Disorders of Iron metabolism

The impact of the mutations of the HFE gene and of the SLC11A3 gene on iron overload in Greek thalassemia intermedia and β^s/β^{thal} anemia patients

In this study, we evaluated the impact of mutations of the HFE and ferroportin gene on iron overload in thalassemia intermedia and β^s/β^{thal} patients. Neither HFE (C282Y and H63D) nor ferroportin(Val162del) mutations were determinants of total body iron status, as assessed by ferritin levels, in either group of patients.

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Hereditary hemochromatosis (HH) is a common genetic cause of non-transfusional iron overload in Northern European countries. The majority of the cases are due to mutations of the HFE gene.[1] Ferroportin is a molecule that mediates iron export from the cells (macrophages and duodenal epithelial cells). Mutations of the ferroportin gene have been described in patients with HH type 4, an atypical form of hemochromatosis characterized by iron deposition prevalent in the reticuloendothelial system. The Val162del mutation is the most frequent since it has been identified in 4 families from different ethnic backgrounds.[2,3]

In Greece, like in other Mediterranean countries, thalassemia is the major cause of iron overload while HH represents a rare cause of iron overload.[4]

Although thalassemia intermedia patients are transfusion independent, they do become iron loaded to a varying extent because of increased iron absorption secondary to ineffective erythropoiesis. Compound heterozygotes for β^s/β^{thal} do not normally develop iron overload. In addition, iron-induced tissue damage was hypothesized to be unusual in sickle cell disease, because inflammation associated with this disorder sequesters a large proportion of iron in reticuloendothelial cells. Lately, the indications for transfusions in β^s/β^{thal} patients have increased and iron overload has complicated the course of many patients.[5]

In this study, we evaluate the impact of H63D mutation of the HFE gene but also of the Val162del of the SLC11A3 gene on iron overload in 25 unrelated Greek patients with thalassemia intermedia (13 M/12 F) and in 16 β^s/β^{thal} patients (5M /11F). At the time of the evaluation none of the patients was on a regular transfusion treatment or following chelation therapy. Iron overload was assessed by serum ferritin and transferrin saturation levels (Table 1A).

C282Y and H63D mutations of the HFE gene were detected as described elsewhere.[4] The Val162del mutation of the SLC11A3 gene was detected by SSCP analysis of polymerase chain reaction (PCR) amplified products (169bp) using the following primers:

Forward 5'-TCCTGCTATATCCTGATCATCACT–3'
Reverse 5'-TGAATCCTAACATGCTCATTT CAT-3'

β-thalassemia mutations were detected by ASO hybridization. Four out of sixteen of the β^s/β^{thal} patients and 6/25 of the thalassemia intermedia patients were heterozygotes for the H63D mutation with an allele frequency of 0.125 and 0.120, respectively (not statistically different from the frequency among the Greek population 0.145).

Table 1A. Demographic and genetic characteristics of the patients.

Age (yrs)	*Gender*	*β-globin genotype*	*HFE genotype*		*Val162del*	*Serum ferritin (ng/mL)*
			C282Y	*H63D*		
51	F	β^-/β^s	-/-	-/-	-/-	139
29	F	β^-/β^s	-/-	-/-	-/-	189
35	M	β^-/β^s	-/-	-/-	-/-	169
55	M	β^-/β^s	-/-	-/-	-/-	37.1
40	F	β^-/β^s	-/-	-/-	-/-	869
43	F	β^-/β^s	-/-	-/-	-/-	77.19
55	F	β^-/β^s	-/-	+/-	-/-	98
52	M	β^s/Hb Lepore	-/-	-/-	-/-	26
40	F	β^+/β^s	-/-	-/-	-/-	102
45	F	β^+/β^s	-/-	-/-	-/-	294
28	F	β^+/β^s	-/-	-/-	-/-	126
23	F	β^+/β^s	-/-	-/-	-/-	54
24	M	β^+/β^s	-/-	-/-	-/-	193
67	M	β^+/β^s	-/-	+/-	-/-	14.9
38	F	β^+/β^s	-/-	+/-	-/-	65.5
63	F	β^+/β^s	-/-	+/-	-/-	28
45	M	β^+/β^+	-/-	-/-	-/-	1520
32	F	β^+/β^+	-/-	-/-	-/-	524
40	M	β^+/β^+	-/-	-/-	-/-	3161
45	F	β^+/β^+	-/-	-/-	-/-	809
65	M	β^+/β^+	-/-	-/-	-/-	3628
49	F	β^+/β^+	-/-	-/-	-/-	289.3
62	M	β^+/β^+	-/-	+/-	-/-	436
25	F	β^+/β^+	-/-	+/-	-/-	899
52	M	β^0/β^+	-/-	-/-	-/-	892
45	F	β^0/β^+	-/-	-/-	-/-	754
42	M	β^0/β^+	-/-	-/-	-/-	1769
48	M	β^0/β^+	-/-	-/-	-/-	850
38	F	β^0/β^+	-/-	-/-	-/-	840
42	F	β^0/β^+	-/-	-/-	-/-	898
35	M	β^0/β^+	-/-	-/-	-/-	3527
44	F	β^0/β^+	-/-	-/-	-/-	1584
50	F	β^0/β^+	-/-	-/-	-/-	754
50	M	β^0/β^+	-/-	-/-	-/-	951
52	M	β^0/β^+	-/-	-/-	-/-	801
50	F	β^0/β^+	-/-	+/-	-/-	294
42	F	β^0/β^+	-/-	+/-	-/-	604
40	M	β^+/Hb Lepore	-/-	+/-	-/-	585
48	M	$\beta^+/^-$	-/-	-/-	-/-	885
46	F	$\beta^+/^-$	-/-	-/-	-/-	1370
68	M	$\beta^-/^-$	-/-	+/-	-/-	2187

(β^+: IVS1-110(G→A), IVS1 6(T→C), 87(C→G), 101(C→T). β^0:cd39(C→T), IVS2 1(G→A), $^-$: deletion Sicilian Type).

The presence of the H63D mutation did not seem to influence iron overload either in the thalassemia intermedia or in the β^s/β^{thal} anemia patients (Table 1B). Our data are in agreement with those of different groups that have reported that the presence of a single mutation in the HFE gene does not influence iron overload in β thalassemia major or minor.[6] In contrast, when the H63D mutation is found in the homozygote state, it may influence the ferritin levels of β-thalassemia carriers.[7]

Table 1B. Comparison of ferritin levels of H63D$^{-/-}$ and H63D$^{+/-}$ patients in the β thalassemia intermedia and β^{thal}/β^{S} group of patients.

β globin genotype	*H63D genotype +/-*	*-/-*	*p value*
β^{thal}/β^{S}			
Serum ferritin levels (ng/mL)	51.6±18.8	189.6±65.6	NS
Number of patients n=16	4	12	
β^{thal}/β^{thal}			
Serum ferritin levels (ng/mL)	834.2±282.6	1358±228.6	NS
Number of patients n=25	6	19	

The differences in ferritin levels between patients were evaluated by the non-parametric Man-Whitney test.

None of our patients carried the C282Y mutation of the HFE gene. The role of heterozygosity for C282Y in iron overload in thalassemia intermedia patients remains controversial.[8,9] The various β thalassemia mutations do not seem to influence the serum ferritin levels of the patients.

It has been proposed that iron metabolism is regulated by two regulators, namely the *erythroid* regulator and the *stores* regulator. Our findings in the thalassemia intermedia and β^{S}/β^{thal} patients, along with the observations from other groups, indirectly support the hypothesis that the erythroid regulator (β thalassemia) might represent a stronger stimulus than the stores regulator (defective HFE gene) in determining the degree of iron absorption, also taking into account that the H63D mutation leads to a lesser loss of function of the HFE protein. Among other genetic factors that modulate iron accumulation, we investigated the presence of a defective ferroportin gene because of the similarity of the mode of iron accumulation in HH type 4 and thalassemia or β^{S}/β^{thal} anemia (reticuloendothelial system).

It has been proposed that heterozygosity for the Val162del represents the prototype of selective reticuloendothelial iron-overload.[10] None of our patients carried the Val162del mutation. The presence of other SLC11A3 mutations in our patients cannot be excluded but these preliminary results indicate that ferroportin mutations are unlikely to play a significant role in iron overload in these patients.

Furthermore, the SSCP method described here is a valuable, reproducible, inexpensive technique, suitable for population screening for the prevalence of the Val162del mutation of the SLC11A3 gene. Based on our results we conclude that neither the common HFE mutations (C282Y and H63D) nor the Val162del mutation of the SLC11A3 gene are major determinants of total body iron status in patients with thalassemia intermedia and β^{S}/β^{thal} anemia.

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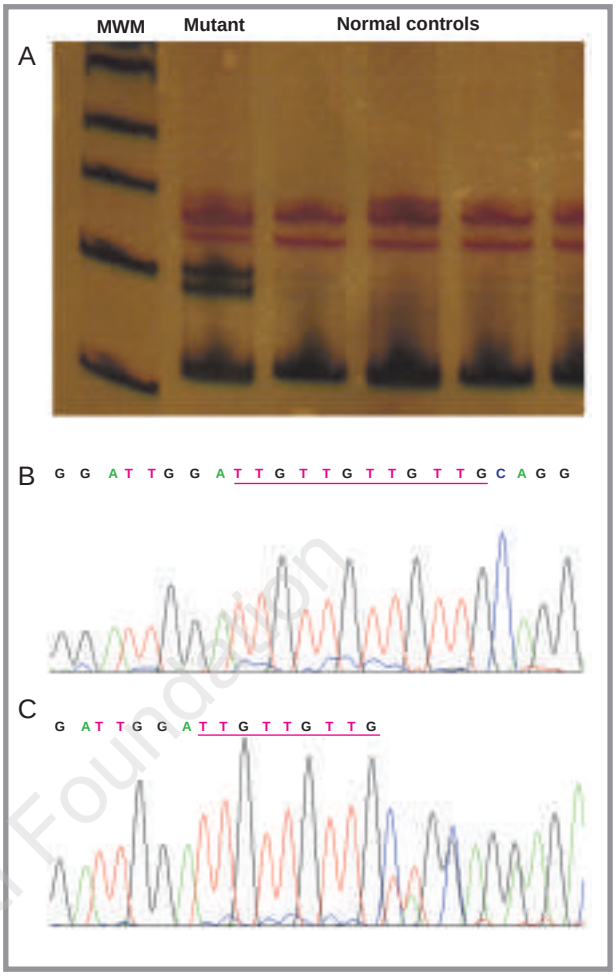

Figure 1. Detection of the Val162del mutation of the SLC11A3 gene. 1A. PCR products were denaturated at 95°C for 10 min and run in 10% non-denaturing acrylamide gel with 10% glycerol at 4°C. Acrylamide gels were stained by silver staining. Distinct electrophoretic patterns for the normal and the mutated Val162del allele were obtained by SSCP analysis of PCR amplified products. The deletion of a TTG triplet between nucleotides 780 and 791 was confirmed by direct sequencing. 1B. Normal. 1C. Mutated.

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Red Cell Disorders

Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia

Malarial anemia involves destruction of parasitized and non-parasitized red blood cells and dyserythropoiesis. Malarial pigment, hemozoin (HZ), is possibly implicated in dyserythropoiesis. We show that supernatants of HZ and HZ-fed-monocytes, and 4-hydroxynonenal generated by them, inhibited progenitor growth.

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Malarial anemia involves destruction of parasitized and non-parasitized red blood cells (RBC), and dyserythropoiesis.[1-3] Dyserythropoiesis is common and severe, but poorly understood.[1-3] Malarial pigment, hemozoin, (HZ) is possibly implicated in dyserythropoiesis. First, HZ-containing trophozoites and schizonts as well as HZ and HZ-containing macrophages are abundantly present in the bone-marrow of malaria patients.[2] Secondly, HZ and HZ-laden monocyte/macrophages generate inhibitory lipid-peroxidation derivatives.[4,5] Lastly, HZ-laden phagocytes produce potentially inhibitory cytokines.[6]

We show here that supernatants of HZ and HZ-fed monocytes inhibit erythroid-progenitor growth. Inhibition was reproduced by 4-hydroxynonenal (4-HNE), a final product of lipid-peroxidation generated by HZ and present in supernatants.[5] These observations indicate that toxic molecules generated by HZ or HZ-fed monocytes may play a role in malaria dyserythropoiesis.

Trophozoites were isolated from *P. falciparum* cultures (FCR-3 strain, mycoplasma-free) and native HZ prepared as described elsewhere[4,5] and supernatants of HZ/delipidized-HZ were obtained.[4] Anti-D IgG-opsonized RBC, serum-opsonized trophozoites, HZ, or latex beads were fed to adherent human monocytes.[4] After removal of non-ingested meals, monocytes were reincubated at 37°C for a further 6 h before the conditioned medium was harvested, centrifuged, filtered, and added to the progenitor culture medium (see below and legend to Table 1 for details).

BFU-E/CFU-E colonies were obtained from low-density cord or bone-marrow cells cultured as previously described.[7,8] When indicated, 0.1 mL HZ/monocyte-supernatant was added to the progenitor culture medium. Colonies were counted after 14 days (see legend to Table 1 for details). 4-HNE (Biomol, Berlin, Germany) was supplemented to the progenitor culture medium at 0.07-7.0 μM (final concentration). In some experiments, 4-HNE was supplemented with liposomes (12.6 μM phosphatidylcholine, 3.6 vM stearylamine and 1.8 μM cholesterol, final concentrations) and quantified in conditioned supernatants (see legend to Table 2 for details).[5] As shown in Table 1, HZ-supernatants inhibited BFU-E growth by 38±11% (p=0.006; n=16) when supernatant generated by HZ equivalent to 12.5 trophozoites/progenitor was added to the cultures. This compares to the ratio between progenitors and *nursing* macrophages in the erythroblastic islets. Supernatants of

Table 1. Inhibitory effect on erythroid progenitor growth of supernatant of native HZ and supernatants of monocytes fed with serum-opsonized HZ, anti-D IgG-opsonized RBC or serum-opsonized Latex.

Supernatant of	*BFU-E Percent inhibition*	*Significance of difference*
Native HZ	38±11 (n=16)	p<0.006, HZ vs control
Delipidized HZ	13±2.2 (n=9)	p<0.001, HZ vs delipidized HZ; 0.17, delipidized HZ vs control
Monocytes fed HZ	48±8.2 (n=10)	p<0.001 HZ vs control; p<0.005 HZ vs RBC; p<0.006 HZ vs latex
Monocytes fed RBC	2.1±0.4 (n=9)	p>0.5 RBC vs control
Monocytes fed Latex	11.2±2.3 (n=7)	p>0.5 Latex vs control

HZ was prepared from the trophozoite-enriched cultures (>95% trophozoites) by osmotic shock and 4 washes with ice-cold hypotonic phosphate buffer (10 mM potassium phosphate, 1 mM EDTA, 1 mM mannitol, pH 7.2). After the last wash, HZ was suspended in sterile PBS (approx. 20% wt/vol), flushed with nitrogen and frozen at -20°C under nitrogen. Before phagocytosis, HZ was opsonized with an equal volume of fresh human serum for 30 min at 37°C.[4] Frozen HZ suspension (see above) was thawed, homogenized by 10 passages through a fine syringe needle and further diluted with PBS. HZ was quantified according its heme content by measuring heme luminescence. Three hundred micrograms of HZ, corresponding to 50×10⁶ trophozoites were added to 3.7 mL PBS, pH 7.4 and incubated at 37°C for 6 h under agitation. At the end of incubation, HZ-conditioned supernatant was obtained by brief centrifugation (2 min at 12,000g) with an Eppendorf Microfuge, filtered through a sterile filter with a pore diameter of 0.1 μm to exclude debris, and added to the progenitor culture medium (see below). In some experiments, HZ supernatant was obtained from delipidized HZ, prepared as indicated.[4] BFU-E colonies from 10⁵ non-adherent, low density human bone-marrow cells were cultured without or with supernatants or RBC lysate equivalent to 12.5 trophozoites or RBCs per bone-marrow cell. Adherent human monocytes were fed ad libitum with: native HZ prepared from mycoplasma-free trophozoites; Anti-D IgG-opsonized RBC; serum-opsonized Latex beads (2.5% solids, diameter 0.105 μM, Sigma). Monocyte supernatant (0.1 mL) was obtained 24 h after start of phagocytosis of the respective meals and added to the progenitor culture medium. On average, each bone-marrow cell was supplemented with the supernatant produced by 0.2 monocytes. Control bone-marrow cells received 0.1mL medium. Triplicate dishes were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air and colonies were counted after 14 days. Data are expressed as percentage inhibition of growth compared to controls (mean±SD, n=10). Statistical comparisons were by Student's t test on paired samples. The mean (±SD) number of control BFU-E colonies was 123±32 (n=10).