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THE TRANSFERRIN POLYMORPHISM
POPULATION GENETICS AND ASSOCIATIONS WITH
REPRODUCTIVE HAZARDS AND DISEASE

by

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av

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Abstract

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Human serum transferrin (TF) carries iron from the intestine, reticuloendothelial system and liver parenchymal cells to proliferating cells in the body. TF is highly polymorphic, and previous findings of associations between TF types and reproductive hazards in different species suggest that the TF polymorphism may be involved in natural selection and susceptibility to disease.

The aims of this thesis were:
- to examine the TF polymorphism in Finns, Swedes and Swedish Saamis and variations of TF allele frequencies between 23 North-Swedish subpopulations.
- to study TF types in relation to spontaneous abortion and disease.
- to investigate the relationship between TF types, iron-binding capacity and body iron stores in an attempt to elucidate the mechanism through which TF types may be involved in spontaneous abortion and disease.

The following results were found:

1. Significant allele frequency differences were found between Finns, Swedes and Saamis. Finns had a lower TF*C2 frequency compared to Saamis and Swedes and the highest TF*C3 frequency so far observed in the world. Saamis had a very low TF*C3, and originally they probably lacked this allele. A significant heterogeneity between 23 subpopulations in northern Sweden was found for all TF alleles, and the geographical picture of TF*C3 and rare allele frequencies showed clines, which could be interpreted in terms of Finnish influence.

2. A significantly increased frequency of TF*C2 and especially the TF C2 type was found among mothers with a history of previous spontaneous abortion.

3. In patients with occupational photodermatosis of the face a highly significant increase of TF*C2 was found.

4. A highly significant association was found between TF*C3 and myocardial infarction. There was also an association with the TF C2 type, but with marginal significance.

5. In patients with hereditary hemochromatosis and thus extreme iron overload the frequency of TF*C1 was significantly increased, and this association was more pronounced in HLA-A3 associated hemochromatosis.

6. No significant relationship was found between TF types and serum iron, total iron binding capacity (TIBC), transferrin saturation and serum ferritin, thus the adverse effects of TF C2 and C3 appear to be independent of iron-binding and body iron stores.
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by

CARIN SIKSTRÖM
To Roland,
Anna and Anders
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This thesis is based on the following papers which will be referred to in the text by their Roman numerals:


INTRODUCTION AND AIMS

Human serum transferrin (TF) carries iron from the intestine, reticulo-endothelial system and liver parenchymal cells to proliferating cells in the body. The TF gene is located on 3q21, and the transferrin molecule is the result of an ancient intragenic duplication. TF has been shown to be highly polymorphic with distinct ethnic differences. The considerable frequency variations between human populations and previous findings of associations between TF types and reproductive hazards suggest that the TF polymorphism may be involved in natural selection and susceptibility to disease.

The aims of this thesis were:

- to examine the TF polymorphism in Finns, Swedes and Swedish Saamis, and variations of TF allele frequencies between 23 North-Swedish subpopulations.
- to study TF types in relation to spontaneous abortion and disease.
- to investigate the relationship between TF types, iron-binding capacity and body iron stores in an attempt to elucidate the mechanism through which TF types may be involved in spontaneous abortion and disease.
BACKGROUND

Iron metabolism

Iron is essential for the growth of all higher organisms. Transferrin carries iron from the intestine, the reticuloendothelial system and liver parenchymal cells to proliferating cells in the body. The process of uptake of iron into the tissues is now fairly well understood (for review see 1). Cell surface transferrin receptors bind diferric transferrin. The complex is internalized by receptor-mediated endocytosis to form small nonlysosomal vesicular structures called endosomes. As the pH falls below 5.5 the affinity of iron to transferrin is reduced, and iron is released into the cytosol. The endosome is recycled to the cell surface, and the apotransferrin is released on the return to a physiological pH. Less than 10 minutes are required for the cycle. The mechanism by which iron moves from the endosome to the mitochondria or the storage protein, ferritin, is not known.

When a cell is short of iron, it responds by synthesizing more transferrin receptor protein and thereby increasing the capacity for iron uptake. When a cell is replete with iron, it synthesizes more ferritin protein to store more iron. The synthesis of the transferrin receptor and ferritin is regulated at the mRNA level. The mRNAs for these two proteins have a specific sequence, the iron-responsive element (IRE), in common (2). This iron-responsive sequence is recognized by the iron-responsive element binding protein (IRE-BP). Binding of IRE-BP to the IRE upregulates the biosynthesis of the transferrin receptor and downregulates that of ferritin. Recent studies (2) have revealed that IRE-BP is identical to cytosolic aconitase, an iron-containing protein. Iron free cytosolic aconitase is enzymatically inactive, but active as IRE-BP, whereas on the contrary iron-bound aconitase is enzymatically active but inactive as IRE-BP.

The transferrin molecule

There are numerous studies of the structure and function of transferrins in man and other species, which have been summarized in comprehensive reviews by de Jong et al (1) and Bowman et al (3). A brief summary is given below.

The transferrin family is a class of single chain proteins of about 80 kDa, with two binding sites. The most studied transferrins have been isolated from mammalian sera, from milk (lactoferrin) and from egg (ovotransferrin or conalbumin). In human melanomas a membrane-bound glycoprotein of 97 kDa, which shows a high degree of sequence homology with transferrin, has been found. The human transferrin molecule is a polypeptide chain of 679 amino acid residues and encoded by a gene on chromosome
3q21 close to the gene for the transferrin receptor. There are two homologous domains, the N-terminal domain (residues 1-336) and the C-terminal domain (residues 337-679) with the carbohydrate moieties in the C-terminal domains at positions 413 and 611. A high degree of internal homology between the two domains has led to the hypothesis that vertebrate transferrins originated by gene duplication during evolution.

**Transferrin polymorphism**

Genetic transferrin polymorphism was first described by Smithies (4) using starch gel electrophoresis. In addition to the common serum transferrin variant designated TF C, a series of anodal (B) and cathodal (D) variants have been reported in different populations.

Compilations of population data have shown that C is the most common variant in all populations. Of the B- and D-variants only D1, D CHI, B 0-1 and B 2 have been found in polymorphic frequencies (5). By isoelectric focusing TF C subtypes have been discovered (6, 7). There are three common subvariants TF C1, TF C2 and TF C3 and a series of additional variants, which are relatively rare and show a restricted geographic or ethnic distribution (review Kamboh and Ferrell, 8). TF*C1 is the most frequent allele in all populations and varies between 75 and 79 % in European populations. TF*C2 has been found to vary from 1 % in Apache and Navajo Amerindians to over 30 % in some Indonesian and Asiatic Indian populations. In Europe TF*C2 frequencies between 9 and 20 % have been observed. The range of TF*C3 frequencies in Europeans is 3-7 %, while in most African and Asiatic populations the C3 allele is rare or absent.

![Fig. 1 Schematic representation of transferrin types after PAGIF in the pH range 5-7.](image)

The anode is at the top. The TF types are from left to right: C1, C2, C2-1, C3-1, C3, C3-2, C1-D CHI and B 0-1-C1.
Transferrin types and iron parameters in relation to reproductive hazards and disease

Transferrin types apparently play a selective role in reproduction, and TF alleles have been found to be associated with reduced fertility and spontaneous abortion in mice (9), pigs (10), horses (11), rhesus monkeys (12, 13) and humans (14). In humans an association between TF*C3 and spontaneous abortion has been found (14), and a significantly increased frequency of the TF*C2 allele has been observed among prematurely born infants (15).

A number of statistically significant associations between transferrin types and different disorders have been found in previous investigations. Lange et al (16) observed an increased frequency of TF B types among schizophrenic patients. In patients with rheumatoid arthritis the TF C2 type was significantly increased, and this association was stronger in male patients and patients with a family history of polyarthritis (17). An excess of the TF C3 variant has been observed in patients with renal cell carcinoma, especially in patients with diploid tumor DNA content and clinical stage I (18, 19).

Mitchell and Carcino (20) reported a significantly increased relative risk of multiple myeloma in patients with the TF C1 type. They referred to a study showing an increased iron level in TF C1 individuals (21), and speculated that the increased iron level in TF C1 may increase the risk for immunoproliferative diseases like multiple myeloma.

In a number of epidemiological studies, results have been found which are consistent with the hypothesis that increased body iron stores increase the risk of cancer and general mortality (for review see Stevens, 22). In a recent investigation from Finland Salonen et al (23) reported an association between iron overload and coronary heart disease.

Extreme iron overload has been found in hereditary hemochromatosis, an autosomal recessive disorder characterized by a number of clinical manifestations like hepatic cirrhosis and carcinoma, arthropathy, cardiomyopathy, diabetes mellitus, hypogonadism and skin pigmentation (Ritter, 24). Hereditary hemochromatosis has been found to be associated with HLA-antigens A3 and B14 (24-27). HLA-linked hemochromatosis is a rather common disease, and the gene frequency has been estimated to 7 % in northern Sweden (28).
Transferrin types have been studied in relation to iron-binding in a number of previous investigations. No differences with respect to iron-binding and transport have been found between the electrophoretic variants B, C and D (29). In a study by Eckfeldt et al (30) no significant differences were found between transferrin C subtypes and serum iron, iron-binding capacity, transferrin concentration and percentage transferrin saturation. Wong and Saha (31), studying transferrin types in relation to transferrin levels and total iron-binding capacity (TIBC), found a significantly higher TIBC in individuals with the TF Cl type compared to those of the TFC 2-1 and TFC 2 types. Cleve et al (32) examined transferrin types and iron-binding in two population samples; one from Europe and the other from Africa. In the European sample a significantly higher TF concentration was found in the TFC 1 type compared to that of the TFC 2-1 type. The data published so far indicate a possible relationship between TF types and TIBC.
MATERIAL AND METHODS

Population samples and patients

The samples of conscripts, mothers and newborn infants studied in paper I were all collected at the Umeå University Hospital.

The blood samples from Norrbotten and Västerbotten counties studied in paper III were obtained from conscripts and blood donors. The individuals were distributed according to their place of birth into 23 subpopulations (regions) consisting of one or a group of parishes. The Finnish blood samples were obtained from blood donors from Uleåborg in northern Finland, and the Saamish blood samples from children attending the nomad schools.

The control material in paper IV was conscripts and blood donors from Västerbotten county presented in paper III.

The material of 1330 individuals studied in paper V with respect to TF types and iron parameters was originally sampled in 1986 in connection with the northern Sweden MONICA project (33).

The control sample in paper VI were blood donors from the county of Jämtland collected at the Östersund Hospital.

Descriptions of patients with occupational photodermatosis (II), myocardial infarction (V) and hereditary hemochromatosis (VI) are given in the respective papers.

Laboratory methods

In paper I transferrin C subtypes were studied by polyacrylamid gel isoelectric focusing (PAGIF) in the pH range 3.5-10.0 after desialylation. In the other papers (II-VI) transferrin types were examined by PAGIF at the pH range 5-7 after dilution of the serum samples 10 times with 0.25 % ferrous ammonium sulphate solution. After focusing, the gels were stained with Comassie blue R-250.

Methods for study of other genetics markers, aspartate aminotransferase (ASAT), cholesterol, Lp(a) protein, serum iron, total iron binding capacity (TIBC) and serum ferritin are given in the respective papers.
Statistical methods

Gene frequencies were calculated by gene counting, and departures from the Hardy-Weinberg equilibrium were calculated by the chi square test. In the analysis of differences in phenotype and allele frequencies between population samples and between patients and controls, the chi square with or without Yates correction was used. Differences in quantitative variables were evaluated with the t-test or ANOVA.
RESULTS

Spontaneous abortion (I)
Transferrin C subtypes were studied in 144 women with a history of previous abortion, 347 women without previous abortion and 302 military conscripts. An increased frequency of the TF*C2 allele and especially of the TF C2 type was found among the mothers with a history of previous abortion. There was a significant correlation between the TF C2 type and the placental alkaline phosphatase variant F, which in previous studies has been found to be associated with spontaneous abortion.

Occupational photodermatosis (II)
Among 120 workers at a factory in northern Sweden exposed to photoactive substances 73 developed occupational facial eczema, while 47 showed no such reaction. In an attempt to identify disposing factors 16 genetic marker systems thereamong TF were examined. Among the reactors a highly significant increase of the TF*C2 allele and the C2 variant was found. The association with TF C2 remained significant also after correction for the number of significance tests performed.

Gene frequency variations and ethnic heterogeneity in northern Sweden (III)
Frequencies of TF types and alleles were studied in 315 Finns from northern Finland, 222 Swedish Saamis and 4157 conscripts and blood donors from the counties of Norrbotten and Västerbotten in northern Sweden.

Significant ethnic differences with respect to TF allele frequencies were found in northern Sweden. The frequency of the C1 allele in the Swedish Saamis was significantly higher than that in Finns and in Norrbotten county. Finns had a significantly lower C2 allele frequency compared to that in Saamis and in Norrbotten and Västerbotten counties. The C3 allele displayed highly significant ethnic differences with frequencies of 14 % and 3 % in Finns and Saamis respectively and intermediate values in Norrbotten (9 %) and Västerbotten (5 %) counties. The frequencies of rare alleles, predominantly D CHI and B 0-1, were significantly higher in Finns than in Saamis and in the Norrbotten and Västerbotten counties.

A significant heterogeneity between the 23 regions in northern Sweden was observed for all transferrin alleles. TF*C1, TF*C3 and rare alleles showed clineal variation in northern Sweden. The frequencies of the TF*C1 and TF*C3 alleles were increasing in the southwestern and northeastern direction respectively and that of the rare TF alleles in the northern direction.
Transferrin C subtypes and myocardial infarction (IV)

Transferrin C subtypes were studied in 124 patients (97 males and 27 females) who had survived myocardial infarction and population controls to test the hypothesis that TF C2 is associated with an increased risk for myocardial infarction. The TF C2 showed a significant increase, which however, was rather marginal, while a highly significant increase of the TF*C3 allele and the types carrying the TF C3 variant (C3-1 and C3-2) was found. There were no significant differences between TF types with respect to the levels of the Lp(a) protein and cholesterol. Significantly lower asparte aminotransferase (ASAT) peak values were found in individuals with the TF types C3-1 and C3-2.

Transferrin types, iron-binding capacity and body iron stores (V)

Transferrin types were studied in relation to serum iron, total iron binding capacity (TIBC), transferrin saturation and serum ferritin in a sample of 691 males and 639 females from northern Sweden in an attempt to elucidate whether the adverse somatic and reproductive effects of the TF C2 and C3 variants were associated with increased body iron stores. A highly significant sex difference was found, males showing indications of increased body iron stores, viz. increased levels of serum iron, transferrin saturation and serum ferritin and a decreased TIBC. There was no significant association between the TF C2 variant and parameters indicating iron-binding and storage. In females the TF C3 variant was associated with a significantly lower TIBC value, but this decreased TIBC value was not accompanied by an increased ferritin value and thus no unequivocal indicator of increased body iron stores. Thus the adverse somatic and reproductive effects of the TF C2 and C3 variants appear to be independent of iron-binding and body iron stores.

Transferrin types and expression of hereditary hemochromatosis (VI)

Transferrin types were studied in 68 unrelated patients with hemochromatosis (51 males and 17 females) and 297 blood donor controls. The distribution of TF phenotypes and alleles in the patients were significantly different from those in the controls. The TF C1 type was significantly increased in the patients and more pronounced among HLA-A3 positive patients. Thus it appears that the TF C1 type may promote the clinical expression of hereditary hemochromatosis, and that effect is more pronounced in HLA-A3 associated hemochromatosis.
DISCUSSION

The variable frequencies of transferrin alleles between human populations suggest that the transferrin polymorphism is balanced and maintained by natural selection.

Some transferrin variants with restricted geographical or ethnic distribution are useful markers for studies of migration and admixture. The electrophoretic variants TF DCHI and B 0-1 have e.g. been used as markers of Finnish influence in northern Sweden (34, 35). In the course of this work we have discovered another useful Finnish marker, the TF*C3 allele (III). Most European populations have TF*C3 allele frequencies between 5 and 7 %. We found a high TF*C3 frequency in northern Finland (14 %), and Tenkanen et al (36) reported a TF*C3 frequency of 13 % from southern Finland. Thus the Finns have the highest TF*C3 frequency so far in Europe and the world. In the regions in northern Sweden along the Finnish border high TF*C3 frequencies were found in agreement with the Finnish influence known to exist in these regions. Other transferrin markers of eastern influence e.g. TF*DCHI and B 0-1 appear to be of Asiatic Mongoloid origin. TF*C3 on the other hand is absent or almost absent in Asiatic Mongoloid populations and may therefore be a marker for Finno-Ugrian populations. To elucidate this question further studies of other Finno-Ugric speaking peoples like the Estonians are desirable.

The low TF*C3 frequency among the Swedish Saamis (0.0315) found in this study (III) has been confirmed by Sikström and Nylander (37) in an additional sample of 153 Saamis. They found a still lower TF*C3 frequency (0.0131) and concluded that the very low TF*C3 frequency in the Saamis suggests that they originally lacked this allele. The present low frequency is readily explained by the Finnish admixture known to exist in Saamis (38).

The frequencies of all transferrin alleles showed significant variations between the 23 regions, but only those of TF*C3 and of the rare alleles TF*D CHI and B 0-1 were explainable in terms of ethnic influence. These alleles are markers of Finnish influence in northern Sweden, and significant correlations (P < 0.001) have been found between the frequencies of these markers in the 23 regions in northern Sweden (35).

Among women with a history of previous spontaneous abortion we observed an increased frequency of the TF*C2 allele and the TF C2 variant (I). Both the TF C2-1 and the TF C2 type were increased, but the increase of TF C2 was more pronounced statistically. Furthermore a correlation was found between the maternal TF C2 type and
the placental alkaline phosphatase variant F, which in previous studies has been found to be associated with spontaneous abortion. Thus there appears to exist an intrauterine selection mainly against fetuses with the TF C2 type, and perhaps to some extent also against fetuses with the TF C2-1 type. When this study was performed the technique for separation of the TF C3 variant was not known. On retyping of this material for TF C3, I found no significant association between this variant and spontaneous abortion. The association between TF C2 and spontaneous abortion has been confirmed by Saha et al (39) in a Chinese population. The negative effect of TF C2 in pregnancy is further substantiated by the finding by Auconi et al (15) of a highly significant increase of this phenotype among extremely premature infants. No correlations have, however, been found between TF types and birth weight variations in the normal range (39, 40). The associations with spontaneous abortion and prematurity in humans and the findings of reproductive hazards associated with TF types in other species (9-13) provide considerable evidence for the involvement of TF types in intrauterine selection.

The finding of an association between TF C2 and occupational facial eczema (III) was unexpected. The fact that only some of the workers exposed to phototoxig substances developed occupational facial eczema suggested that they had a susceptibility of some kind. Altogether 16 different genetic marker systems were examined in an explorative study with no explicit hypotheses other than that HLA- and complement factors might be involved in an underlying allergic mechanism. A strong association with TF C2, was found which remained significant also after correction for the number of associations tested. In our study individuals with or without eczematous reaction were compared. If the reactors are compared with a population control (the Västerbotten material from paper III) with respect to the frequencies of the TF C2 type and the TF*C2 allele highly significant differences ($P = 1 \cdot 10^{-4}$ and $4 \cdot 10^{-5}$, respectively) are found. On the basis of the findings in this study a hypothesis was formulated that the transferrin variant C2 may act as an enhancer of cyto- and genotoxic damage, possibly by influencing the concentration or distribution of iron, and thereby the formation of oxygen free radicals. Since oxygen free radicals are believed to play a role in the pathogenesis of rheumatoid arthritis, it was predicted that the TF C2 type would be associated with rheumatoid arthritis, which also turned out to be the case (17). Furthermore it was predicted that individuals with the TF C2 type would be more susceptible to chromosomal damage when exposed to ionizing radiation. A study of chromosomal aberrations in irradiated short-term cultivated lymphocytes gave same suggestive evidence to support this hypothesis (41).
The study of transferrin C subtypes in myocardial infarction (IV) was undertaken in an attempt to test the hypothesis that TF C2 is associated with an increased risk for myocardial infarction. In agreement with the hypothesis an increased frequency of the TF C2 was found among the patients with myocardial infarction. However, in addition an unexpected and highly significant increase of the TF*C3 allele and of individuals carrying the TF C3 variant (TF C3-1 and TF C3-2) was observed. Furthermore the over-represented TF types had significantly lower ASAT peak values which indicates that they had smaller infarctions. As discussed in paper IV this is, however, unlikely to be due to an increased survival of individuals carrying the TF C3 variant. Another interesting circumstance was that the highest frequency of the TF*C3 so far has been found in the Finnish population, which is known to have a high rate of cardiovascular disease.

In hereditary hemochromatosis the excessive iron overload is caused by an enhanced iron absorption from the small intestine, but the exact nature of the mechanism has not been clarified. The phenotypic expression of the underlying mechanism can apparently be modified by a number of factors like diet, sex and conceivably also by variations in the transferrin-transferrin receptor mechanism. When patients with hereditary hemochromatosis from the county of Jämtland were compared with blood donor controls from the same population a significant increase of the TF C1 type was found among the patients (VI). These results indicate that the TF C1 type may promote the clinical expression of hereditary hemochromatosis.

As the pathogenic effect of increased body iron stores is well established, it has been hypothesized that the adverse somatic and reproductive effects of TF C2 and TF C3 may be due to increased iron transportation and body iron stores (II, IV, VI). The results from previous investigations (31, 32) suggested that TF C2 may be associated with a lower TIBC, which is an indicator of increased body iron stores. The previous studies have, however, not been conclusive and there have been no attempts to study the relationship between transferrin types and ferritin levels. Our study (V) included the serum levels of iron, transferrin and ferritin in a large material, and males and females were analyzed separately. Thus the power to detect an association between TF types and body iron stores was considerable, but in spite of this we failed to demonstrate any consistent and statistically significant association between TF types and parameters indicating iron-binding and storage. The significantly decreased TIBC levels in females carrying the TF C3 variant are hard to explain. Since they were not accompanied by increased ferritin values they can not be interpreted as signs of increased body iron...
stores. Altogether the results indicate that the differences between TF variants with respect to iron-binding and body iron stores are small or non-existent. Even rather small differences between transferrin variants in iron transporting capacity may, however, over long periods of time lead to differences in iron storage, especially in combination with a drastically increased iron absorption as in hereditary hemochromatosis. Thus results from the study of hereditary hemochromatosis indicate that the TF C1 variant may have a slightly increased transporting capacity.

The adverse somatic and reproductive effects associated with the TF variants C2 and C3 are apparently not caused by increased body iron stores. The pathogenic mechanism may therefore depend of some hitherto unidentified function of TF. Another hypothesis is that the TF*C2 and C3 alleles are in linkage disequilibrium with one or a couple of pathogenic loci (e.g. the TF receptor locus). Future studies of associations between TF and TF receptor alleles will be facilitated by our recent discovery of DNA markers at the TF receptor locus (Sikström and Beckman, 42).
SUMMARY

Human serum transferrin carries iron from the intestine, reticuloendothelial system and liver parenchymal cells to proliferating cells in the body. TF is highly polymorphic, and previous findings of associations between TF types and reproductive hazards in different species suggest that the TF polymorphism may be involved in natural selection and susceptibility to disease.

The aims of this thesis were:

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The following results were found:

1. Significant allele frequency differences were found between Finns, Swedes and Saamis. Finns had a lower TF*C2 frequency compared to Saamis and Swedes and the highest TF*C3 frequency so far observed in the world. Saamis had a very low TF*C3, and originally they probably lacked this allele. A significant heterogeneity between 23 subpopulations in northern Sweden was found for all TF alleles, and the geographical picture of TF*C3 and rare allele frequencies showed clines, which could be interpreted in terms of Finnish influence.

2. A significantly increased frequency of TF*C2 and especially the TF C2 type was found among mothers with a history of previous spontaneous abortion.

3. In patients with occupational photodermatosis of the face a highly significant increase of TF*C2 was found.
4. A highly significant association was found between TF*C3 and myocardial infarction. There was also an association with the TF C2 type, but with marginal significance.

5. In patients with hereditary hemochromatosis and thus extreme iron overload the frequency of TF*C1 was significantly increased, and this association was more pronounced in HLA-A3 associated hemochromatosis.

6. No significant relationship was found between TF types and serum iron, total iron binding capacity (TIBC), transferrin saturation and serum ferritin, thus the adverse effects of TF C2 and C3 appear to be independent of iron-binding and body iron stores.
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