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#### Genetic determinants of iron metabolism plasma phenotypes and their relationship with risk of thrombosis

This study evaluates the relative contributions of genetic and environmental factors to 6 iron-related phenotypes and the relationship between these phenotypes and the risk of thrombosis. All of these phenotypes were influenced significantly by genetics, with heritability ranging between 18% for transferrin saturation to 55% for soluble transferrin receptor (sTfR). Only sTfR exhibited a significant genetic correlation with thrombosis.

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The physiologic and biochemical pathways involved in iron metabolism are very complex. Given the spectrum of iron metabolism-related phenotypes, it is likely that there are a number of interacting genetic and environmental factors that jointly determine the variable expression. The primary objective of the present investigation was to examine the relative roles of genetic and environmental factors in determining iron-related phenotypes. To this end, we studied a series of extended Spanish kindreds, half of which were enrolled through individuals with thrombophilia. They were recruited in a project called GAIT (Genetic Analysis of Idiopathic Thrombophilia) designed to look for new genetic risk factors for thrombosis through the analysis of intermediate phenotypes.<sup>1-3</sup> Recent data on a possible association between the hemochromatosis gene mutation HFE Cys282Tyr and cardiovascular diseases<sup>4-6</sup> motivated us to include iron metabolism-related phenotypes in the GAIT Project. In 1981, Sullivan proposed a possible relationship between iron and ischemic heart disease. He hypothesized that iron depletion protects against myocardial infarction and could explain the sex differences in the rates of heart disease.<sup>7</sup> The ascertainment criteria for our families also allowed us to study the potential relationship of iron metabolism phenotypes with thromboembolic disease.

The details on the enrollment of families and statistical methods used in the GAIT Project can be found elsewhere.<sup>1-3</sup> Plasma iron, unsaturated iron binding capacity (UIBC) and soluble transferrin receptor (sTfR) were measured in a Hitachi 911 biochemical analyzer by colorimetric and immunoassay methods, respectively (Fe, UIBC and sTfR Tinaquant). Total iron binding capacity (TIBC) was obtained as the sum of plasma iron and UIBC. Circulating ferritin was evaluated using an electrochemiluminescent immunoassay (Ferritina) in a biochemical analyzer (ELECSYS). All assays and the analyzer were from Roche Diagnostics (Mannheim, Germany). Transferrin saturation (SAT) was obtained by dividing iron values by TIBC values and was expressed as a percentage. Intra- and inter-assay coefficients of variation were less than 10% for all of the phenotypes. We logarithmically (ln) transformed the values of sTfR and ferritin to obtain normal distributions.

Table 1 presents the estimated components of the residual variance for the iron metabolism-related phenotypes. All of the traits had significant heritability ( $h^2$ ), ranging between 18% and 55% of the residual phenotypic variability. The proportion of the residual phenotypic variability accounted for by shared household effects ( $\chi^2$ ) tended to be considerably smaller than that accounted for by genetic effects. Household effects were significant for only 2 traits: ferritin and SAT. The remaining variance (not accounted for in Table 1) was attributed to individual-specific random environmental influences and random error.

Table 2 shows the results of bivariate genetic analyses of thrombosis with each of the quantitative iron metabolism-

**Table 1. Components of variance from the most parsimonious model ± standard errors.**

Variable	Heritability ( $h^2$ )	Household ( $\chi^2$ ) effect
Ln sTfR	0.55±0.10*	
TIBC	0.39±0.09*	
UIBC	0.33±0.09*	
Ln ferritin	0.20±0.12 <sup>‡</sup>	0.23±0.08 <sup>#</sup>
Plasma iron	0.19±0.08 <sup>‡</sup>	
SAT	0.18±0.09 <sup>‡</sup>	0.11±0.07 <sup>†</sup>
Liability to thrombosis <sup>†</sup>	0.61±0.16 <sup>#</sup>	

\* $p < 0.000001$ ; <sup>†</sup> $p < 0.001$ ; <sup>‡</sup> $p = 0.001$ ; <sup>§</sup> $p < 0.05$ ; <sup>††</sup> $p = 0.05$ .  
<sup>†</sup>See Souto et al., reference 3.

**Table 2. Phenotypic, genetic and environmental correlations of the iron metabolism-related phenotypes with a liability to thrombosis.**

Phenotype	$\rho_p$	$p$	$\rho_g$	$p$	$\rho_e$	$p$
Ln sTfR	0.316	0.0003	0.415	0.05	0.212	0.28
TIBC	0.146	0.11	0.069	0.77	0.212	0.26
UIBC	0.210	0.02	0.229	0.31	0.207	0.27
Ln Ferritin	-0.156	0.12	0.108	0.69	-0.334	0.04
Plasma iron	-0.205	0.03	-0.217	0.47	-0.220	0.19
SAT	-0.241	0.008	-0.241	0.38	-0.257	0.12

$\rho_p$ : phenotypic correlation;  $\rho_g$ : genetic correlation;  $\rho_e$ : environmental correlation. The phenotypic correlation ( $\rho_p$ ) was derived from the two constituent correlations ( $\rho_g$  and  $\rho_e$ ) and the heritabilities ( $h^2_1$  and  $h^2_2$ ) of the traits, where trait 2 is liability - or risk - of thrombosis:

$$\rho_p = \rho_g \sqrt{(h^2_1 h^2_2)} + \rho_e \sqrt{(1 - h^2_1) \div (1 - h^2_2)}$$

For a detailed explanation see references 2 and 3.

related traits. We had previously estimated that liability or susceptibility to thrombosis is a quantitative phenotype with a high heritable component of 61%.<sup>3</sup> It is noteworthy that sTfR was genetically correlated with liability to thrombosis ( $\rho_g = 0.415$ ,  $p = 0.05$ ). Significant environmental correlation was observed only for ferritin ( $\rho_e = -0.334$ ,  $p = 0.04$ ). The significant genetic correlation provides evidence for pleiotropic genes contributing to the covariation between sTfR and thrombotic risk.

This systematic family study was dedicated to assessing the role of genes on quantitative variability of iron-related phenotypes. Very few data on this topic have been published. Previously, using segregation analysis in African families, Moyo et al.

demonstrated a genetic influence on serum ferritin, SAT and UIBC. However, they did not report heritability estimates.<sup>8</sup> Our study clearly demonstrates that genetic factors influence the variability of all the iron-related phenotypes. In fact, we found that heredity was the largest determinant of the quantitative variation in these traits. In the GAIT Project, we found that several hemostasis-related phenotypes were genetically correlated with thrombotic risk.<sup>2,3</sup> The present report adds another plasma phenotype - the sTfR - to those described previously. However, the positive genetic correlation between sTfR and thrombosis and the negative environmental correlation between ferritin and thrombosis suggest a relationship between iron deficiency and thromboembolic disease - just the opposite of the previous hypothesis.<sup>7</sup> In any case, the genetic correlation that we found between sTfR and thrombotic risk is thought-provoking because it opens a new door in the investigation of the pathophysiology of thrombosis. Armed with these results, a search for genes that determine variability in sTfR should yield new candidate genes to explain idiopathic thromboembolic disease. Beyond the hypothetical implications for cardiovascular disease, knowledge of the genetic basis of iron metabolism-related phenotypes should be useful in managing a broad spectrum of iron-related diseases.

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### Feasibility of idiotype vaccination in relapsed B-cell malignancies

Feasibility of idiotype vaccination was statistically compared among five different B-cell malignancies in first relapse. When based on hybridoma production techniques, idiotypic vaccination for relapsed B-cell malignancies was consistently feasible only in follicular lymphoma patients, whereas the main cause of failure in other settings was the short survival of idiotype-producing hybridomas.

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With two ongoing, phase-III clinical trials enrolling newly-diagnosed follicular lymphoma (FL) patients, idiotypic vaccination<sup>1</sup> is approaching the final stage of its clinical development, that of demonstrating a possible benefit to patients. However, even in the event that either or both ongoing clinical trials succeed, a number of relevant questions would still remain unanswered, in particular whether idiotype (Id) vaccines may be feasible for most if not all relapsed FL and for some other B-cell malignancies.

An interim analysis was performed of all Id vaccine clinical trials currently ongoing at our institution based on a single, major endpoint, that is actual ability to administer an Id vaccine according to intention-to-treat. Inclusion criteria common to all cases were that the Id vaccine production attempt was carried out only at the time of pathologically-confirmed first relapse and that there was a prior, formal demonstration of the presence of a complete, clonal and tumor-specific immunoglobulin on the tumor cell surface. Furthermore, all Id vaccine production attempts were carried out by the same personnel and always using the same fusion partner (K6H6/B5, i.e. ATCC number: CRL-1823), according to the standard tumor/hetero-hybridoma fusion-based method previously described.<sup>2-5</sup> All patients received the chemotherapy regimen currently in use at our institution for their respective disease in first relapse (Table 1). Patients with FL, mantle cell lymphoma and small lymphocytic lymphoma were supposed to receive Id vaccine treatment if they achieved either complete (CR) or partial (PR) response, while patients with either diffuse large cell or Burkitt's lymphoma were supposed to receive Id vaccine treatment only if they achieved a CR.

Id vaccine treatment unfeasibility was evaluated as (i) related to induction treatment, if the salvage therapy did not induce a response sufficient to proceed with Id vaccination, (ii) fusion-related, if sufficient Id could not be generated to make the vaccine, or (iii) overall. Fusion-related feasibility was evaluated by taking into consideration both its potential causes of failure: short hybridoma survival and loss of Id production.

The feasibility of Id vaccination in relation to induction treatment was markedly different, being 80%-100% in indolent NHL subtypes and 40%-50% in aggressive ones. This difference was not, however, due to an overall lack of efficacy of the respec-

**Table 1. Induction treatment-related Id vaccine feasibility.**

Diagnosis	Number of patients	Induction treatment	Number of CR/PR	Induction treatment-related feasibility
FL	15	CHOP×6	<u>9/5</u>	14/15 (93%)
MCL	5	R-HyperCVAD ×8	<u>2/2</u>	4/5 (80%)
SLL	5	FMC×6	<u>4/1</u>	5/5 (100%)
DLCL	5	mini BEAM×3 + BEAM + ABMT	<u>2/2</u>	2/5 (40%)
BL	4	mini BEAM×3 + BEAM + ABMT	<u>2/1</u>	2/4 (50%)
All but FL	19	See above	<u>10/3</u> + 3	13/19 (68%)
MCL+SLL	10	See above	<u>6/3</u>	9/10 (90%)
DLCL+BL	9	See above	<u>4/3</u>	4/9 (44%)

*Underlined CR and PR numbers refer to the cases for which subsequent Id vaccination was ethically acceptable according to the respective clinical trial protocols.*

tive chemotherapy regimens, but rather to the different eligibility criteria for Id vaccination following chemotherapy. In fact, the overall response to induction treatment for aggressive NHL was 75%-80% (CR+PR), but in this group only patients achieving CR were considered eligible to receive Id vaccination. A far more important factor that halted treatment was Id-secreting hybridoma production (Table 2). Fusion experiments were successful in most FL cases at the very first attempt, whereas in other NHL cases, irrespective of the ultimate Id production outcome, as many as 5 attempts had to be carried out most of the time. Similarly, in most FL cases, the average number of successful fusion wells per 96-well plate was well above 15, whereas that of most of the other NHL cases was typically lower than five.

Statistically significant differences in fusion-related and overall feasibility were found between cases of FL and those of all other NHL, indolent and aggressive lymphoma, respectively (Table 2). Both fusion-related and overall feasibility of the Id vaccine treatment for FL in first relapse were comparable with those already described for both newly-diagnosed and relapsed patients with the same disease.<sup>3-5</sup> Interestingly, the Id vaccine production success rate was substantially low in cases of mantle cell lymphoma (MCL), as opposed to what has been preliminarily described with the very same method in newly-diagnosed MCL patients.<sup>6</sup> This apparent discrepancy could be due, at least in theory, to MCL cells at first relapse biologically resembling those of aggressive NHL rather than FL clones, with obvious possible repercussions on the fusion process.