The -582A>G variant of the *HAMP* promoter is not associated with high serum ferritin levels in normal subjects

In a recent issue of this Journal, Andreani *et al.*[1] reported a potential role of iron loading modifier in poly-transfused beta-thalassemic patients for the -582 A>G polymorphic change (rs10421768)[2] in the 5' flanking region of the *HAMP* (Hepcidin AntiMicrobial Peptide) gene encoding hepcidin. According to these Authors, the presence of the less common "G" at -582 was associated with higher liver iron concentrations (LIC), as determined at liver biopsy, and with higher serum ferritin in patients as compared with the presence of "A" at the same position. This difference was observed only in patients (52 males and 45 females) irregularly treated by iron chelators, since regular iron chelation treatment is likely able to override possible differences. Obviously several determinants may contribute to the clinical condition of iron overload in beta-thalassemia patients, including blood transfusions, chelation regimen and also iron absorption secondary to erythropoietic expansion. However, after considering several variables, the Authors speculate that these patients might theoretically have lower hepcidin levels than the corresponding patients without the polymorphic change.

Although the paper does not report measurements of serum or urinary hepcidin of the patients studied, it indirectly suggests that the presence of the "G" nucleotide substitution makes these patients more prone to iron loading. As the Authors pointed out, this SNP affects a conserved non-coding transcriptional box of the *HAMP* gene promoter and might change its affinity binding to transcriptional factors.[3,4]

Table 1. Frequency of the -582 A->G polymorphism in Italian blood donors compared to Caucasians (CEU) from NCBI database.

		N.	Italian healthy blood donors(N. 263) Frequency (%)	Caucasians (CEU) from NCBI database Frequency (%)
Allele	A	423	80.4	83.6
	G	103	19.6	16.4
	Total	526	100.0	100.0
Genotype	A/G	87	33.1	29.3
	G/G	8	3.0	1.7
	A/A	168	63.9	69.0
	Total	263	100.0	100.00

Table 2. Transferrin saturation and serum ferritin levels according to *HAMP* promoter variant in 105 male blood donors.

	HFE wt and -582A/A	HFE wt and -582A/G or G/G
Males N. (%)	70 (67)	35 (33)
TS (%) mean ± SD	35.70±11.63	36.84±15.78
TS (%) median	35.00	35.00
FS (ng/ml) mean±SD	131.56±89.00	92.23±63.20*
FS (ng/ml) median	109.00	74.00

*TS: transferrin saturation; FS: serum ferritin; SD: standard deviation; *P=0.011 vs. HFE wt and –582A/A.*

These results prompted us to evaluate our analysis of the same SNP in a population of 263 healthy blood donors (196 males and 67 females), previously studied for mutations in the *HFE* gene.[5] Informed consent was available for all the studied individuals.

The analysis of the -582A>G polymorphism of *HAMP* promoter was performed with the NMW 1000 NanoChipTM Molecular Biology Workstation (Nanogen®, Inc. San Diego, CA), previously used in several genetic applications.[6] Oligonucleotide sequences of PCR primers, reporter probes, stabilizers and amplification conditions are available on request. Conditions for PCR product purification, addressing the chip, denaturation, hybridization and thermal stringency have been previously described.[6] Assay conditions were validated in a blinded fashion on 226 DNA samples who had been found to be either -582G/A heterozygous or homozygous (A/A or G/G) by direct sequencing.[7]

The allele frequency of -582 A->G change in the cohort of healthy blood donors is similar to that reported in the database (Table 1) and in thalassemic patients.[1] Table 2 shows transferrin saturation and serum ferritin levels in the 143/263 blood donors with normal *HFE* genotype, either carriers or non-carriers of the -582 A->G polymorphism. To avoid the bias of physiologically lower serum ferritin levels in females, statistical calculations on iron parameters were performed considering only male subjects (105/143). A t-test for independent samples after log transformation showed that in the absence of *HFE* mutations, serum ferritin levels were significantly lower in subjects who carried the "G" allelic variant compared to subjects homozygous for the "A" allelic variant. Conversely, there was no difference in transferrin saturation levels between the two groups.

The difference between our results and those by Andreani and co-workers, who found an association between the less common "G" variant in the *HAMP* promoter gene and higher serum ferritin levels, could be explained mainly on the basis of the different phenotype of the populations evaluated in the two studies: poly-transfused thalassemic patients versus healthy blood donors. Although the presence of "G" at -582 in normal conditions causes even a reduction in serum ferritin levels, when the hepcidin transcription must be reduced, as in thalassemic patients, the "G" variant could favor a stronger hepcidin inhibition, increasing iron absorption and ferritin levels. We can speculate that the mutant promoter could abnormally respond to both activatory and inhibitory signals. This hypothesis should be confirmed by direct hepcidin measurements in groups of individuals/patients with different -582 *HAMP* genotypes.

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A novel telomeric (~285 kb) α-thalassemia deletion leading to a phenotypically unusual HbH disease

Many large deletions removing the entire α-globin gene cluster on the short arm of the human chromosome 16 (16p13.3) have been described.[1-3] At the heterozygous state, the resulting phenotype consists in α-thalassemia (α-thal) for relatively short deletions (100 to 356 kb) while an α-thalassemia mental retardation syndrome (ATR-16 syndrome) is observed for larger deletions (> 1 Mb) which generally include the 16p telomere.[4,5] We report here a new large telomeric deletion (~285 kb) associated with the common alpha-thalassemia $-\alpha^{3.7}$ deletion in *trans*. This genotype led to a phenotypically unusual HbH disease.

The proband was a 14-year old girl (French Caucasian mother and Algerian father) with a marked hypochromic and microcytic anemia (Hb: 9.2 g/dL; MCV: 55.0 fL; MCHC: 29.9 g/dL; MCH: 16.5 pg and reticulocyte count: 1.7%). Physical examination was normal (without hepatosplenomegaly or subicterus) except for a marked scoliosis for which surgery was considered. She presented no developmental delay and had a normal school education. The presence of HbH (~8%) was detected at routine hemoglobin analysis using isoelectric focusing and cation-exchange liquid chromatography (Variant I, Bio-Rad). Unfortunately, a new blood sample to identify Heinz inclusion bodies could not be obtained. The search for the common α-thal deletions was carried out by multiplex PCR[6] and the common $-\alpha^{3.7}$ deletion was found at the homozygous state. This result could not be accepted for two reasons: (i) the father carried the $-\alpha^{3.7}$ deletion at the heterozygous state but the mother did not; (ii) a homozygosity for the $-\alpha^{3.7}$ deletion is not associated with Hb disease. We thus performed an MLPA analysis (Salsa MLPA kit P140-B2 HBA, MRC Holland) which identified, for both the proband and her mother, a large deletion of the α-globin gene cluster (Figures 1 and 2). A CGH-array analysis was then carried out to gap the deletion which appeared to be approximately 285 kb in length, spanning from the telomeric region in 5' to the *AXIN1* gene in 3' (Figure 1). We could finally determine, by semi-quantitative PCR assays,[8] that the deletion removes exons 5 to 10

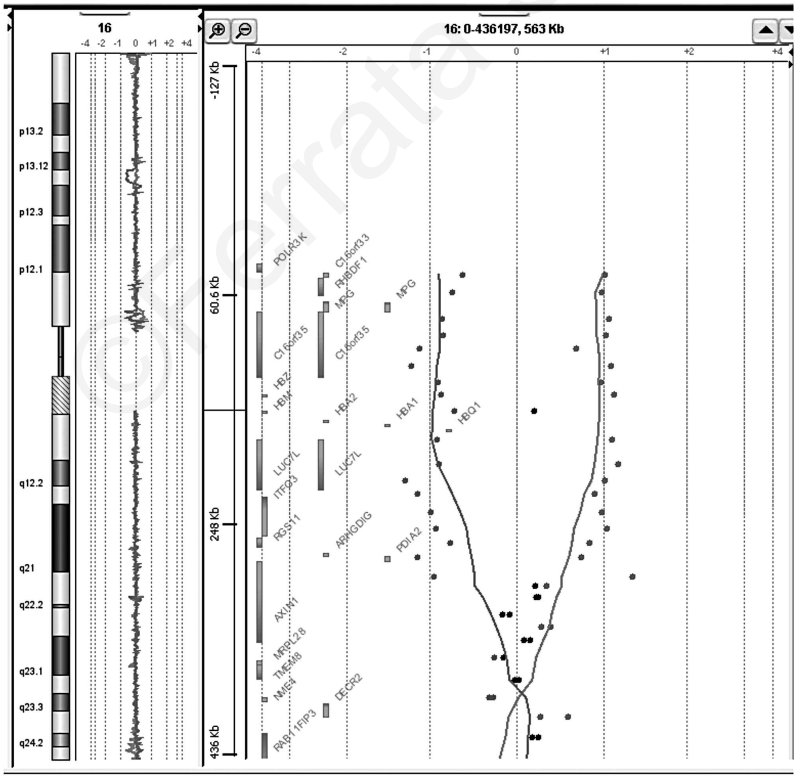

Figure 1. CGH array analysis. Experiment was performed using an Agilent Technologies 180,000-oligonucleotide array (SurePrint G3 Human CGH 4X180K). The patient's DNA and the reference DNA were digested with *Rsa*I and *Alu*I (Promega, Madison), respectively. The digestion products were labeled by random priming with Alexa Fluor 5 or Alexa Fluor 3 according to the Bioprime Total Labeling Kit protocol (Invitrogen, California). Each probe was purified by passage through a column, denatured and annealed with 50 µg of human Cot-1 DNA (Invitrogen, California). Hybridization was carried out at at 65°C for 24 hours. The array was then washed and analyzed with Feature Extraction® 10.5.1.1 software. The results were interpreted with the DNA Analytics® 4.0.85 software, with the following parameters: ADM-2, threshold: 6.0, window: 0.2 Mb. The control DNA consisted of DNA from 2 other patients presenting different diseases, in accordance with the loop model.[7] A copy number variation was noted if at least 3 contiguous oligonucleotides presented an abnormal $\log_2$ ratio (> + 0.5 or < -0.5 according to the Alexa 5 deviation) with a mirror image. For our proband, a 16pter deletion was identified. The last deleted oligonucleotide has coordinates 289,205 pb to 289,264 pb, and the first non-deleted one has coordinates 297,397 pb to 297,456 pb (NCBI reference sequence: NT_010393.16).