

Tumor Necrosis Factor- α –308G>A Allelic Variant Modulates Iron Accumulation in Patients with Hereditary Hemochromatosis

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Background: In vitro and animal studies suggest that tumor necrosis factor α (TNF- α) modulates intestinal iron transport. We hypothesized that the effect of TNF- α might be particularly relevant if iron absorption is not effectively controlled by the HFE gene.

Methods: In patients with homozygous C282Y hemochromatosis, we investigated the influence of TNF- α –308G>A allelic variant on total body iron overload, determined in all patients by measuring iron removed during depletion therapy, and hepatic iron index and need for phlebotomy to prevent iron reaccumulation, measured in patient subgroups.

Results: Of 86 patients with hereditary hemochromatosis, 16 (19%) were heterozygous carriers and 1 (1%) was a homozygous carrier of the TNF- α promoter –308A allele. Mean (SD) total body iron overload was increased 2-fold in TNF- α –308A allele carriers [10.9 (7.6) g] compared with homozygous carriers of the G allele [5.6 (5.0) g, $P < 0.001$]. Hepatic iron index differed markedly between TNF- α –308A allele carriers [5.6 (3.5) $\mu\text{mol/g/year}$] and homozygous G allele carriers [3.1 (2.2) $\mu\text{mol/g/year}$, $P = 0.040$, $n = 30$]. After iron depletion, the need for phlebotomy to prevent iron reaccumulation (maintenance therapy) was substantially higher in TNF- α –308A allele carriers than in homozygous G allele carriers ($P = 0.014$, $n = 73$). We used multiple regression analyses to exclude possible confounding

effects of sex, age, family screening, body-mass index, and meat or alcohol intake.

Conclusion: TNF- α –308G>A allelic variant modulates iron accumulation in patients with hereditary (homozygous C282Y) hemochromatosis, but the effect of the TNF- α –308A allele on clinical manifestations of hemochromatosis was less accentuated than expected from the increased iron load associated with this allele.

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Hemochromatosis, one of the most frequently occurring inherited diseases, is characterized by progressive iron overload in parenchymal tissues. Clinical manifestations include hepatic fibrosis, arthropathy, diabetes mellitus, cardiopathy, and hypogonadism. After the *HFE* gene⁴ was identified in 1996 (1), several studies showed that ~90% of patients with typical clinical features of this metabolic disease are homozygous for a G>A variation at nucleotide 845 of the *HFE* gene that leads to a substitution of tyrosine for cysteine at amino acid 282 (C282Y) (2). Because epidemiologic studies indicate low clinical penetrance and variable expression of the homozygous *HFE* C282Y variation (3–7), we suspect that environmental and/or genetic factors play a role in modifying the phenotypic expression of *HFE* C282Y homozygosity. The *HFE* gene is located on chromosome 6 in the vicinity of genes encoding MHC class I molecules (8), such as tumor necrosis factor- α (TNF- α)⁵.

TNF- α participates in the regulation of ferritin and transferrin receptor gene expression in vitro (9, 10) and is involved in iron metabolism of macrophages (11–13) and of cell lines resembling hepatocytes or intestinal epithelial cells (14, 15). A possible role of the TNF- α –308G>A

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⁴ Human gene: *HFE* gene, hemochromatosis.

⁵ Nonstandard abbreviations: TNF- α , tumor necrosis factor- α .

allelic variant has been investigated in several diseases. The TNF- α -308A allele was positively associated with manifestations of inflammatory processes such as septic shock (16), cerebral malaria (17), Crohn disease (18), and asthma (19); however, not all studies confirmed these associations (20–23). No significant association or controversial results have been reported in studies investigating the role of the TNF- α -308A>G allelic variant in coronary artery disease (24–26), type 2 diabetes (27), or hypertension (28, 29). An inverse association between the TNF- α -308A allele and disease manifestation was found in Gaucher disease (30) and primary biliary cirrhosis (31, 32). Various effects have been reported for TNF- α -308A allele carrier status in hemochromatosis, including lower prevalence of liver cirrhosis (33), no effect on liver cirrhosis, siderosis, or serum ferritin concentration (34), and slightly increased collagen concentrations in liver tissue (35).

Transcription assays have been used to quantify the transcriptional activity of the TNF- α -308A allele compared with the common G allele. Some studies (36–39) indicated that the TNF- α -308A allele is a more powerful activator of TNF- α gene transcription than the common allele, and others (40–42) that this is not the case. The data suggest that, under certain circumstances, the TNF- α -308A allele is associated with increased transcriptional activity. Moreover, the TNF- α gene is located within the MHC region, and there is strong linkage disequilibrium between alleles across MHC. Accordingly, in Epstein-Barr virus-transformed human B cells, the TNF- α -308A allele was shown not to be associated with increased transcriptional activity but to reside in linkage with another functional variant (43).

TNF- α inhibits iron transport and divalent metal transporter-1 expression in Caco-2 cells, a cell line resembling human intestinal epithelial cells (44). Studies in animal models further support the theory that the control of iron absorption is associated with secretion of TNF- α , probably by lymphocytes in the small intestinal epithelium (45–46). We hypothesized that the effect of TNF- α might be particularly relevant if intestinal iron absorption is not effectively controlled by the HFE gene, that is, in patients with hereditary hemochromatosis.

Patients and Methods

STUDY DESIGN

In a retrospective association study, we investigated the possible influence of TNF- α -308G>A allelic variant on total body iron overload (primary study parameter), hepatic iron index, and the need for phlebotomy to prevent iron reaccumulation (secondary parameters assessed in subgroups) in patients with hereditary hemochromatosis.

STUDY PATIENTS

This study was approved by the ethics committee of the University Hospital of Zurich. All participating patients gave written informed consent. Inclusion criteria were

homozygous C282Y variation of the HFE gene, serum ferritin concentration >300 $\mu\text{g/L}$ at diagnosis, and completed iron depletion therapy. Exclusion criteria were chronic hepatitis B or C infection, treatment with deferoxamine, or blood donation before hemochromatosis diagnosis. Of 86 hemochromatosis patients included in the study, 73 presented with clinical manifestations and 13 were identified by family screening. Some of the hemochromatosis patients were previously included in a study investigating the iron isotope composition of blood (47).

ASSESSMENT OF VARIABLES REFLECTING IRON ACCUMULATION

Total body iron overload was assessed from the amount of blood removed by phlebotomy during depletion therapy, assuming that 500 mL of blood contains 250 mg of elemental iron (47, 48). To evaluate all patients in the same way, although iron depletion therapy had been carried out according to different threshold values (≤ 300 $\mu\text{g/L}$) in our study patients, we used a ferritin concentration of 300 $\mu\text{g/L}$ as the threshold of depletion therapy. We used atomic-absorption photometry to measure iron concentration in dry liver tissue from 30 patients and assessed hepatic iron index as iron concentration of dry liver tissue ($\mu\text{mol/g}$) divided by the age of the patient (years). A total of 73 patients had completed iron depletion therapy at least 2 years before the study, and their need for iron removal to prevent reaccumulation was assessed from mean amounts of blood removed by phlebotomy per year during maintenance therapy (47).

We used a standardized questionnaire to assess habitual meat and alcohol intake. Meat intake was assessed as the number of meat meals and the amount of meat consumed weekly. Considerable meat intake was defined as either ≥ 5 meat meals or ≥ 500 g meat taken in per week. We calculated alcohol intake from the amount of beer, wine, and liquor consumed weekly, assuming alcohol content of 4%, 12%, and 20%, respectively. Considerable alcohol intake was defined as consumption of ≥ 140 g (males) or ≥ 70 g (females) per week.

ASSESSMENT OF BIOCHEMICAL VARIABLES AND CLINICAL MANIFESTATIONS

We assumed that liver disease was present if histologic examination of a liver biopsy specimen showed substantial portal fibrosis and/or septal/bridging fibrosis or cirrhosis (49), or in patients without liver biopsy, if the serum concentration of alanine aminotransferase was above the reference interval at diagnosis. We tested all patients with liver disease for hepatitis B virus surface antigen (HBsAg), hepatitis B virus core antibodies (anti-HBc), and hepatitis C virus antibodies (anti-HCV). Arthropathy of metacarpophalangeal joints was assessed from clinical and/or radiologic examinations. Diabetes mellitus, cardiopathy, and hypogonadism were assessed from the medical records as established diagnoses. Serum

concentrations of iron, transferrin, ferritin, and alanine aminotransferase were determined at the Institute of Clinical Chemistry (University Hospital of Zurich) with commercial assays from Roche Diagnostics. HBsAg, anti-HBc, and anti-HCV were determined at the Institute of Immunology with commercial assays from Abbott.

GENETIC ANALYSES

TNF- α promoter c.-308G>A allelic variant and *HFE* gene c.845G>A (C282Y) variation were determined by Light-Cycler PCR and melting curve analyses (Roche Molecular Biochemicals), with ToolSets containing specific primers and fluorescent probes (Genes-4U AG) according to the manufacturers' instructions.

STATISTICAL ANALYSIS

We used Statistica version 6 for analyses. We used the Student *t*-test, Mann-Whitney *U*-test, χ^2 test, or Spearman rank order correlation to compare variables. We performed additional multiple regression analyses on the study parameters to consider possible confounding effects, and during analyses, we used a stepwise backward elimination procedure to exclude variables with uncertain influence ($P > 0.10$). The threshold of significance was defined with $\alpha = 0.05$.

Results

PATIENT CHARACTERISTICS

At diagnosis, serum ferritin concentration was $>300 \mu\text{g/L}$ in 86 patients with hemochromatosis homozygous for the 845G>A (C282Y) variation of the *HFE* gene. Of these patients, 16 (19%) were heterozygous carriers and 1 (1%) was a homozygous carrier of the TNF- α promoter -308A allele (TNF- α promoter c.-308A allele). This corresponds to an allele frequency of 10%; similar values were previously found in healthy persons at our hospital (15%, $n = 55$), in Spain (12%), and in France (16%) (50, 26). The study groups were well balanced with regard to sex, age at time of diagnosis, and body-mass index (Table 1). Neither meat nor alcohol intake differed substantially between the 2 study groups.

VARIABLES REFLECTING IRON ACCUMULATION

Total body iron overload (primary parameter) was assessed as total amount of iron removed by phlebotomy during depletion therapy (Table 2). Mean (SD) iron overload was 10.9 (7.6) g in carriers of the TNF- α -308A allele (genotypes AA+AG) and 5.6 (5.0) g in homozygous carriers of the G allele (genotype GG; $P < 0.001$). This corresponds to a 2-fold increase of iron accumulated in TNF- α -308A allele carriers compared with homozygous G allele carriers. In the only homozygous carrier of the TNF- α -308A allele, however, removal of more than 30 g of iron during 2 years of depletion therapy did not bring about serum ferritin concentrations within the reference interval.

Investigation of secondary parameters provided additional evidence for increased iron accumulation in TNF- α -308A allele carriers. Iron concentration of liver tissue had been determined in 30 study patients (Table 2). Mean (SD) hepatic iron index, i.e., iron concentration of liver tissue divided by the age of the patient, was 5.6 (3.5) $\mu\text{mol/g/year}$ in TNF- α -308A allele carriers and 3.1 (2.2) $\mu\text{mol/g/year}$ in homozygous G allele carriers ($P = 0.040$). The need for phlebotomy to prevent iron reaccumulation (maintenance therapy) was assessed in 73 patients who had completed iron depletion therapy at least 2 years before this study (Table 2). Iron removal during maintenance therapy was substantially higher in TNF- α -308A allele carriers ($<0.5 \text{ g/year}$ in 0%, 0.5–1 g/year in 58%, $>1 \text{ g/year}$ in 42%) than in homozygous G allele carriers ($<0.5 \text{ g/year}$ in 33%, 0.5–1 g/year in 47%, $>1 \text{ g/year}$ in 20% of patients; $P = 0.014$), see Table 2.

POSSIBLE CONFOUNDING EFFECTS ON IRON ACCUMULATION

Possible confounding effects on iron accumulation were considered in multiple regression analyses (Table 3). The TNF- α -308A allele continued to markedly influence total body iron overload ($P < 0.001$), hepatic iron index ($P = 0.040$), and iron removal during maintenance therapy ($P = 0.025$). Iron removal during maintenance therapy was also associated with sex ($P = 0.033$) and age at

Table 1. Baseline characteristics of 86 hemochromatosis patients homozygous for the C282Y variation of the *HFE* gene as a function of the TNF- α -308G>A allelic variant.

Characteristics	TNF- α -308G>A allelic variant		P value ^b
	A allele carriers ^a (n = 17)	Homozygous G allele carriers (n = 69)	
Male sex, n (%)	13 (77)	44 (64)	0.32
Age at diagnosis, year	48 (13)	49 (13)	0.90
Body mass index, kg/m ²	24.7 (4.6)	24.6 (3.4)	0.85
Considerable meat intake ($\geq 500 \text{ g}$ meat or 5 meat meals per week), n (%)	4 (24)	21 (30)	0.57
Considerable alcohol intake (males $\geq 140 \text{ g}$, females $\geq 70 \text{ g}$ per week), n (%)	5 (29)	15 (22)	0.50

Values are numbers of patients (%) or mean (SD).

^a Sixteen heterozygotes (AG), 1 homozygote (AA).

^b P values were calculated using χ^2 test or Student *t*-test.

Table 2. Variables reflecting iron accumulation in 86 patients with hereditary hemochromatosis as a function of the TNF- α -308G>A allelic variant.

Variables	TNF- α -308G>A allelic variant		Difference mean (95% CI)	P value ^b
	A allele carriers ^a (n = 17)	Homozygous G allele carriers (n = 69)		
Total body iron overload				
Iron removed by phlebotomy during depletion therapy, g (SD)	10.9 (7.6)	5.6 (5.0)	5.3 (2.3–8.3)	<0.001
Secondary parameters investigated in subgroups				
Hepatic iron index	(n = 6)	(n = 24)		
Iron concentration of liver tissue divided by the age, $\mu\text{mol/g/year}$ (SD)	5.6 (3.5)	3.1 (2.2)	2.5 (0.1–4.7)	0.040
Maintenance therapy	(n = 12)	(n = 61)		
Iron removed by phlebotomy to prevent reaccumulation				
<0.5 g/year, n (%)	0 (0)	20 (33)		
0.5–1 g/year, n (%)	7 (58)	29 (47)		0.014
>1 g/year, n (%)	5 (42)	12 (20)		

Values are means (1SD), mean of the difference (95% confidence interval), number of patients (%).

^a Sixteen heterozygotes (AG), 1 homozygote (AA).

^b P values were calculated using Student t-test or Spearman rank order correlation.

time of diagnosis ($P = 0.059$). Other possible confounding effects were not associated with study parameters and were therefore excluded during the stepwise regression procedure (Table 3). The 13 patients identified by family screening were likely to demonstrate a TNF- α genotype and concordance of hemochromatosis expression similar to other members of their family. Therefore, we stratified the single group analyses, including only the proband of each family. The TNF- α -308A allele continued to show marked influence on total body iron overload ($P = 0.007$), hepatic iron index ($P = 0.042$), and iron removal during maintenance therapy ($P = 0.037$). Moreover, a possible confounding effect of family screening on variables reflecting iron accumulation was excluded in multiple regression analyses (Table 3).

BIOCHEMICAL AND CLINICAL MANIFESTATIONS

Transferrin saturation was 83% in both study groups, a value characteristic for patients with hereditary hemochromatosis. Mean serum ferritin concentration was higher in TNF- α -308A allele carriers (2414 $\mu\text{g/L}$) than in homozygous G allele carriers (1841 $\mu\text{g/L}$; Table 4); however, the differences in ferritin concentrations between the

study groups did not fully reflect differences in total body iron overload (Table 2). This suggests that the ferritin concentration reflects variables in addition to iron load in hereditary hemochromatosis.

Liver disease, arthropathy of metacarpophalangeal joints, diabetes mellitus, and cardiopathy were observed more frequently in TNF- α -308A allele carriers than in homozygous G allele carriers, whereas hypogonadism was found exclusively in a few patients of the latter group (Table 4). In spite of markedly increased iron load associated with the TNF- α -308A allele, clinical expression of hemochromatosis did not differ substantially between the 2 study groups, considering the total number of clinical manifestations per patient ($P = 0.34$; Table 4). Further insight is provided by Fig. 1. The toxic effect of iron, although associated with iron overload in both study groups, was less accentuated in TNF- α -308A allele carriers than in homozygous G allele carriers. From linear regression analyses (Fig. 1), we can estimate that additional accumulation of 4–5 g iron is needed in TNF- α -308A allele carriers to induce clinical manifestations of hemochromatosis similar to those in homozygous carriers of the G allele.

Table 3. Multiple regression analyses considering possible confounding effects on variables reflecting iron accumulation.

Multiple regression model ^a	Total body iron overload, P values	Hepatic iron-index, P values	Maintenance therapy, P values
TNF- α -308A allele	<0.001	0.040	0.025
Male gender	0.33	0.49	0.033
Age at time of diagnosis	0.43	0.15	0.059
Inclusion by family screening	0.52	0.37	0.37
Body mass index	0.83	0.63	0.43
Considerable meat intake	0.56	0.82	0.84
Considerable alcohol intake	0.73	0.52	0.54

^a Variables remaining in the model are written in bold.

Table 4. Biochemical and clinical manifestations in 86 patients with hereditary hemochromatosis as a function of the TNF- α -308G>A allelic variant.

Manifestations	TNF- α -308G>A allelic variant	
	A allele carriers	Homozygous G allele carriers
Transferrin saturation, % (SD)	83 (14)	83 (15)
Serum ferritin concentration, $\mu\text{g/L}$ (SD)	2414 (2072)	1841 (1520)
Clinical manifestations		
- Liver disease, n (%) ^a	10 (67)	32 (46)
- Arthropathy of metacarpophalangeal joints, n (%)	7 (41)	23 (33)
- Diabetes mellitus, n (%)	3 (18)	0 (0)
- Cardiopathy, n (%)	1 (6)	0
- Hypogonadism, n (%)	0	3 (4)
Total number of clinical manifestations per patient (median range) ^b	1 (0-4)	1 (0-3)

Values are means (SD), numbers of patients (%), or median (range).

^a Substantial portal fibrosis and/or septal/bridging fibrosis and/or cirrhosis in histological examination or, in patients without biopsy, elevated serum alanine aminotransferase concentration at time of diagnosis.

^b Difference not significant ($P = 0.34$ by Mann-Whitney U -test).

Discussion

This study provides evidence that the TNF- α -308G>A allelic variant (TNF- α c.-308G>A allelic variant) modulates iron accumulation in patients with hereditary hemochromatosis. The association of the TNF- α -308A allele (genotypes AA+AG) with iron accumulation was demonstrated in TNF- α -308A allele carriers compared with homozygous G allele carriers (genotype GG) by a 2-fold higher total body iron overload, by a higher hepatic iron index, and by a markedly higher iron removal during maintenance therapy, measured in a steady state with continuous intestinal iron (hyper) absorption. These 3 variables were assessed independently, and their relevance was confirmed by significant correlations of total body iron overload with hepatic iron index ($P = 0.033$), as well as iron removal during maintenance therapy ($P < 0.001$).

The TNF- α -308A allele was also positively associated

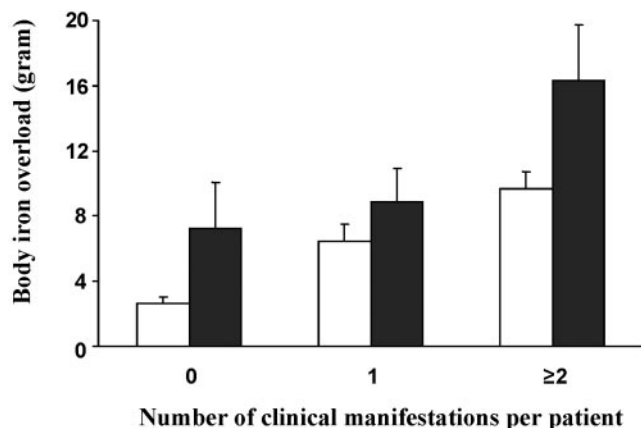


Fig. 1. Correlation of total body iron overload with the number of clinical manifestations in TNF- α -308A allele carriers (black bars, $P = 0.035$) and homozygous G allele carriers (white bars, $P < 0.001$) with hereditary hemochromatosis.

Values are provided as means (SEM, signified by error bars).

with clinical expression of hemochromatosis in our study population, but this association was less pronounced than expected from the increased iron load and did not reach statistical significance. This discrepancy is documented in Fig. 1 and corresponds to a smaller relative toxicity of iron in TNF- α -308A allele carriers than in homozygous G allele carriers. Our study shows an association of the TNF- α -308A allele with iron accumulation on the one hand and with decreased relative sensitivity to iron toxicity on the other hand. We do not have evidence regarding the underlying pathophysiologic mechanisms for these clinical observations, but in light of previous investigations, both are attributable to decreased activity of TNF- α . In vitro and animal experiments suggest that intestinal iron absorption is controlled (inhibited) by TNF- α (44-46), suggesting that increased iron accumulation in our study patients might be related to decreased intestinal activity of TNF- α . The cytotoxic activity of TNF- α is well documented (51), and therefore, decreased relative iron toxicity might be explained by decreased activity of TNF- α in parenchymal tissues of our patients.

We cannot draw a general conclusion from our study regarding transcriptional activity and biological effects of the TNF- α -308A allele. Transcription assays have shown that, in vitro and under certain circumstances, the TNF- α -308A allele can increase transcription of the TNF- α gene (36-42, 52). Variables such as the length of the promoter sequence used, the presence or absence of the 3' untranslated region (UTR), or the cell type used for transfection can affect the results of these experiments. However, the TNF- α gene is located within the MHC region, and there is a strong linkage disequilibrium between alleles across MHC. Thus, expression of TNF- α may not be regulated by the TNF- α -308G>A allelic variant itself, but by variation of a linked gene (43, 52, 53).

Overall, TNF- α activity seems to be regulated by complex and multifactorial processes, and clinical manifestations of the TNF- α -308A allele do not follow a fixed

pattern. In inflammatory processes, such as septic shock or cerebral malaria, mainly positive associations between the TNF- α -308A allele and disease manifestation have been reported (16–23), whereas in diseases in which inflammation is less accentuated, such as coronary artery disease or type 2 diabetes, nonsignificant or inconstant associations between the TNF- α -308A allele and clinical manifestations were found (24–29). Inverse associations between the TNF- α -308A allele and disease expression were reported in Gaucher disease (30) and in primary biliary cirrhosis (31, 32). Both Gaucher disease and hereditary hemochromatosis are metabolic diseases, and both primary biliary cirrhosis and hemochromatosis are characterized by altered production of TNF- α in monocytes (13, 33, 54).

With regard to clinical expression of hemochromatosis, our findings agree with previous studies showing only nonsignificant (favorable or unfavorable) effects of the TNF- α -308G>A allelic variant (33–35). In one study only, association between the TNF- α -308A allele and iron removed during depletion therapy was investigated and not found to be significant. However, the population included in this study was relatively small and heterogeneous, i.e., only some of the patients were homozygous for the C282Y variation of the HFE gene (33).

In conclusion, this study shows that the TNF- α -308G>A allelic variant modulates iron accumulation in patients with hereditary hemochromatosis. The result is based on investigation of a considerably large and homogeneous study population carrying homozygous C282Y variations of the HFE gene. Further genetic and/or environmental factors must be postulated that modify the penetrance of the homozygous C282Y variation, considering that only a minor part of HFE C282Y homozygotes develop clinical hemochromatosis.

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