Common Variants in the *BMP2, BMP4,* and *HJV* Genes of the Hepcidin Regulation Pathway Modulate HFE Hemochromatosis Penetrance

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Most cases of genetic hemochromatosis (GH) are associated with the *HFE* C282Y/C282Y (p.Cys282Tyr/p.Cys282Tyr) genotype in white populations. The symptoms expressed by C282Y homozygotes are extremely variable. Only a few suffer from an overt disease. Several studies have suggested that, in addition to environmental factors, a genetic component could explain a substantial part of this phenotypic variation, although very few genetic factors have been identified so far. In the present study, we tested the association between common variants in candidate genes and hemochromatosis penetrance, in a large sample of C282Y homozygotes, using pretherapeutic serum ferritin level as marker of hemochromatosis penetrance. We focused on two biologically relevant gene categories: genes involved in non-*HFE* GH (*TFR2, HAMP,* and *SLC40A1*) and genes involved in the regulation of hepcidin expression, including genes from the bone morphogenetic protein (BMP) regulatory pathway (*BMP2, BMP4, HJV, SMAD1, SMAD4,* and *SMAD5*) and the *IL6* gene from the inflammation-mediated regulation pathway. A significant association was detected between serum ferritin level and *rs235756,* a common single-nucleotide polymorphism (SNP) in the *BMP2* genic region ($P = 4.42 \times 10^{-5}$). Mean ferritin level, adjusted for age and sex, is 655 ng/ml among TT genotypes, 516 ng/ml in TC genotypes, and 349 ng/ml in CC genotypes. Our results further suggest an interactive effect on serum ferritin level of *rs235756* in *BMP2* and a SNP in *HJV,* with a small additive effect of a SNP in *BMP4*. This first reported association between common variants in the BMP pathway and iron burden suggests that full expression of *HFE* hemochromatosis is linked to abnormal liver expression of hepcidin, not only through impairment in the HFE function but also through functional modulation in the BMP pathway. Our results also highlight the BMP regulation pathway as a good candidate for identification of new modifier genes.

Genetic hemochromatosis (GH) is a group of hereditary disorders proceeding from an impairment in the production of the key regulator of plasma iron, hepcidin,[1] and resulting in progressive iron loading of parenchymas. Four recessive forms of GH are currently described.[2] Two late-onset, adult forms (HFE [MIM 235200] and HFE3 [MIM 604250]) are related to mutations in *HFE* (MIM 235200) or *TFR2* (MIM 604720), the gene encoding the receptor transferring 2 protein. Two early-onset, juvenile forms (JH [MIM 602390]) are secondary to mutations in either the gene encoding hemojuvelin (*HJV* [MIM 608374]) or the gene encoding hepcidin (*HAMP* [MIM 606464]). The "ferroportin disease" (HFE4 [MIM 606069]), related to mutation in the *SLC40A1* gene (*SLC40A1* [MIM 604653]) coding for the metal-transporter ferroportin regulated by hepcidin, is a closely related but not classic form of GH, because of a dominant transmission usually with predominant mesenchymal iron deposition.[3]

HFE hemochromatosis accounts for >95% of GH cases in white populations.[4] It is related to one major mutation, C282Y, with a reported frequency of 5%–15%.[5–7] The biochemical or clinical symptoms expressed by C282Y homozygotes are extremely variable.[8] Only a few suffer from an overt disease consisting of various associated symptoms, including osteoarticular damage, cirrhosis, diabetes, hypogonadism, arrhythmia, and heart failure. Most C282Y homozygotes display a mild disease limited to biochemical abnormalities (increased transferrin saturation, with or without elevated serum ferritin), with either absent or mild clinical symptoms. Moreover, some homozygotes may show no expression throughout their lives.[9] As a whole, estimates of the penetrance of C282Y homozygosity by studies have ranged from 1% to 90%.[5,10–13] This wide range reflects the variability in the definition of disease penetrance, with some studies referring to the level of iron burden (serum ferritin, liver iron concentration…) using disparate thresholds, others to organ damage, and most to mixed criteria. These results are complicated by the absence of a strict correlation between the level of iron burden and organ-damage expression. How-

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Table 1. Single SNP Genotypic and Allelic Association with Ferritin Level for All 75 SNPs

Gene (Chromosome) and SNP	Location (bp)	MAF	P^a for Association Test		Allele (Frequency) Associated with Higher Ferritine Level
			Genotypic	Allelic	
HAMP (19):					
rs916145	40,459,724	.113	.7637	.5016	
rs10405246	40,460,789	.221	.1045	.0423	A (.779)
rs1882694	40,463,222	.376	.3376	.1576	
rs7251432	40,467,281	.491	.8527	.5838	
rs12971321	40,471,262	.376	.8367	.9815	
rs10402233	40,472,691	.347	.2110	.1180	
rs17705188	40,473,156	.084	.9412	.8520	
BMP2 (20):					
rs2206917	6,683,511	.434	.9867	.9516	
rs235730	6,684,189	.418	.9146	.9172	
rs6077060	6,688,317	.099	.1262	.0498	T (.099)
rs235710	6,688,366	.439	.5832	.3072	
rs1980499	6,694,498	.495	.5092	.3742	
rs3178250	6,708,201	.173	.4460	.2532	
rs235772	6,710,719	.409	.2757	.1124	
rs6117432	6,712,536	.233	.9039	.6946	
rs173107	6,713,841	.380	.0026	.0052	T (.380)
rs235757	6,714,019	.374	.1994	.0933	
rs235756	6,715,111	.364	1.80×10^{-4}	4.42×10^{-5}	T (.636)
rs910141	6,715,642	.255	.0038	.0010	G (.745)
rs235753	6,717,533	.348	.2473	.0950	
rs6054514	6,719,370	.052	.4355	.4865	
rs235704	6,720,263	.120	.9306	.7748	
rs17804639	6,721,316	.092	.5071	.3447	
BMP4 (14):					
rs10498464	53,441,332	.193	.3857	.2666	
rs1951865	53,442,591	.372	.5052	.5954	
rs11157990	53,453,695	.303	.3464	.1881	
rs3742555	53,455,219	.058	.1838	.0760	
rs2147105	53,475,815	.448	.7883	.5060	
rs4444235	53,480,669	.492	.1852	.6077	
rs762642	53,492,803	.409	.7235	.5443	
rs1957860	53,499,105	.467	.8738	.8229	
rs6572927	53,503,140	.081	.2345	.1097	
rs11157994	53,521,052	.076	.0766	.1883	
rs1957844	53,527,828	.230	.1068	.5648	
rs4901474	53,539,487	.429	.0050	.0054	C (.429)
HJV (1):					
rs16827043	144,106,797	.102	.8254	.9153	
rs7536827	144,109,299	.452	.9444	.8876	
SMAD1 (4):					
rs6537355	146,622,042	.149	.2329	.7810	
rs2118438	146,647,834	.187	.1893	.0839	
rs714195	146,665,130	.408	.9119	.7041	
rs1016792	146,698,229	.200	.3142	.1397	
rs2036138	146,704,312	.467	.7503	.8810	
rs11939979	146,707,777	.469	.6741	.7925	
SMAD4 (18):					
rs606073	46,749,479	.440	.3876	.7981	
rs10163789	46,749,360	.070	.7042	.7042	
rs10502913	46,822,269	.269	.9241	.9260	
rs17663887	46,843,716	.098	.5783	.3150	
rs17663994	46,920,796	.329	.3553	.8460	
rs9304408	46,928,354	.362	.0510	.3874	
rs9963878	46,933,520	.089	.1652	.0655	
rs7242459	46,935,348	.396	.6693	.3740	
SMAD5 (5):					
rs2346361	135,476,404	.459	.2769	.2209	
rs9327744	135,501,661	.236	.4137	.2589	
TFR2 (7):					
rs7812235	100,049,422	.195	.5966	.3716	
rs10247962	100,057,865	.155	.2861	.7993	
rs4434553	100,078,127	.492	.0920	.0315	G (.492)

(*continued*...)

Table 1. (continued)

Gene (Chromosome) and SNP	Location (bp)	MAF	P^a for Association Test		Allele (Frequency) Associated with Higher Ferritine Level
			Genotypic	Allelic	
SLC40A1 (2):					
rs12693541	190,126,935	.134	.1990	.0757	
rs11884632	190,133,087	.244	.6722	.4917	
rs2304704	190,138,422	.354	.1747	.1206	
rs10188230	190,140,858	.020	.3613	.3698	
rs16831659	190,141,534	.103	.2471	.1823	
rs10202029	190,154,529	.025	.0677	.0677	
rs2352267	190,157,210	.395	.7741	.4807	
rs1123109	190,162,978	.208	.8950	.6928	
IL6 (7):					
rs1880242	22,726,132	.494	.4962	.8671	
rs10499563	22,727,013	.238	.2078	.0782	
rs2056576	22,727,727	.307	.2417	.0924	
rs12700386	22,729,534	.177	.4979	.5244	
rs2069827	22,731,981	.106	.8888	.6857	
rs1800795	22,733,170	.441	.9075	.7436	
rs2069837	22,734,552	.068	.2151	.0796	
rs2069840	22,735,097	.344	.8775	.6220	
rs2069860	22,737,563	.088	.2467	.1021	
rs2069849	22,737,681	.007	.0051	.0051	T (.007)
rs2069861	22,738,179	.018	.3537	.3537	

[a] *P* value uncorrected for multiple testing.

ever, they strongly support the involvement of factors modulating disease expressivity. With respect to iron loading, environmental factors have been poorly investigated in humans, although it is likely that some may be relevant, such as alimentary regimen and blood donation.[11] Several results also suggest the role of additional genetic factors. The incidence of GH-related conditions is higher in relatives of clinically affected probands than in relatives of probands with only elevated transferrin saturation,[14] and concordance of iron store indices in GH-affected families between same-sex siblings homozygous C282Y is high.[15] On the basis of a twin study, Whitfield et al.[16] estimated that *HFE* explains only part of the genetic component of iron-store variation. In mice, differences in hepatic iron loading in two *Hfe*-deficient mice strains have been associated with a polygenic pattern of inheritance.[17]

Apart from sex, only a few genetic factors have been identified as putatively modifying disease expression. A mitochondrial polymorphism was reported as more frequent in C282Y homozygotes with hemochromatosis than in nonexpressing C282Y homozygotes.[18] However, in another study, no association has been observed between the ferritin level of C282Y homozygotes and the same polymorphism.[19] Mutations in two other genes involved in iron metabolism, *HJV* and *HAMP,* have been clearly associated with higher iron indices in a French cohort of C282Y homozygotes,[20–22] but the mutations identified are rare, with a frequency of heterozygous carriers <2% among C282Y homozygotes.

Hepcidin is a peptide hormone produced by the liver that controls plasma iron concentration and iron-tissue distribution by inhibiting intestinal iron absorption, iron recycling by macrophages, and iron mobilization from the liver. Interestingly, hepcidin levels are abnormally low in patients with adult or juvenile forms of GH. Babitt et al.[23] recently demonstrated that hepcidin expression is induced by the bone morphogenetic protein (BMP)–signaling pathway. This pathway involves BMP2 and BMP4 proteins and the BMP coreceptor HJV, which phosphorylates receptor-activated Smad proteins (R-Smads) 1 and 5. Phosphorylated R-Smads form a complex with the common mediator Smad4 (Co-Smad), which allows signal transduction into the nucleus for hepcidin gene induction.

The aim of the present study was to search for relatively frequent variants (as opposed to the rare mutations already known) in genes that modify the serum ferritin levels in C282Y homozygotes. Given these recent molecular data, we focus on variants in two pathophysiologically relevant gene categories: genes involved in non–*HFE* hemochromatosis and genes involved in the regulation of hepcidin expression. In a large sample of C282Y homozygotes, we tested for association between initial serum ferritin levels and SNPs located within or near 10 genes: 3 non–*HFE*-GH genes (*TFR2, HAMP,* and *SLC40A1*), 6 BMP signaling pathway genes (*BMP2* [MIM 112261], *BMP4* [MIM 112262], *HJV, SMAD1* [MIM 601595], *SMAD4* [MIM 600993], and *SMAD5* [MIM 603110]), and the *IL6* gene (*IL6* [MIM 147620]) coding for an inflammatory cytokine known to increase inflammation-mediated hepcidin expression. We detected a significant association between a SNP in the *BMP2* genic region and serum ferritin level, adjusted for age and sex. Given the biologically relevant gene interactions along the BMP regulatory pathway, our data suggest an additive effect on serum ferritin level of the *BMP2* SNP and of a SNP in *BMP4,* with an interaction effect between the *BMP2* SNP and a SNP in *HJV.*

The database of the Family Screening Centre for Hemochromatosis[8] comprises all C282Y homozygous probands referred to the Liver Unit in Rennes, France, since 1990 and their relatives who received diagnoses through a systematic family-screening policy. At the time of the study, 1,319 C282Y homozygotes were recorded, among whom 729 unrelated probands fulfilled inclusion criteria: availability of (i) sex, (ii) age at diagnosis, (iii) serum ferritin level at diagnosis before any venesection therapy, and (iv) DNA sample stored at −20°C. Of these 729 subjects, 592 gave their written informed consent to participate in the study, in accordance with the protocol validated by the committee of ethics of Rennes on November 10, 2004.

Of these 592 subjects, 262 were women and 330 were men. Mean (SD) age was 46 (14) years for women and 44 (13) years for men. Median value (25th–75th percentile range) for ferritin levels was 1,040.5 ng/ml (582–2,356) in men and 400.5 ng/ml (186–699) in women. Ferritin data were normalized using a $\log_e$ transformation. Log-transformed ferritin levels were adjusted for age, with consideration of age groups of 10 years, and sex. The final multiple-regression model includes a parameter for each of the seven independent age groups, a parameter for sex, and an interaction parameter, Age × Sex, for each age group.

Genomic DNA was extracted from peripheral blood cells by the phenol-chloroform method or by use of the Flexigen DNA kit (Qiagen). SNPs in the 10 candidate genes were selected from the CEU HapMap database. For each gene, a region including the complete genic sequence and the upstream and downstream intergenic sequences was delimited. The set of tag SNPs was identified for each region, so that all the SNPs with a minor-allele frequency (MAF) ⩾5% in the database have a pairwise $r^2 \geqslant 0.8$ with at least one tag SNP. Tagging was performed using the algorithm implemented in Tagger.[24] Two coding SNPs located within the *IL6* gene were added to the list. A total of 81 SNPs were included in the study.

SNP genotyping followed Custom SNP Genotyping Assays consisting of a mix of unlabeled PCR primers and TaqMan minor groove binder (MGB) SNP-allele-specific probe. PCR primers and probes used for allelic discrimination were designed and purchased from the Applied Biosystems "assay on demand" or "assay by design." Ge-

Table 2. Haplotype Association with Ferritin Level

Gene	No. of SNPs in the Gene	Global *P*[a]
Genes with <10 SNPs:		
HJV	2	.951
SMAD1	6	.571
SMAD4	8	.792
SMAD5	2	.516
HAMP	7	.106
SLC40A1	8	.290
TFR2	3	.128
Genes with >10 SNPs[b]:		
BMP2	5	.0098
BMP4	5	.052
IL6	5	.013

[a] Global *P* values obtained by permutation are not corrected for multiple testing across genes but are corrected for the number of SNPs included in each haplotype.

[b] Global *P* value for the best five-SNP combination (*BMP2, rs235756* to *rs235704*; *BMP4, rs762642* to *rs1957844*; *IL6, rs12700386* to *rs2069840*).

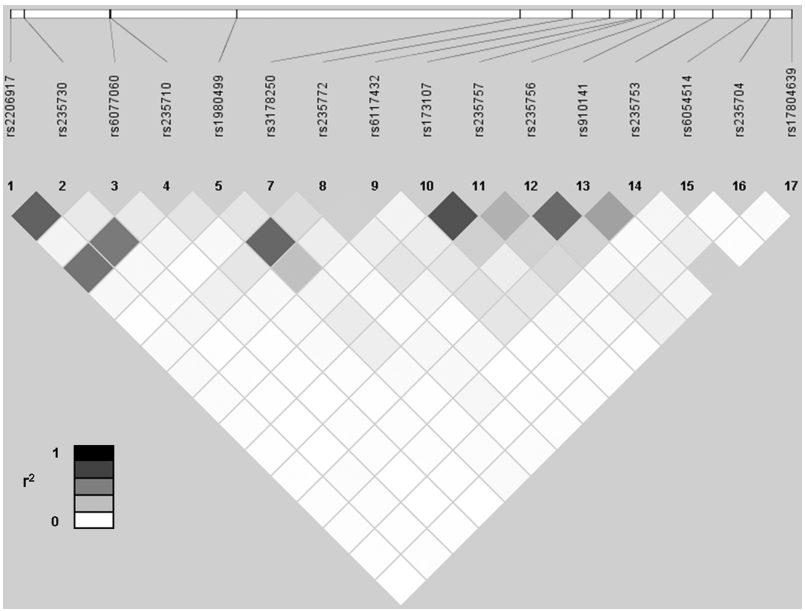

Figure 1. LD (r^2) between SNPs of the *BMP2* gene

Table 3. Comparison between Models of Gene Combinations Implicated in the Regulation of Hepcidin Expression

Model and Gene Combination	*P* Value of Model versus					
	Null Model	No Interaction	M1[a]	M2[a]	M3[b]	M4
M1:						
BMP2	4.42×10^{-5}	...	...	...	...	...
M2:						
BMP2+*BMP4*	1.68×10^{-6}	...	.0031	...	...	...
BMP2+*BMP4*+*BMP2* × *BMP4*[c]	6.10×10^{-6}	.550	...	...	...	...
M3:						
BMP2+*HJV*	2.19×10^{-4}	...	.923	...	...	...
BMP2+*HJV*+*BMP2* × *HJV*	1.64×10^{-5}	.0046	.0179	...	...	...
M4:						
BMP2+*BMP4*+*HJV*+*BMP2* × *HJV*	8.41×10^{-7}	...	.0013	.0153	.0046	...
M5:						
BMP2+*BMP4*+*HJV*+*BMP2* × *HJV*+*SMAD*	4.46×10^{-7}	...	.0021	.0135	.0070	.0708
BMP2+*BMP4*+*HJV*+*BMP2* × *HJV*+*SMAD*+ *BMP2* × *SMAD*[c]	7.93×10^{-7}	.281	...	...	...	...

[a] Models without interaction.
[b] Model including interaction term.
[c] Interaction not significant. Model not contrasted with simpler models.

notyping followed the Applied Biosystems protocol. Briefly, PCR was performed in a final volume of 5 μl containing 10 ng of sample DNA, 0.625 μl of custom SNP-specific Assay Mix, and 2.5 μl of Universal Master Mix no AmpErase UNG. Amplification was allowed to proceed for 40 cycles of 15 s at 95°C and 60 s at 60°C. Automatic genotype call was performed <24 h after PCR, by scanning microtitration plates on the 7900HT Fast Real-Time PCR, which provides the SDS2.3 software (Applied Biosystems).

Assays for *rs1880241* (in *IL6*) and *rs6596286* (in *SMAD5*) were unsuccessful. Of the 79 SNPs genotyped, 4 SNPs (*rs1005464* in *BMP2, rs10498466* and *rs4901473* in *BMP4,* and *rs3764942* in *SMAD5*) were not in Hardy-Weinberg equilibrium and therefore were excluded from the analyses. Mean genotyping success rate for the 75 remaining SNPs was 99.1%. Correlation between allele frequencies of the 75 SNPs in our sample and allele frequencies in the CEU HapMap data was very high (regression $r^2 = 0.94$).

The linkage disequilibrium (LD) structure among SNPs was examined with Haploview.[25] The mean r^2 between our markers, computed on the whole sample, was 0.10. All subsequent statistical analyses were performed using R.[26] A linear regression was used to test for association between each individual SNP and ferritin. Both allelic and genotypic associations were considered. The sensitivity of association results to the inclusion of phenotypic outliers was evaluated. Because the results were fully concordant, we present and discuss only results based on the whole sample. To correct for multiple testing, the effective number of independent tests was assessed using the method of Li and Ji[27] as implemented in the SNPSpd software.[28] Following this procedure, our set of 75 SNPs is equivalent to 62 independent tests. When a Bonferroni correction is applied, an individual significance threshold of 8×10^{-4} should be used to control a global 5% type I error.

Association between haplotypes and ferritin was tested using the efficient score statistic proposed by Schaid et al.[29] and implemented in haplo.stats R-Package v1.2.2 (Schaid Lab Web site) with a permutation-based assessment of the *P* values. We favored this relatively simple regression-based approach over more sophisticated ones based on population history modeling,[30] because our sample is a subsample selected from the general population with an unknown selection scheme. In the absence of a clear evaluation of the consequences of such a selection scheme on sophisticated haplotype tests, we favored a robust approach.[31] Haplotypes comprising all SNPs were considered for genes with <10 SNPs. Otherwise, haplotypes comprising five SNPs were considered, with a sliding window of one SNP to browse the gene. Sensitivity to window size was evaluated. Because results were fully concordant, we present and discuss only results based on five-SNP windows. Association of SNP combinations within genes was further analyzed using unphased, multimarker data. A stepwise linear regression starting from the model including only the SNP with the lowest individual *P* value was performed to determine the subset of SNPs with the strongest association.

In a final, exploratory stage, we evaluated the association of several biologically relevant gene combinations with ferritin level. To limit the number of combinations tested, we considered only those for which a molecular interaction between gene products has been described and which include one gene showing significant association at the single-gene level. Again, we used multiple-regression models. To evaluate whether more-complex models were significantly better predictors of phenotype than simpler ones, we compared nested models (simpler models are particular cases of the more complex models) using F statistics. These results are exploratory, in the sense that we drop the requirement for multiple-comparison adjustment while assessing significance. For each gene combi-

nation, a "best model" was estimated with a backward regression, starting from a full model that included the three SNPs displaying the strongest individual association for each gene and two SNP-interaction terms.

SNP *rs235756,* located in the 3′ region of *BMP2,* is significantly associated with ferritin level in our C282Y homozygote sample (table 1), after correction for multiple testing (uncorrected $P = 4.4 \times 10^{-5}$; corrected $P = .002$). Note that the association would still have been significant if a more conservative Bonferroni correction for 75 tests had been applied (corrected *P* value would then be .003). The T allele associated with higher ferritin level has a frequency of 0.64 (HapMap CEU frequency is 0.58). Mean ferritin level, adjusted for age and sex, is 654.66 ng/ml among TT genotypes, 516.39 ng/ml among TC genotypes, and 349.11 ng/ml among CC genotypes. A neighboring SNP in the gene (*rs910141*) also displays a suggestive association (corrected $P = .062$), but *rs235756* and *rs910141* are in LD ($r^2 = 0.528$), as can be seen from figure 1. Interestingly, SNP *rs235756* has no impact on the age at diagnosis (mean age at diagnosis, adjusted for sex, is 44.7 years among TT genotypes, 45 years among TC genotypes, and 45.8 years among CC genotypes; *P* value of the analysis of variance [ANOVA] is .82). Suggestive but nonsignificant associations are also observed for one SNP in *BMP4* (*rs4901474,* uncorrected $P = .0054$, corrected $P = .335$) and one synonymous SNP in *IL6* (*rs2069849*; uncorrected $P = .0051$, corrected $P = .316$). Because the results for the allelic and the genotypic tests were very similar, the following analyses were performed considering the allelic model only.

Results for haplotype association are presented in table 2. In *BMP2,* the best haplotype combination provided a *P* value of only .0098, which did not improve the single-SNP association. Similar results were observed for *BMP4* and *IL6.* Tests based on unphased genotype data did not detect any additional interesting SNP combination (results not shown). However, the tagging strategy chosen leads to a low LD among SNPs in each gene (mean $r^2 = 0.1$), a situation in which multimarker approaches (either phased or unphased) are expected to be less powerful.[32]

As *BMP2* was the only gene found significantly associated with ferritin in the previous analyses, we focused on the hepcidin expression-regulation pathway to explore possible effects of gene combinations. Analyses of gene

Table 4. Genotype Frequencies and Counts for the Three SNPs Best Explaining the Ferritin Level (*rs235756* in *BMP2, rs4901474* in *BMP4,* and *rs16827043* in *HJV*)

BMP2 Genotype (Frequency[a]) and *HJV* Genotype	Frequency[a] (*n*)			
		BMP4 × *BMP2* × *HJV* for *BMP4* Genotype		
	BMP2 × *HJV*	C/C	T/C	T/T
T/T (40.4, *n* = 237):				
A/A	33.7 (198)	6.0 (35)	16.2 (95)	11.6 (68)
A/G + G/G	6.6 (39)	1.2 (7)	3.6 (21)	1.9 (11)
T/C (46.5, *n* = 273):				
A/A	37.0 (217)	8.0 (47)	16.4 (96)	12.6 (74)
A/G + G/G	9.5 (56)	1.4 (8)	4.3 (25)	3.9 (23)
C/C (13.1, *n* = 77):				
A/A	10.2 (60)	1.2 (7)	6.3 (37)	2.7 (16)
A/G + G/G	2.9 (17)	1.2 (7)	1.2 (7)	.5 (3)

[a] Genotype frequencies calculated for the 587 individuals fully genotyped for the three polymorphisms.

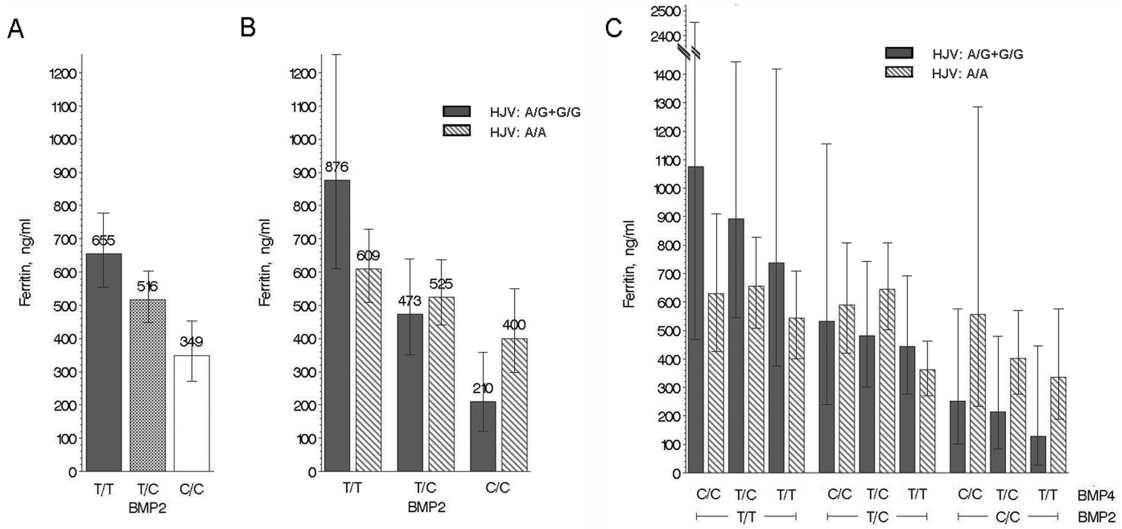

Figure 2. Mean ferritin levels at diagnosis, adjusted for age and sex, in the different *BMP2, HJV,* and *BMP4* genotype categories. *A,* Means classified by *rs235756* genotype (*BMP2*). *B,* Means classified by both *rs235756* (*BMP2*) and *rs16827043* (*HJV*) genotypes with pooling of the A/G and G/G genotypic categories for the latter. *C,* Means classified by *rs235756* (*BMP2*), *rs16827043* (*HJV*) and *rs4901474* (*BMP4*) genotypes. Mean values are reported in panels A and B but have been omitted for the sake of clarity in panel C. 95% variation intervals are reported as vertical bars.

combinations were restricted to biologically relevant combinations, including *BMP2*. Four combinations were analyzed: *BMP2* and *BMP4* (molecular interaction of the two BMP proteins); *BMP2* and *HJV* (molecular interaction of HJV and BMP2); *BMP2, BMP4,* and *HJV* (molecular interaction); and *BMP2, BMP4, HJV, SMAD1, SMAD4,* and *SMAD5* (genes implicated in the whole BMP-signaling pathway). The three most-associated SNPs (in the single-SNP analyses) for each gene were potentially included in the models, as well as interactions involving SNPs from *BMP2*. Starting from the simple M1 model, in which only *rs235756* is included, table 3 presents the comparison with more-complex models of gene combinations, expressed as *P* values of model comparison tests. Models M2, M3, M4, and M5 correspond to the "best models" of the gene combination considered. In our case, the best M2, M3, and M4 models included only one SNP per gene (M2, *rs235756* in *BMP2* and *rs4901474* in *BMP4*; M3, *rs235756* in *BMP2* and *rs16827043* in *HJV*; M4, *rs235756* in *BMP2, rs4901474* in *BMP4,* and *rs16827043* in *HJV*). In model M5, only one SNP in *SMAD4* remained (*rs235756* in *BMP2, rs4901474* in *BMP4, rs16827043* in *HJV,* and *rs9963878* in *SMAD4*).

Interestingly, an additive effect of *BMP4* and *BMP2* was detected (M1 vs. M2 comparison $P = .0031$), as well as an interaction between *BMP2* and *HJV* (M1 vs. M3 comparison $P = .0179$). These two effects added up, so that the model best explaining the ferritin level (model 4) included an interaction between *BMP2* (SNP *rs235756*) and *HJV* (SNP *rs16827043*) and an additive effect of *BMP4* (SNP *rs4901474*) (M2 vs. M4 comparison $P = .0153$). Including SNPs from the *SMAD* genes did not improve the association (M4 vs. M5 comparison $P = .071$). Figure 2 presents the ferritin levels, adjusted for age and sex, in the different *BMP2, HJV,* and *BMP4* genotype categories, and table 4 presents the frequencies of these three SNP genotype categories in our sample. The interaction effect of BMP2 and HJV was such that the two-locus genotype combination at greater risk (TT at *rs235756* and A/G or G/G at *rs162703*) involved the same HJV genotypes as the two-locus genotype combination at lower risk (AA at *rs235756* and A/G or G/G at *rs162703*). Again, no effect of this gene combination on age at diagnosis was detected.

In this study, we report the first association between relatively common variants in genes of the BMP pathway and iron burden, considering the pretherapeutic serum ferritin level as a marker of hemochromatosis penetrance. Although it may lead, in a given patient, to overestimate iron burden in case of associated excessive alcohol consumption, metabolic syndrome, or inflammation, it is a good marker of body iron stores in large populations.[33] Hepatic iron concentration, determined on liver biopsy, or the amount of iron removed to obtain low body iron stores would have been better markers, but such data are difficult to reliably obtain for all subjects from a large population.

Our sample of C282Y homozygotes from the Family Screening Centre is not strictly representative of the population of C282Y homozygotes. It is rich in individuals with serious symptoms. Conducting an association study on such a selected sample tends to lower the power to detect genetic factors modifying the effect of C282Y. However, the phenotypic variation in our sample seems to be large enough to detect the effect of genetic variants. Note that the associated allele of SNP *rs235756* in *BMP2* has a frequency slightly higher in our sample (0.64) than in the HapMap CEU sample (0.58), as would be expected for an associated allele in a sample rich in extreme phenotypes. Note also that the mean ferritin levels estimated for the different genotypic categories are specific to our sample and would only be biased estimates of the levels in the whole C282Y homozygous population. The initial targeting of the biologically relevant BMP signaling pathway is definitively key to our results. By narrowing the sets of gene tested, we increased our power to detect the association, counterbalancing the inevitable limits of a selected database with an indirect marker of penetrance.

In this association study, we used a tag SNP strategy in which only a subset of SNPs, representative of the common polymorphism variability, were tested. This strategy enabled us to fully cover the genes selected and the genic regions upstream and downstream of the genes with respect to SNP information available in the HapMap database. However, association results should be interpreted cautiously. A direct effect of SNP *rs235756* on ferritin level cannot be ruled out. But this polymorphism with no evident biological role on ferritin level is more likely a proxy for one or several functional polymorphisms in the region, yet to be identified.

Our results further suggest a possible additive effect between *rs235756* in *BMP2* and *rs4901474* in *BMP4*. This effect is particularly interesting, because both proteins are able to activate the regulatory pathway and subsequent hepcidin expression. Further, the interaction effect between *rs16827043* in *HJV* and *rs235756* in *BMP2* is in line with the biological demonstration that BMP2 exhibits more affinity for HJV than BMP4 does.[23]

Further studies will also be necessary to evaluate whether SNP *rs235756* (or the functional polymorphism for which *rs235756* is a proxy) has an additive effect on the C282Y homozygote genotype or a specific modifying effect. Testing the effect of SNP *rs235756* on serum ferritin level in the general population should help with deciphering the model. An additive effect of *rs235756* should also be detected in the general population, whereas a specific modifying effect should not (the effect of *rs235756* is restricted to C282Y homozygotes).

This first association between relatively common variants in genes of the BMP pathway and iron burden suggests that full expression of *HFE* hemochromatosis is linked to abnormal liver expression of hepcidin, not only through impairment in the HFE function but also through functional modulation in the BMP pathway. These results support the idea that all the genes currently described in the BMP signaling pathway are good candidates as mod-

ifier genes in C282Y homozygotes. This includes *BMP9* (*GDF2* [MIM 605120]), because Truska et al.[34] recently showed that the liver-specific BMP9 is the most potent inducer of hepcidin expression through the BMP pathway in mouse, but also includes *SMAD8* and *BMP* type I and type II (*BMPR1A* [MIM 601299], *BMPR1B* [MIM 603248], and *BMPR2* [MIM 600799]) receptors.[23] With respect to other, non-*HFE* hemochromatosis genes and the *IL6* gene, no strong SNP association was observed in our patients, which is in accordance with the findings of Truska et al.[34] that *TFR2* and *IL6* act independently of the BMP pathway to regulate hepcidin expression. However, the lack of association with *IL6* alone does not exclude the involvement of other genes from the inflammation-mediated hepcidin regulation pathway, including STAT3[35,36] (MIM 102582).

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for HFE, HFE3, *HFE, TFR2, JH, HJV, HAMP,* HFE4, *SLC40A1, BMP2, BMP4, SMAD1, SMAD4, SMAD5, IL6, GDF2, BMPR1A, BMPR1B, BMPR2,* and STAT3)

Schaid Lab Web site, http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm?CFID=7459420&CFTOKEN=67925359 (for haplo.stats software)

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