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Low serum transferrin levels in *HFE* C282Y homozygous subjects are associated with low CD8$^+$ T lymphocyte numbers

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Abstract

Hereditary hemochromatosis (HH) is a genetic iron overload disease, in the majority of cases associated with homozygosity for the C282Y mutation of the *HFE* gene. In spite of this genetic homogeneity, there is a great clinical heterogeneity among HH patients. Low CD8$^+$ lymphocyte numbers have been associated with a more severe expression of iron overload in HH patients, and in experimental models of iron overload. HH patients present low serum transferrin levels. Transferrin is an indispensable resource for lymphopoiesis. Lymphocyte homeostasis follows general ecology rules of population dynamics that involve competition for limiting resources. In the present study, we questioned whether transferrin levels could be associated with the anomalies seen previously in lymphocyte subset numbers in HH patients. Transferrin levels, total and subset T lymphocyte counts were done in 426 apparently healthy subjects genotyped for *HFE.* All *HFE* C282Y carriers presented significantly lower serum transferrin levels than the wild type group, a difference that could not be explained solely by the degree of iron overload. Significant differences were also seen in transferrin levels between males and females, with females presenting higher average serum Transferrin levels. In the population of subjects with Transferrin levels lower than 248 mg/dl, a positive correlation was seen between the peripheral CD8$^+$ lymphocyte numbers and serum transferrin levels ($R^2 = 2.41$; $r = 0.16$; $P = 0.018$). To test the possible limiting resource effect of transferrin, the correlation between transferrin levels and CD8$^+$ lymphocyte numbers was scrutinized in 34 HH patients, homozygous for the C282Y mutation. In the homozygous males, where the lowest average transferrin levels were seen, another highly significant correlation was observed between Transferrin levels and CD8$^+$ numbers. This correlation points to a possible role of transferrin as a limiting resource for MHC class I dependent lymphocyte proliferation, an effect that was not observed in C282Y homozygous female patients.

Keywords: Transferrin; HFE; CD8$^+$ T lymphocytes

Introduction

Hereditary hemochromatosis (HH), a genetic iron-overload disease, is associated with *HFE*, a gene that codes for a non-classical MHC class I protein [1]. In spite of the fact that one mutation, the C282Y, is found in homozygozity in the majority of Caucasian patients, there is a great clinical heterogeneity in expression of the resulting iron overload [2–4]. The severity of iron overload assessed by iron removed by phlebotomy, serum ferritin and Transferrin (Trf) saturation, is consistently greater in men than in women [5–7]. Low CD8$^+$ T and/or total lymphocyte numbers have been reported to correlate significantly with higher iron stores in HH [4,8–10]. Selective abnormalities in CD8$^+$ T lymphocytes have been consistently described in HH patients. These include a defective p56-lck kinase activity, decreased cytotoxic activity, a decreased number of

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$CD8^+$ T cells expressing the CD28 molecule, abnormalities in the T cell receptor repertoire and cytokine production [11–14]. In addition, studies of double knockout mice indicate that the iron load in Hfe−/− mice is higher when crossed to β2m−/− [15] or to Rag1−/−[16].

Lymphocyte homeostasis follows general ecology rules of population dynamics that involve competition for limiting resources [17]. Trf is a well-known resource for the growth of numerous cell types including lymphocytes [18–20]. Moreover, anti-Trf receptor antibodies inhibit lymphocyte and thymocyte proliferation and differentiation in vitro [21,22]. Quimeric animals for a deletion in the Trf receptor gene revealed the importance of the Trf receptor for the differentiation of bone marrow hematopoietic precursors into erythroid and thymus dependent lymphoid lineages [23]. The hypotransferrinemic mice permitted to go further dissecting the role of Trf in hematopoiesis. These animals present a defective T cell differentiation at a specific early stage, during the thymocyte differentiation from TN3 to the TN4 stage [24]. The same specific defect was observed in mice heterozygotes for the Trf receptor 1 deficiency, indicating that the Trf dependent thymus effect is mediated by its receptor [24].

Abnormally low serum Trf levels are commonly observed in C282Y homozygous subjects [25,26]. These are generally attributed to the effect of severe iron overload, in contrast with the finding of increased serum Trf concentration in iron deficiency [27]. Decreased Trf values were also reported in large populations of C282Y carriers with iron parameters within the normal range [25 26]. The objective of the present study was threefold: (1) to examine Trf levels in a large apparently healthy population of 426 *HFE* genotyped subjects; (2) to examine the existence of possible associations between lymphocyte numbers and Trf in groups selected for distinct average Trf serum levels and (3) to examine the same question in a group of 34 C282Y homozygous HH patients.

Material and methods

Population Studied

C282Y homozygous HH patients

Thirty-four hereditary hemochromatosis (HH) patients homozygous for the C282Y mutation of the *HFE* gene were included in this study. Patients are regularly followed up at the Hemochromatosis Outpatient Clinic of Santo António General Hospital, Porto. Nineteen were males (mean age 47 ± 14) and 15 were females (mean age 44 ± 13). All patients had Trf saturation greater than 50% at the time of the diagnosis. Patients are regularly treated by weekly phlebotomy (intensive treatment) until iron depletion is achieved (Trf saturation below 10% and/or ferritin levels below 10 ng/ml). After intensive treatment, patients are phlebotomized between 4 and 12 times per year, depending on individual profile (maintenance treatment) [9]. Because of the impact of liver disease on Trf levels, patients with cirrhosis and/or any degree of fibrosis in liver biopsy were excluded.

Healthy population

Four hundred and twenty-six apparently healthy subjects previously studied as part of the routine screening program for hemochromatosis at the Hemochromatosis Outpatient Clinic of Santo António General Hospital and Predictive and Preventive Genetic Center (IBMC), Porto, were also analyzed. These include 279 family members of HH patients with no evidence of iron overload or other related pathologies, and 147 unrelated normal subjects. This healthy population included 181 males (mean age 40 ± 15) and 245 females (mean age 44 ± 15). All subjects had been previously *HFE* genotyped as described before [28]. Exclusion criteria for the healthy population study were age <20 years old and evidence of viral infection (HBV, HCV, HTLV, HIV1 and HIV2).

Clinical and laboratory parameters included in this work were reviewed from the clinical files or determined in the course of the present study, with the informed consent of the subjects according to the Helsinki declaration. Clinical and laboratory data from the present study population have been previously published [4,9,13,28].

A final group of 10 subjects with previously detected Trf saturation lower than 20% was used as controls to establish reference values for serum Trf levels in iron deficient conditions.

Biochemical and hematological parameters

Biochemical parameters of iron metabolism (serum iron, serum Trf, Trf saturation and serum ferritin) and hemoglobin were routinely determined in the Clinical Chemistry and Hematology Laboratories of Santo António General Hospital, Porto by standard techniques. Serum iron was analyzed in a Cobas INTEGRA 800 Analyzer (Roche, Switzerland). Serum ferritin and Trf were determined by immunoturbidimetric tests in the same Analyzer. Trf saturation was calculated from the formula: (serum iron × 100) / (serum Trf × 1.41).

Peripheral blood T cell phenotyping

Total lymphocyte counts were determined in an automatic blood cell counter (Coulter Gen S). Leukocytes were stained from samples of freshly collected peripheral blood. A volume of 3 ml of whole blood was fixed with an equal volume of formaldehyde (0.4%) for 4 min at 37°C. The red blood cells were then lysed with 50 ml of lyses solution (10 mM Tris, 0.15 M NH_4Cl, pH 7.4) for 10 min at 37°C. Cells were washed twice in phosphate-buffered saline (PBS) supplemented with 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA). After the final wash, approximately 5×10^6 cells were stained in round-bottom

96-well plates (Greiner-Nurtingen, Germany), at 4°C for 30 min in the dark. The following monoclonal antibodies (moABs) were used: CD3-PerCP, CD4-PE and CD8-FITC (Dako, Copenhagen, Denmark). After the staining, cells were washed twice with PBS-BSA and fixed in a final volume of 500 μl with PBS containing 0.1% paraformaldehyde. At least 2×10^4 lymphocytes were acquired in a FACScan (Becton Dickinson, San Jose, CA, USA) and analyzed using the Lysis II or Cell Quest programs (Becton Dickinson, San Jose, CA, USA). Percentages of $CD3^+CD4^+$and $CD3^+CD8^+$ cells within the lymphocyte population were obtained. The absolute numbers of $CD4^+(CD3^+CD4^+)$ and $CD8^+(CD3^+CD8^+)$ lymphocytes were then calculated from the total lymphocyte counts by the formula: $CD4^+$(or $CD8^+$) cells = $\%CD3^+CD4^+$(or $CD3^+CD8^+$) × total lymphocyte counts ($\times 10^6$/ml) / 100.

Statistical methods

For the purpose of statistical analysis, subjects were divided according to the *HFE* genotype. All values tested represent the results obtained at diagnosis, unless otherwise stated. Group means were compared by the one-way analysis of variance test (ANOVA). Multiple comparisons were performed using the Scheffe test. Student's *t* test was used whenever comparisons were done between two groups.

Trf longitudinal study

Serial data from 16 hemochromatosis patients regularly treated at the Hemochromatosis Outpatient Clinic were used for Trf longitudinal studies. Serum iron Trf and hemoglobin levels were measured regularly from the beginning to the end of the intensive treatment and for at least 2 years thereafter. Presentation of results (Fig. 1A) was done by calculating the average of serum iron and Trf as well as hemoglobin levels at diagnosis, one third and two thirds of the intensive treatment period, at the end of intensive treatment and 6 months, 1 and 2 years after the end of the intensive treatment.

Trf versus lymphocyte numbers

The relationship between Trf and lymphocyte populations was assessed by regression analysis. In addition, a stepwise multiple regression model was used to test the relative impact of other iron related variables, namely Trf saturation, serum iron and serum ferritin, on $CD8^+$ T lymphocyte numbers. Data were analyzed by Statgraphics software (Statgraphics Graphics System, version 7.0).

Results

Serum Trf and HFE mutations

A summary of iron biochemical parameters according to gender and *HFE* genotypes is shown in Table 1. As expected, serum iron and Trf saturation were higher in all *HFE* mutation groups in both genders. Serum Trf levels were significantly lower in all C282Y carriers in comparison to *HFE* wild type subjects, both in males and females. The lowest average values were observed in the groups of C282Y homozygous (180 ± 32 in males and 191 ± 36 in females). In all groups analyzed, serum Trf levels are higher in females than in males (Table 1).

In order to evaluate the impact of iron burden on Trf values, a serial analysis of serum Trf was performed in 16 C282Y homozygous HH patients during the course of phlebotomy treatment (Fig. 1A). In all cases, Trf serum values are low and do not change significantly with the diminishing serum iron levels during the course of intensive phlebotomy treatment. The end of the intensive

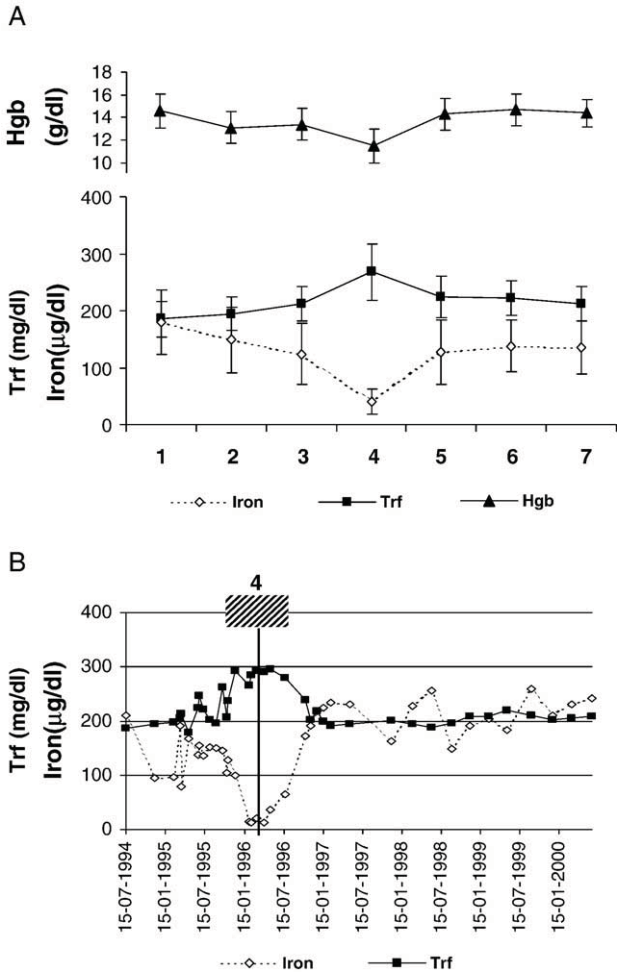

Fig. 1. Variation of serum Trf, iron and hemoglobin levels in C282Y homozygous subjects during the course of intensive and maintenance phlebotomy treatment. (A) Average of Trf, iron and hemoglobin (Hgb) levels ($n = 16$) at 7 time points during phlebotomy treatment: (1) diagnosis; (2) one third of the intensive treatment period; (3) two thirds of the intensive treatment period; (4) end of intensive treatment; (5) 6 months after the end of the intensive treatment; (6) 1 year after the end of the intensive treatment; (7) 2 years after the end of the intensive treatment. (B) Representative example out of 16 cases studied. ▨ Characteristic Trf values of non-hemochromatosis subjects with iron deficiency 368 ± 45.

Table 1
Iron biochemistry according to *HFE* genotype and gender

HFE genotype	Males						Females					
	n	Age	Fe (μg/dl)	TrfSat (%)	Ferritin (ng/ml)	Trf (mg/dl)	*n*	Age	Fe (μg/dl)	TrfSat (%)	Ferritin (ng/ml)	Trf (mg/dl)
C282Y/C282Y	19	47 ± 14	213 ± 37[a]	89 ± 13[a]	1658 ± 455[a]	180 ± 32[a]	15	44 ± 13	192 ± 44[a]	79 ± 15[a]	225 ± 79[a]	191 ± 36[a]
C282Y/wt	53	36 ± 13	113 ± 32	35 ± 11[a]	128 ± 19	233 ± 36[a]	77	41 ± 15	94 ± 36	27 ± 12[a]	46 ± 7	257 ± 44[a]
C282Y/H63D	12	42 ± 18	142 ± 39[a]	50 ± 17[a]	180 ± 16	211 ± 36[a]	13	43 ± 16	118 ± 31[a]	38 ± 9[a]	35 ± 17	224 ± 34[a]
H63D/wt	48	44 ± 16	118 ± 46[a]	35 ± 14[a]	127 ± 22	245 ± 25	60	49 ± 14	96 ± 35	27 ± 12	48 ± 14	263 ± 42[a]
H63D/H63D	12	47 ± 15	149 ± 31[a]	47 ± 18[a]	167 ± 80	242 ± 51	6	47 ± 15	109 ± 21	32 ± 11	27 ± 26	252 ± 47
Wt/wt	56	40 ± 13	100 ± 35	29 ± 11	113 ± 22	253 ± 36	89	44 ± 15	89 ± 31	23 ± 9	34 ± 9	278 ± 49
P (ANOVA)		0.0265	<0.0000	<0.0000	<0.0000	<0.0000		0.0634	<0.0000	<0.0000	<0.0000	<0.0000

Mean ± standard deviation for all parameters except serum ferritin, which is geometric mean ± standard error.
[a] Statistically significant differences between groups of subjects carriers of *HFE* mutations and the group without *HFE* mutations by the multiple range analysis.

treatment in the protocol used is characterized by a decrease in hemoglobin (Hgb) due to iron deficient erythropoiesis. At this point, serum iron levels drop and Trf concentration peaks (see Fig. 1A), but average Trf (268 ± 49) never attains levels comparable to those seen in iron deficient subjects (368 ± 45) at comparable low Trf saturation levels (<20%). During maintenance, lasting on average more than 2 years, Trf levels drop again to levels significantly lower than the ones observed in wild type subjects ($P = 0.00001$). A representative individual example is given in Fig. 1B.

Serum Trf and lymphocyte numbers

When the overall, male and female, population was analyzed, no correlation could be found between serum Trf levels and lymphocyte numbers (both total lymphocyte and $CD8^+$ or $CD4^+$ T lymphocytes). If Trf acts as a limiting resource, however, one could expect that a result would only be seen in the subjects with the lower Trf values. Thus, the population studied was divided into two subpopulations, including males and females according to serum Trf levels. A statistically significant positive correlation was observed between serum Trf and $CD8^+$ lymphocyte numbers for Trf levels less or equal to 248 (median of the Trf levels of the studied population) (Fig. 2A; $R^2 = 2.41$; $r = 0.16$; $P = 0.018$; $n = 233$). No correlation was found between Trf values higher than 248 and $CD8^+$ lymphocyte numbers (Fig. 2B). No correlation was observed for the $CD4^+$ or total lymphocyte numbers (data not shown). In order to clarify the possible effect of other iron related variables in the correlation found between Trf and $CD8^+$ T lymphocyte numbers, a stepwise multiple regression model was run taking as dependent variable the $CD8^+$ T lymphocyte numbers and as independent variables, besides Trf, Trf saturation, serum ferritin and serum iron. The only variable entering this model was Trf ($F = 6.93$; $P = 0.00372$). To confirm further a limiting effect of Trf in the $CD8^+$ T lymphocyte numbers, the subpopulation with the lowest Trf levels namely, male subjects homozygous for the C282Y mutation (Trf: 180 ± 32, Table 1), was also examined. A highly significant positive correlation was observed between serum Trf and $CD8^+$ lymphocyte numbers

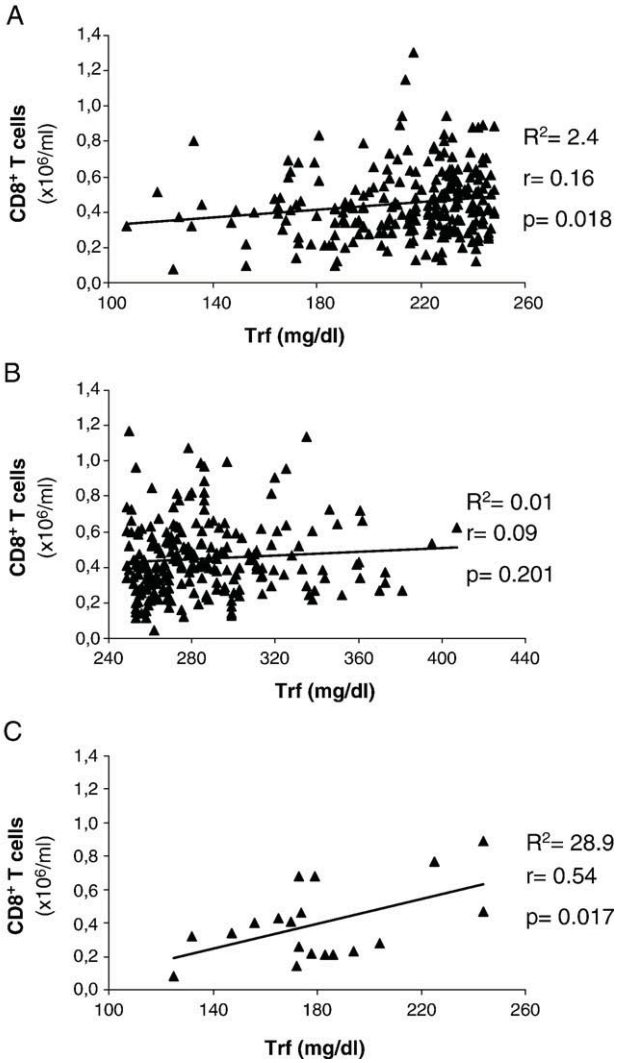

Fig. 2. Regression of $CD8^+$ lymphocyte numbers with serum Trf levels in: (A) subjects with Trf values ≤ 248; (B) subjects with Trf values > 248; (C) male HH subjects C282Y homozygous.

(Fig. 2C, R^2 = 28.9; r = 0.54; P = 0.017). No significant correlations were found, however, between Trf values and either $CD4^+$ or total lymphocyte numbers or in females (data not shown).

Discussion

The question of genotype–phenotype discrepancies in *HFE* hemochromatosis has been the subject of considerable discussion [29–32]. Both from clinical and specifically from experimental work, there is mounting evidence that low $CD8^+$ T lymphocytes could act as indicators of severe iron overload expression [4,8–10,13,14]. Several animal models of increased iron absorption with defective $CD8^+$ T cell or total lymphocyte numbers have demonstrated the modifier effect of those cells in the severity of iron overload, namely single or double knockout mice (MHC class I−/−; β2m−/−Rag1−/−; Hfe−/−Rag1−/−; Hfe−/−β2m−/−) [15,16,33]. $CD8^+$ knockout mice alone do not develop spontaneous iron overload [33]. A more probable link might involve MHC class I expression itself [33,34]. No clues have been offered so far to explain the modifying effect of $CD8^+$ cells in iron overload in humans or explain the lack of correlations seen in females. There is growing evidence, both in human and animal models, that the numbers of lymphocytes are genetically determined [28,35–41]. Recent work has shown that the MHC class I genetic region is involved in this regulation [28]. Other nutritional but at present unidentified factors may be involved that might contribute to the persistent failure to observe any correlation between lymphocyte numbers and iron stores in homozygous female patients.

Trf, however, is a well-known resource for the proliferation of numerous cell types including lymphocytes [20,21]. The effect of a limited resource in vivo in lymphocyte numbers is compatible with the statistically significant positive correlation between serum Trf and numbers of circulating $CD8^+$ cells for subjects with Trf levels lower than 248 mg/dl, described in this paper. Male patients homozygous for the C282Y mutation consistently present lower levels of serum Trf in comparison to female patients. Therefore, it is reasonable to assume that if Trf acts as a limiting factor for lymphocyte numbers, this effect would be particularly manifested in this subgroup. This was the case. It remains unsubstantiated and unexplained in the female patient group. The significantly higher serum Trf levels seen in females, however, may help to explain the differences in iron overload expression between males and females [5–7]. Surprisingly, no correlation between serum Trf levels and $CD4^+$ or total lymphocyte numbers was seen. Such a result brings to light once more the involvement of MHC class I in this disorder [10,28,33]. $CD8^+$ but not $CD4^+$ T lymphocytes are activated and proliferate in the context of MHC class I molecules (reviewed in [17]). Recent results of a separate study of MHC class I expression in C282Y homozygous patients show that the mutant C282Y *HFE* peripheral blood mononuclear cells fail to express MHC class I as abundantly as cells from wild type subjects [34].

The lower serum Trf values observed in the C282Y *HFE* mutant groups could be a result of decreased Trf synthesis. It is classically assumed that this could be due to down-regulation evoked by iron overload. It does not explain, however, the finding that the decreased levels found in HH patients do not correct after treatment. Moreover, lower mean values of Trf are found also in C282Y carriers, without severe iron overload, as shown here and before by other groups [25,26]. HFE itself could mediate this effect. We would like to consider, however, an additional explanation. Trf has been shown sometime ago to circulate from the extracellular pool to the “interstitial fluid pool” and enter the lymph [27]. Passage to the lymph of serum proteins was thought to depend exclusively on protein molecular weight [42] at a time when Trf receptors had not been discovered [27]. More recent work has demonstrated that murine brain and lymph node derived endothelial cell lines have receptors for Trf. The lymph node endothelial cell line was shown to have twice as many Trf receptors as brain derived endothelium [43]. Trf traffic seems therefore to coincide with lymphocyte traffic from blood to lymph [44]. The lymph nodes provide a preferred environment for lymphocyte growth and division restricted in the case of $CD8^+$ lymphocytes by MHC class I alleles. This could provide an explanation for the apparent preferential correlation found in this and previous reports [8,9] of low $CD8^+$ rather than $CD4^+$ cells with severity of iron overload.

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