo, 2004; Yen et al, 2006). A

threshold of 45% or more identifies individuals with potential iron overload and is an indication for genetic testing (Yen et al, 2006).

Though this proband did not show the accepted biochemical criterion, a mild form of HH could not be ruled out due to the marked increase of ferritin levels, the clinical and laboratory signs, the MRI scan, suggestive of iron deposition on liver and spleen, and the reduced BMD. In confirmation of this, genetic testing described a heterozygosity for the H63D mutation that usually has low penetration and, consequently, a minor phenotypic expression.

Differently from C282Y homozygosity, clinical effects of the H63D mutation and its role in the pathogenesis of the disease are still unclear and appear to be limited (Carella et al, 1997), because the expression of a given mutation can be influenced by combined heterozygous or homozygous mutations of multiple hemochromatosis genes. Unfortunately, we do not have the possibility of performing genetic testing to evaluate the presence of mutations in ferroportin, TFR2, HAMP, and HJV, which could explain the gradual and highly variable progression of the disease.

In relation to our data, the history of our proband is not in agreement with the conclusions of several reports that had highlighted the lack of clinical manifestations of iron overload both in homo- (Gochee et al, 2002) and heterozygous (Barosi et al, 2002; Gochee et al, 2002) H63D mutations.

The presence in our proband of normal transferrin-saturation values, and persistently elevated serum ferritin could be explained by ferroportin-associated iron overload, currently classified as HH type 4 (Pietrangelo et al, 1999; Montosi et al, 2001), which results in a genetic disorder with 2 different clinical manifestations. In fact, the subjects with mutant ferroportin proteins with intracellular localization show typical ferroportin disease with low to normal transferrin-saturation and early Kupffer cell iron loading, while the patients with mutant proteins with plasma membrane localization exhibit high transferrin saturation and early hepatocyte iron loading, similar to classic HH (De Domenico et al, 2006). These 2 classes, after phlebotomy, respond differently because patients with mutations that lead to Kupffer cell iron loading do not gain from treatment (Pietrangelo, 2004; De Domenico et al, 2006), showing a rapid decrease of transferrin saturation with persistently high serum ferritin (Pietrangelo, 2004). Therefore, in this case a ferroportin disease can be excluded because our proband, before therapy, had normal transferrin saturation, and in response to phlebotomy exhibited, in absence of anemia, a decrease of serum ferritin in parallel with transferrin saturation. A likely dysmetabolic hepatosiderosis has

been excluded because this new condition is characterized by a mild to moderate hepatic iron overload associated with a concomitant metabolic disorder and insulin resistance, in absence of other causes of iron overload, particularly genetic hemochromatosis and chronic alcoholism in almost all patients (Moirand et al, 1997). High-ferritin diabetes can be excluded by the history of our proband, who shows a mild elevation of fasting glycemia, a normal value of HbA_{1c}, and impaired glucose tolerance at 2 hours following oral glucose-tolerance test. Moreover, the normal value (<45%) of serum transferrin saturation, and/or the milder symptomatic organ involvement, can almost certainly exclude the juvenile-onset phenotype (HAMP and HJV mutations) as well as TFR2 and Cybrd1 mutations. The absence of anemia can exclude, with a good degree of certainty, the rare DMT1 mutation.

Differently from the classic description of HH, which, in symptomatic patients, generally presents generalized cutaneous hyperpigmentation (69%–75%) associated, in its most extreme form, with hepatomegaly (70%–95%), osteoarticular polyarthropathy (20%–70%), diabetes mellitus (10%–60%), ECG abnormalities (up to 35%), and hypogonadism (up to 35% of males) (Chalès and Guggenbuhl, 2003; Limdi and Crampton, 2004), these findings are only partly present in our proband. In fact, he exhibited an unusual presentation of the disease, with partial hypopituitarism associated with reduced BMD.

To confirm the unusual presentation of this case, the largest detailed study of hypogonadism reported in HH highlighted a low prevalence of male hypogonadotropic hypogonadism (6.4%) that was related to the severity of iron overload (McDermott and Walsh, 2005). In fact, in this paper, patients with a lower degree of hepatic siderosis on liver biopsy and without hepatic cirrhosis, diabetes mellitus, or markedly elevated serum ferritin levels rarely manifested hypogonadism, which was associated with hepatic cirrhosis, diabetes mellitus, and ferritin levels greater than 1500 ng/mL, at diagnosis, in 89%, 33%, and 77% of the subjects, respectively (McDermott and Walsh, 2005).

The association of hemochromatosis and osteoporosis is well established, but it is unclear whether this is due to iron overload, hypogonadism, hypoparathyroidism, liver disease, or diabetes mellitus. In HH the prevalence of osteoporosis, as defined by bone density measurements, is 25%–33%, and the prevalence of spinal fractures ranges from 1.8% to 18% (Chalès and Guggenbuhl, 2001).

Considering that the influence of bone iron deposition in the mineralization process remains an issue of debate, and in light of the history of our proband, the presence of reduced BMD could be predominantly due to hypogonadism, which can lead to increased rates of

bone resorption, and bone loss, as well as observed in the first years after menopause. In fact, our patient referred to a recent clinical deterioration of erectile dysfunction that could be related to a parallel rapid decline in androgen production, even if it is not clear whether the bone loss seen immediately after testosterone withdrawal mimics what is seen with estrogen deficiency. In this respect several authors (Stepan et al, 1989) observed in young men who had undergone bilateral orchiectomy because of sexual delinquency a bone loss of 7% per year in the first 2 years of lumbar bone density, evaluated by dual photon absorptiometry, as a function of time after orchiectomy.

Differently from the increase observed immediately after estrogen deficiency of both markers of bone formation and resorption, our proband highlighted a slight disassociation in the levels of the markers of bone turnover. The reason for this is unclear and could be explained by a subtle longstanding hypogonadism.

This deleterious effect on bone is a further confirmation of the atypical presentation of this case that was not associated with a severe form of HH.

The reduction of androgenic requirements, in response to therapeutic phlebotomy, is probably due to a partial normalization of the hypothalamic-pituitary-gonadal function. This result, if confirmed in following evaluations, is important because there is evidence that arthropathy, cirrhosis, and hypogonadism would not seem to be affected by treatment, unlike malaise, fatigue, skin pigmentation, abdominal pain, and insulin requirements in diabetes (Tavill, 2001).

In patients with suspected HH (transferrin saturation values greater than 45%), a genetic screening is generally recommended (EASL, 2000). Nonetheless, in view of the low frequency of compound heterozygotes in patients with HH (Bomform, 2002) and the minor phenotypic expression in heterozygosity for the H63D mutation (Carella et al, 1997), it is conceivable to propose a genetic testing in symptomatic men with high values of ferritin (≥ 300 $\mu\text{g/L}$) not due to secondary causes of hyperferritinemia, even if normal values of transferrin saturation are present. This behavior is suggested in order to identify, in time, individuals with HH to prevent or minimize iron-related organ injury (EASL, 2000; Chalès and Guggenbuhl, 2001), and because a timely treatment with phlebotomy, before onset of cirrhosis or insulin-requiring diabetes, is associated with a normal life expectancy (Wojcik JP et al, 2002).

Moreover, since there is the risk that overreliance on HFE mutations for a diagnosis of hemochromatosis can lead to an incorrect assessment of iron stores, a careful assessment of cofactors for liver damage in hemochromatosis has to be performed, because a proportion of patients could develop additional disease processes.

The history of this proband represents an atypical case of HH (heterozygosity for the H63D mutation of HFE) in which the best single screening test failed to identify the correct diagnosis.

To conclude, if clinical and/or laboratory test abnormalities suggestive for HH are found, a genetic screening should be considered in male adults of all age groups, above all to exclude heterozygous H63D mutation and to prevent or reduce iron-related organ injury.

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