ELECTRONIC LETTER

Genetic correlation between plasma levels of C4BP isoforms containing β chains and susceptibility to thrombosis

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Thrombosis is a complex trait with both genetic and environmental components.¹ In general, disease status is a qualitative trait where individuals are diagnosed as affected or unaffected, but it is now accepted that underlying the disease there is a continuum trait termed liability, susceptibility, or risk. Liability cannot be measured directly, but it can be modelled and estimated. Disease results when an individual's liability is above a critical value or threshold, whereas liability values below the threshold correspond to healthy individuals. These threshold models of an underlying continuous scale of risk allow inferences that are compatible with current models of gene action.²

Quantitative plasma phenotypes, such as those related to haemostasis, are also complex traits, whose regulation depends on multiple genetic and environmental factors.³ Classical statistical methods are unable to quantify or partition the genetic and environmental factors determining the variability in such complex traits. However, variance component methods have been developed which allow the examination of sources of correlation between quantitative physiological measures and disease outcomes.⁴ These statistical genetic methods also permit the localisation and evaluation of the relative effects of the genes involved.⁵

We have applied these methods to the Genetic Analysis of Idiopathic Thrombophilia Project (GAIT Project) to characterise the genetic determinants responsible for idiopathic thrombophilia. Using a family based approach, we estimated that idiopathic thrombophilia has a heritability of 0.61, indicating that 61% of variation in liability to thrombosis at the population level can be attributed to genetic factors.⁶ Phenotypic correlations were also evaluated between 27 plasma phenotypes related to haemostasis and thrombosis liability.⁶ It was found that genetic factors were mainly responsible for these phenotypic correlations,⁶ indicating that some of the genes that regulate quantitative variation in these plasma phenotypes also affect the risk of thrombosis. These pleiotropic effects have been successfully exploited to improve the power to detect the QTL contributing to thrombotic risk.⁷⁻⁹

C4BP is a plasma protein composed of two polypeptides (α and β chains) which form three plasma oligomers ($\alpha_7\beta_1$, $\alpha_7\beta_0$ and $\alpha_6\beta_1$) with different subunit compositions.¹⁰ The C4BP β^+ isoforms bind free protein S (PS) with high affinity, blocking the PS anticoagulatory cofactor activity of the activated protein C (APC) pathway.¹⁰⁻¹² Thus, free PS, but not C4BP β^+ bound PS, is functional as an APC cofactor. Since free PS deficiency is a risk factor for thrombosis, it has been suggested that increased levels of C4BP β^+ isoforms might affect the susceptibility to thrombosis by decreasing the plasma levels of free PS.¹¹⁻¹⁴ We have reported previously that the variation in the C4BP β^+ isoforms¹⁵ and the free PS¹⁶ plasma levels are genetically regulated, with heritabilities of 42% and 46%, respectively. Moreover, the free PS plasma levels show genetic linkage (lod score = 4.07; nominal

Key points

- We designed a family based study using a variance component method that allows the examination of sources of correlation between continuous physiological measures and discrete disease outcomes. The method also allows for the quantification of the genetic and environmental factors underlying the phenotypic correlation between traits.
- We applied these methods to the Genetic Analysis of Idiopathic Thrombophilia Project (GAIT) to characterise the genetic determinants responsible for idiopathic thrombophilia.
- The C4BP β^+ isoforms exhibited significant phenotypic ($\rho_p = 0.27$; $p = 0.03$) and genetic ($\rho_g = 1$; $p < 0.0001$) correlations with thrombosis. Interestingly, free and functional protein S (PS) showed no correlation with the disease.
- Our data demonstrate the existence of a phenotypic correlation between C4BP β^+ and thrombosis that is most probably due to common genetic factors regulating both traits rather than a consequence of an effect of C4BP β^+ on the free and functional PS plasma levels.
- The C4BP β^+ trait is a new thrombosis related phenotype that should help to identify the quantitative trait locus (QTL) influencing the genetic liability to thrombotic disease.

$p < 0.0001$) with the chromosome region 1q32, which contains the genes encoding the α and β chains of C4BP.¹⁷

In this study we have applied the variance component method to assess the phenotypic, genetic and environmental correlations between total C4BP, C4BP β^+ isoforms, total PS, free PS, functional PS plasma levels, and the thrombotic liability. The analyses revealed that plasma levels of C4BP β^+ isoforms show a phenotypic correlation with thrombosis, and that this correlation is due to a pleiotropic regulation on both traits rather than an effect of C4BP β^+ on the plasma levels of free PS.

METHODS

Sample and phenotypes

The GAIT Project is composed of 21 extended families, 12 of which were ascertained through a proband with idiopathic

Abbreviations: APC, activated protein C; GAIT Project, Genetic Analysis of Idiopathic Thrombophilia Project; PS, protein S; QTL, quantitative trait locus

thrombophilia and nine of which were obtained randomly. Due to the lack of plasma samples in one of our thrombophilic pedigrees we only analysed 20 GAIT families, consisting of 358 individuals. Thrombophilia was defined as multiple thrombotic events, a single spontaneous episode with a first degree relative also affected, or onset of thrombosis younger than 45 years. Diagnoses of the thrombophilic probands were verified objectively. Thrombosis in an individual was considered idiopathic because of the exclusion of all biological causes of thrombosis at the time of recruitment (1995–97), including antithrombin deficiency, PS and PC deficiencies, activated PC resistance, plasminogen deficiency, heparin cofactor II deficiency, Factor V Leyden, dysfibrinogenemia, lupus anticoagulant, and antiphospholipid antibodies. In this sample, 45 individuals had venous or arterial thrombosis, 39 of them in the families ascertained through a thrombophilic proband and six of them in the randomly ascertained families. There were more affected women ($n = 24$, 53.3%) than men ($n = 21$, 46.7%) and the age at diagnosis of first thrombosis ranged from 12 to 76 years, with a mean of 44.8 years. Deep vein thrombosis was the most common condition ($n = 23$) and superficial thrombophlebitis was the second most common ($n = 10$) (table 1).

The recruitment, sampling, collection of medical history and lifestyle, and phenotyping methods used in the GAIT Project have been extensively described in previous publications.^{6–16} Total and free PS were measured with ELISA kits from Diagnostica Stago (Asnieres-Sur-Seine; France). Functional PS (APC cofactor activity) was assayed in an STA automated coagulometer (Boehringer Mannheim) with a kit from Diagnostica Stago. Total C4BP and C4BP β^+ isoforms plasma levels were measured with a home made ELISA method, as previously described.¹⁸

All procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau. Adult subjects gave informed consent for themselves and for their children.

Statistical analysis

The susceptibility to thrombosis was modelled as a threshold process using a pedigree based maximum likelihood method.¹⁹ The threshold is placed in an age and sex specific manner to produce the appropriate population prevalence. To use a threshold model, some weak assumptions regarding the form of the underlying continuous process are necessary. For genetic modelling, we assumed that the underlying liability distribution was normal, and we calculated the joint probability of observing the disease statuses of family

members by using a multivariate normal distribution that allowed for correlations among family members.

To study the relationships between thrombosis liability and quantitative variation in the plasma parameters, we used a mixed discrete/continuous trait variance component analysis.⁴ This analysis allowed the decomposition of phenotypic correlations into factors caused by common genetic influences (pleiotropy) and common environmental influences on the two traits. All statistical genetic analyses were performed using the computer package SOLAR.⁵ All hypotheses were tested using likelihood ratio test statistics.^{20–21} Age, sex, smoking habits, and the use of oral contraceptives were used as covariates in the analyses, based on our previous reports.^{15–17}

Because 11 of the 20 pedigrees were ascertained through a thrombophilic proband, an ascertainment correction was included to allow an unbiased estimation of parameters relevant to the general population. To achieve this, the likelihood for each family ascertained through a thrombophilic proband was conditioned on the phenotype of the proband.^{22–23}

RESULTS

This is the first study that evaluated the phenotypic, genetic, and environmental correlations between thrombosis susceptibility and total C4BP, C4BP β^+ isoforms, total PS, free PS and functional PS plasma levels. The results are summarised in table 2. The C4BP β^+ plasma levels and the thrombosis liability showed a positive phenotypic correlation ($\rho_p = 0.27$; $p = 0.03$), indicating that the C4BP β^+ plasma levels tend to be increased in affected individuals. More importantly, the genetic correlation between C4BP β^+ and thrombosis was very high ($\rho_g = 1$; $p < 0.0001$) (ρ_g between 0.55 and 1 for the 95% confidence interval). C4BP β^+ and thrombosis showed a marginally significant negative environmental correlation ($\rho_e = -0.27$; $p = 0.09$), which suggests that there are common environmental factors exerting opposite effects in the C4BP β^+ levels and the thrombotic susceptibility. Total C4BP plasma level showed no statistically significant phenotypic correlation with thrombosis susceptibility, but it was genetically correlated with the disease ($\rho_g = 0.54$; $p = 0.02$).

Another important observation is that total, free and functional PS plasma levels lack phenotypic, genetic and environmental correlation with thrombosis (table 2). These data suggest that variation in the free PS plasma levels and in the PS APC cofactor activity (functional PS) are unrelated to thrombotic susceptibility in our sample.

Table 1 Number and percent of individuals in each diagnostic category of thrombosis and age at diagnosis

Diagnosis	N (% of individuals)	Mean age at diagnosis (years)
Venous thrombosis		
Deep vein thrombosis	23 (51.1)	41.0
Pulmonary embolism	8 (17.8)	46.6
Superficial thrombophlebitis	10 (22.2)	38.8
Other venous thrombosis	3 (6.7)	58.0
Any venous thrombosis	33 (73.3)	40.5
Arterial thrombosis		
Myocardial infarction	3 (6.7)	65.3
Angina pectoris	4 (8.9)	57.3
Ischaemic stroke	6 (13.3)	61.0
Transient ischaemic attack	4 (8.9)	52.3
Any arterial thrombosis	15 (33.3)	59.9
Any thrombosis	45 (100)	44.8

Note: some individuals are included in more than one diagnostic category.

Table 2 Phenotypic, genetic and environmental correlations between quantitative plasma phenotype and thrombotic liability

Phenotype	ρ_p (p value)	ρ_g (p value)	ρ_e (p value)
Total C4BP	0.18 (0.07)	0.54 (0.02)	-0.13 (ns)
C4BPβ ⁺	0.27 (0.03)	1.00 (<<0.0001) [†]	-0.27 (0.09)
Total PS	0.12 (ns)	0.26 (ns)	-0.02 (ns)
Free PS	0.07 (ns)	0.35 (0.07)	-0.29 (ns)
Functional PS	-0.18 (ns)	-0.09 (ns)	-0.30 (ns)
APCR‡	-0.23 (0.0003)	-0.65 (<<0.0001)	0.66 (<<0.0001)
FVIII‡	0.28 (0.0002)	0.68 (0.0005)	-0.12 (ns)
t-PA‡	0.18 (0.0002)	0.75 (0.007)	-0.10 (ns)
vWF‡	0.26 (0.0001)	0.72 (0.0005)	-0.18 (ns)
R1§	-0.31 (0.0006)	-1.00 (<<0.0001)	0.38 (0.06)

P values for $\rho \neq 0$ are shown in parenthesis; ns, non-significant ($p > 0.1$); ρ_p , phenotypic correlation; ρ_g , genetic correlation; ρ_e , environmental correlation; [†]The 95% confidence interval of the genetic correlation between C4BPβ⁺ and thrombosis is (0.55–1). [‡]Data extracted from Souto et al.⁶ showing the four phenotypes with the highest phenotypic and genetic correlations with thrombosis from a panel of 40 haemostasis related phenotypes. [§]Thromboplastin thrombomodulin mediated time²⁰. APCR, Activated Protein C Resistance; FVIII, Factor VIII; t-PA, tissue plasminogen activator; vWF, von Willebrand Factor.

As a whole, these data indicate that there are common genetic factors (pleiotropy) underlying variation of both C4BPβ⁺ plasma levels and thrombotic liability.

DISCUSSION

In this study, we have used a variance component method to decompose the phenotypic correlations between thrombosis liability and the C4BP and PS plasma phenotypes into genetic and environmental components. The positive phenotypic correlation ($\rho_p = 0.27$) between C4BPβ⁺ plasma level and the thrombotic liability that we found indicates that affected individuals tend to have increased levels of C4BPβ⁺, but this does not necessarily imply a causal relationship between both traits. The genetic correlation between C4BPβ⁺ and thrombosis is extremely high ($\rho_g = 1$; 95% confident interval 0.55–1). Notably, this is the highest genetic correlation that we have obtained after testing for pleiotropy among thrombotic liability and 40 quantitative haemostasis phenotypes,^{6, 24} some of which are widely accepted thrombotic risk factors^{25–27} (table 2 includes some of these previously published results for comparison). Thus, the data presented here provide strong evidence that the C4BPβ⁺ trait is a thrombotic related phenotype.

C4BPβ⁺ and thrombosis show a negative environmental correlation ($\rho_e = -0.27$). The correlation between C4BPβ⁺ and thrombosis is a good example of how low phenotypic correlations may misrepresent the true underlying relationships. In this case, the strong positive genetic correlation is attenuated by a low negative environmental correlation, leading to a moderate phenotypic correlation (table 2). Other investigators have reported similar results using bivariate genetic analysis of a variety of traits.^{28–30}

As expected, total PS plasma levels showed no correlation with the disease. Interestingly, free and functional PS plasma levels also lacked phenotypic, genetic or environmental correlations with thrombosis. These data indicate that the contribution of free and functional PS to thrombosis is probably limited to the PS deficiencies. The risk of thrombosis associated with PS deficiency is a controversial issue.^{31–34} In this respect, it should be remembered that because the GAIT Project attempted to identify genetic factors underlying idiopathic thrombophilia, PS deficient families were excluded from the sample.^{6, 16} This explains the lack of correlations among the PS plasma phenotypes and the thrombotic liability in the present study. Our data fit with previous reports^{15, 35, 36} showing that there is a weak phenotypic correlation between C4BPβ⁺ and free PS ($\rho_p = 0.25$; $p < 0.001$),¹⁵ since increases in C4BPβ⁺ seem to be compensated for by elevated total PS plasma levels ($\rho_p = 0.57$; $p < 0.001$. $\rho_g = 0.49$; $p < 0.01$).¹⁵ Taken together, the genetic

correlation between C4BPβ⁺ and thrombosis points to a pleiotropic regulation of C4BP⁺ plasma levels and susceptibility of thrombosis. If there is a causal relationship between C4BPβ⁺ levels and thrombotic liability, it seems to be independent of C4BPβ⁺ induced variation in the free and functional PS plasma levels.

In conclusion, the present work shows that the C4BPβ⁺ plasma level and the thrombotic liability are phenotypically correlated traits, and that genetic factors are mainly responsible for this correlation. Thus, the C4BPβ⁺ plasma level must be considered a new thrombosis related phenotype. Thrombosis is a complex disease with both genetic and environmental components.⁴ Different studies have identified various haemostatic phenotypes^{21–23} as thrombotic risk factors. In addition, some of these quantitative risk factors exhibited significant genetic correlations with thrombosis,⁶ which has been exploited to improve the power to detect the quantitative trait locus (QTL) contributing to thrombotic risk.^{7–9} The C4BPβ⁺ phenotype shows a sizeable genetic component (heritability = 42%)¹⁵ and a pleiotropic regulation with thrombosis (this work), indicating that it might be an intermediate thrombotic risk factor. Intermediate risk factors tend to be proximal to gene action and, when analysed, provide less attenuated genetic signals than discrete clinical endpoints, such as disease. The pleiotropy observed for C4BP⁺ and thrombosis should improve the power to identify QTLs contributing to thrombotic risk.

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