A Genome-Wide Association Study of the Protein C Anticoagulant Pathway

Georgios Athanasiadis1, Alfonso Buil2, Juan Carlos Souto3, Montserrat Borrell3, Sonia López1, Angel Martinez-Perez1, Mark Lathrop4, Jordi Fontcuberta5, Laura Almasy5, José Manuel Soria1*

1 Unit of Genomics of Complex Diseases, Research Institute, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, 2 Department of Genetics and Development, University of Geneva, Geneva, Switzerland, 3 Haemostasis and Thrombosis Unit, Department of Hematology, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain, 4 Centre National de Génomypage, Évry, France, 5 Department of Population Genetics, Southwest Foundation for Biomedical Research, San Antonio, Texas, United States of America

Abstract
The Protein C anticoagulant pathway regulates blood coagulation by preventing the inadequate formation of thrombi. It has two main plasma components: protein C and protein S. Individuals with protein C or protein S deficiency present a dramatically increased incidence of thromboembolic disorders. Here, we present the results of a genome-wide association study (GWAS) for protein C and protein S plasma levels in a set of extended pedigrees from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project. A total number of 397 individuals from 21 families were typed for 307,984 SNPs using the Infinium® 317 K Beadchip (Illumina). Protein C and protein S (free, functional and total) plasma levels were determined with biochemical assays for all participants. Association with phenotypes was investigated through variance component analysis. After correcting for multiple testing, two SNPs for protein C plasma levels (rs867186 and rs8119351) and another two for free protein S plasma levels (rs1413885 and rs1570868) remained significant on a genome-wide level, located in and around the PROCR and the DNAJC6 genomic regions respectively. No SNPs were significantly associated with functional or total protein S plasma levels, although rs1413885 from DNAJC6 showed suggestive association with the functional protein S phenotype, possibly indicating that this locus plays an important role in protein S metabolism. Our results provide evidence that PROCR and DNAJC6 might play a role in protein C and free protein S plasma levels in the population studied, warranting further investigation on the role of these loci in the etiology of venous thromboembolism and other thrombotic diseases.

Introduction
The protein C anticoagulant pathway is an important physiological mechanism that regulates blood coagulation. It prevents the inadequate formation of thrombi and has two main plasma components: protein C and protein S.

Protein C (PC) is a vitamin K-dependent serine protease, which acts as an anticoagulant by inactivating activated Factors V (FVα) and VIII (FVIIIα). PC is activated by the thrombin/thrombomodulin complex on the surface of endothelial cells, where it binds with endothelial PC receptor (EPCR) [1,2]. EPCR also circulates in a soluble form (sEPCR) with similar affinity to both PC and activated PC (APC). Moreover, sEPCR acts as an inhibitor of APC [3].

Protein S (PS) is also a vitamin K-dependent anticoagulant plasma protein. It has no enzymatic activity, but acts as a cofactor to activated PC in the inactivation of FVα and FVIIIα. Moreover, PS is a cofactor for tissue factor pathway inhibitor (TFPI) for inhibiting FXα [4]. PS circulates either as a free molecule (FPS; ~40% of the total PS) or as a complex with the C4b-binding protein (C4BP-PS; ~60% of the total PS) [5]. Until recently, it was thought that only FPS had cofactor activity; however, now there is growing evidence that the C4BP-PS complex participates directly in FVa and FVIIIa inactivation [6].

Individuals with PC or PS deficiency present a dramatically increased incidence of thromboembolic disorders [7]. Many of the mutations that cause these deficiencies are located in and around the structural genes of PC and PS (PROC and PROS1 respectively) [8–10]. However, a high proportion of families with PC or PS deficiency have no mutations in these genes [11]. Moreover, several polymorphisms in the promoter of the PROC gene account for a mere ~6% of the quantitative variation of PC levels [12]. These observations suggest that the genetic mechanisms underlying PC and PS plasma levels are still largely unknown and that more loci, other than the two structural genes, are involved in the variability of these traits.

A genome-wide linkage analysis using the data from the family-based GAIT Project was performed to find novel loci affecting PC...
and PS plasma levels [13,14]. The analysis showed that genotypic variation in the PROC and PROSI genomic regions is not a primary determinant of the quantitative variation of PC and PS plasma levels. Rather, PC levels showed significant linkage with chromosomal region 1q23. This region contains a candidate gene, XQ01 coding for NAD(P)H:dehydrogenase quinone 1, involved in vitamin K metabolism [13]. In addition, there was strong evidence of linkage between chromosomal region 1q32 and iPS plasma levels. Interestingly, this region contains the genes that code for the \( \alpha \) and \( \beta \) chains of the C4b-binding protein (\( C4BP \alpha \) and \( C4BP \beta \)) [14]. Moreover, using a tagSNP approach, the Cardiovascular Health Study (CHS) reported that polymorphism rs67186 from the gene that codes for EPCR (\( PROCR \)) was associated with higher levels of circulating PC antigen and that polymorphism rs1878672 from the \( IL10 \) gene was associated with higher iPS levels [15].

More recently, a genome-wide association scan for loci affecting PC plasma levels in a large sample of patients and controls of European descent identified three novel loci (\( GCGR \), \( EDEN2 \) and \( B3GAT2 \)), together with two already known (\( PROC \) and \( PROCR \)) [16]. In addition, a genome-wide linkage analysis reported that a quantitative trait locus in chromosomal region 20q11 (including genes \( FOXA2 \), \( THBD \) and \( PROCR \)) influences PC levels in one extended family from the GENES study [17,18]. A subsequent study revealed that \( PROCR \) haplotype 3 and a SNP from \( FOXA2 \) (rs1055080) were associated with PC levels in this family, but only \( PROCR \) haplotype 3 was associated also with plasma levels in healthy individuals [19].

The top (i.e., showing the most significant associations) SNPs from the five genes found in the aforementioned genome-wide association scan explained only a fraction (28.2%) of the variance in PC plasma levels [16]. Since previous studies postulated that \( \sim 50\% \) of the phenotypic variation in PC plasma levels is caused by the additive effect of genes [20] the discovery of more loci is necessary [21]. Bearing this in mind, we carried out the first GWAS that encompasses the two main components of the protein C anticoagulant pathway (PC and functional, free and total PS levels). The objective of this work was to search for SNPs that influence PC and PS plasma levels and potentially increase the risk of venous thrombosis. We were successful in identifying such loci.

**Methods**

**Ethics Statement**

The Institutional Review Board of the Hospital de la Santa Creu i Sant Pau approved all protocols used in the GAIT Project and participants gave their informed consent, in compliance with the Declaration of Helsinki.

**The GAIT Project: a brief description**

The GAIT Project included 397 individuals from 21 extended Spanish families (mean pedigree size = 19) [20–21]. Twelve of these families were selected on the basis of a proband with idiopathic thrombophilia, whereas the remaining nine families were selected randomly. Age ranged from \(<1\) to 88 years (mean = 37.7) and male to female sex ratio was 0.85.

**Plasma measurements**

PC plasma concentrations were measured by a biochemical analyzer (CPA Coulter, Coulter Corp) using chromogenic methods from Chromogenix. Functional PS (funcPS) was assayed with the STA automated coagulometer (Boehringer Mannheim) and determined with a Diagnostica Stago kit. iPS and total protein S (free+CHb-bound) were assayed with an ELISA-based commercual kit (Diagnostica Stago). To reduce experimental error, each assay was performed twice and the average value was calculated for each participant. Intra- and inter-assay coefficients of variation were between 2% and 6%.

**Genotypic determinations and data cleaning**

A genome-wide set of 307,984 SNPs was typed in all of the participants using the Infinium® 517 k Beadchip on the Illumina platform (San Diego, CA, USA). Genotype imputation was performed with Merlin [22] to avoid missing values and all genotypes were checked for Mendelian inconsistencies. In addition, any SNP with call rate\(<95\%\), \( MAF\leq0.025 \) or failing to fit Hardy-Weinberg proportions taking into account multiple testing (\( p\leq5\times10^{-7} \)) was removed from the study. In total, 24,547 SNPs failed to pass the data cleaning criteria, leaving a set of 283,437 SNPs for further analysis.

**Statistical analysis**

Association with phenotypes was investigated through variance component analysis that takes into account the family relationships among individuals. The quantitative phenotype \( y \) was modeled as a linear function of the genetic effect of a SNP (\( snp \)), the polygenic effect \( g \) and a random environmental deviation \( e \):

\[
y = \mu + \beta \cdot snp + g + e
\]

The covariance among phenotypic values \( \Omega \) was modeled using the kinship coefficient matrix \( \Phi \) derived from the family structure:

\[
\Omega = 2\Phi \sigma_g^2 + I \sigma_e^2
\]

whereby \( \sigma_g^2 \) and \( \sigma_e^2 \) are the variances of the polygenic and environmental effects and \( I \) is the identity matrix.

The analysis was performed with the SOLAR v4.0 statistical package [23]. Variance component methods present considerable advantages when combined with extended families for the localization of QTLs, as it is now clear that large complex pedigrees have substantially more power per sampled individual than smaller families do [24–26]. All plasma phenotypes (PC iPS, funcPS and total PS) were log-transformed and adjusted for age and sex. Measured genotype analysis was used for testing association, assuming an additive genetic model [27]. Finally, the Benjamini – Hochberg (B-H) adjustment [28] was applied to the \( p \)-values using the \( p \)-adjust function in R and assuming a 10% false discovery rate.

**Results**

PC, iPS, funcPS and total PS plasma levels in the GAIT sample have been comprehensively described elsewhere [13,14]. In brief, PC plasma levels ranged from 37% to 198% those of healthy donors, with a mean value of 113.8% (standard deviation = 19.5%) adjusted for age and gender. In addition, iPS plasma levels ranged from 54% to 166% those of healthy donors and the mean age-adjusted iPS value was 109.4% for men (standard deviation = 21.3%) and 92.2% for women (standard deviation = 18.0%). Finally, mean funcPS and total PS plasma levels were 96.5% (range: 30%–188%; standard deviation = 21.7%) and 101.5% (range: 60%–176%; standard deviation = 20.7%) those of healthy donors respectively.
Table 1 shows the top SNP associations for each of the four phenotypes (PC, funcPS, IPS and total PS plasma levels). In all plasma phenotypes, association statistics followed the expected χ² distribution under the null hypothesis of no association (Figure 1). From the 283,437 SNPs that were tested, two SNPs for the PC plasma levels (rs867186 and rs8119351) and another two for the IPS plasma levels (rs1413885 and rs1570868) remained significant on a genome-wide level, after applying the B-H adjustment.

Polymorphism rs867186 is located in the PROCR gene and is responsible for a non-synonymous substitution in the amino acid chain of EPCR (S219G), whereas polymorphism rs8119351 is intergenic, located at ~10 Kbp upstream from rs867186, with no apparent function. Each copy of minor allele from rs867186 (G) and rs8119351 (A) seems to increase PC plasma levels by 0.845 and 0.812 standard deviations and to explain 10.27% and 9.56% of the variance in PC plasma levels respectively (Table 2).

Table 1. Top ten SNP associations for PC, IPS, funcPS and total PS plasma levels.

| Phenotype | SNP         | Location       | χ²   | p-values            | Gene   |
|-----------|-------------|----------------|------|---------------------|--------|
| PC        | rs867186    | Chr20:33228215 | 34.69| 3.87 × 10⁻²⁹⁺⁺⁺   | PROCR  |
| PC        | rs8119351   | Chr20:33218066 | 30.24| 3.81 × 10⁻²⁹⁺⁺⁺   | PROCR  |
| PC        | rs11906160  | Chr20:33029416 | 23.26| 1.41 × 10⁻⁰⁵      | MYH7B  |
| PC        | rs13230047  | Chr7:36927845  | 19.93| 8.02 × 10⁻⁰⁵      | ELMO1  |
| PC        | rs1006973   | Chr14:51146839 | 19.86| 8.33 × 10⁻⁰⁵      | FRMD6  |
| PC        | rs6006239   | Chr20:33158241 | 18.74| 1.50 × 10⁻⁰⁵      | C20orf31|
| IPS       | rs6691481   | Chr1:19216466  | 18.67| 1.56 × 10⁻⁰⁵      | RBAF600|
| IPS       | rs884608    | Chr9:36490359  | 18.60| 1.61 × 10⁻⁰⁵      | MELK   |
| IPS       | rs915664    | Chr6:30902596  | 18.46| 1.74 × 10⁻⁰⁵      | DDR1   |
| IPS       | rs780873    | Chr12:114680164| 18.35| 1.84 × 10⁻⁰⁵      | TLRAP2 |
| IPS       | rs1413885   | Chr1:65588247  | 26.02| 3.37 × 10⁻⁰⁷⁺⁺⁺   | DNAJC6 |
| IPS       | rs1570868   | Chr1:65603196  | 25.72| 3.94 × 10⁻⁰⁷⁺⁺⁺   | DNAJC6 |
| IPS       | rs12086738  | Chr1:65580000  | 22.10| 2.58 × 10⁻⁰⁶      | DNAJC6 |
| IPS       | rs2137111   | Chr15:75638590 | 20.07| 7.48 × 10⁻⁰⁶      | LLR6A  |
| IPS       | rs2439430   | Chr15:64704898 | 18.83| 1.43 × 10⁻⁰⁵      | LCTL   |
| IPS       | rs7983232   | Chr13:21635999 | 18.50| 1.70 × 10⁻⁰⁵      | FGF9   |
| IPS       | rs10489924  | Chr1:99231562  | 18.43| 1.76 × 10⁻⁰⁵      | PAP2   |
| IPS       | rs2375699   | Chr1:65580869  | 18.34| 1.85 × 10⁻⁰⁵      | DNAJC6 |
| IPS       | rs1878449   | Chr4:155107054 | 17.39| 3.04 × 10⁻⁰⁵      | SFRP2  |
| IPS       | rs4295666   | Chr8:22618037  | 17.11| 3.53 × 10⁻⁰⁵      | PEBP4  |
| funcPS    | rs13130255  | Chr4:43232357  | 21.63| 3.31 × 10⁻⁰⁵      | KCTD8  |
| funcPS    | rs3829183   | Chr10:70793644 | 19.41| 1.06 × 10⁻⁰⁵      | HK1    |
| funcPS    | rs1013719   | Chr7:10521275  | 19.24| 1.15 × 10⁻⁰⁵      | NDJF4A |
| funcPS    | rs1106523   | Chr2:241900212 | 18.73| 1.50 × 10⁻⁰⁵      | SEPT2  |
| funcPS    | rs6724257   | Chr2:241871070 | 18.73| 1.50 × 10⁻⁰⁵      | HDLBP  |
| funcPS    | rs425459    | Chr17:47409200 | 18.55| 1.66 × 10⁻⁰⁵      | CA10   |
| funcPS    | rs2712001   | Chr4:38823444  | 18.01| 2.20 × 10⁻⁰⁵      | KHL5   |
| funcPS    | rs763773    | Chr6:144434079 | 17.99| 2.22 × 10⁻⁰⁵      | SF3B5  |
| funcPS    | rs6921460   | Chr6:144439852 | 17.90| 2.33 × 10⁻⁰⁵      | SF3B5  |
| funcPS    | rs1413885   | Chr1:65588247  | 17.53| 2.82 × 10⁻⁰⁵      | DNAJC6 |
| Total PS  | rs1401543   | Chr3:137070501 | 19.88| 8.23 × 10⁻⁰⁶      | PPP2RA |
| Total PS  | rs1607504   | Chr3:13707926  | 19.88| 8.23 × 10⁻⁰⁶      | PPP2RA |
| Total PS  | rs2523674   | Chr6:31544768  | 19.60| 9.53 × 10⁻⁰⁶      | HCP5   |
| Total PS  | rs7648592   | Chr3:137075769 | 19.36| 1.08 × 10⁻⁰⁵      | PPP2RA |
| Total PS  | rs6762218   | Chr3:137064983 | 18.20| 1.99 × 10⁻⁰⁵      | PPP2RA |
| Total PS  | rs7029526   | Chr9:119162623 | 18.13| 2.06 × 10⁻⁰⁵      | ASTN2  |
| Total PS  | rs7095655   | Chr10:87294479 | 17.96| 2.25 × 10⁻⁰⁵      | GRID1  |
| Total PS  | rs1372328   | Chr9:118523439 | 17.53| 2.82 × 10⁻⁰⁵      | ASTN2  |
| Total PS  | rs2723603   | Chr11:133174597| 17.32| 3.16 × 10⁻⁰⁵      | SPATA19 |
| Total PS  | rs10241576  | Chr7:37825948  | 16.90| 3.93 × 10⁻⁰⁵      | TXNDC3 |

An asterisk in the p-value indicates significance after B-H adjustment for multiple comparisons.

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However, these observations are not independent as both SNPs belong to the same LD block ($D^* = 0.99; r^2 = 0.91; p = 6.74 \times 10^{-15}$). On the other hand, both significant SNPs for the fPS plasma levels (rs1413885 and rs1570868) are intronic, located in the DNAJC6 gene, and have no known function. In this case, each copy of minor allele from rs1413885 (C) and rs1570868 (T) seems to increase fPS plasma levels by 0.428 and 0.415 standard deviations and to explain 6.24% and 7.53% of the variance in fPS plasma levels respectively (Table 2). Again, these observations are not completely independent as the two SNPs present significant LD ($D^* = 0.78; r^2 = 0.44; p = 1.36 \times 10^{-14}$). It is also important to note that two more SNPs from the DNAJC6 genomic region (rs12086738 and rs2375699) also showed suggestive association with fPS plasma levels (Table 1).

Although none of the SNPs were significantly associated with funcPS or total PS plasma levels (Table 1), one of the significant SNPs for fPS (rs1413885) also ranked among the top hits for funcPS ($p = 2.82 \times 10^{-10}$), suggesting that DNAJC6 might be involved in the PS metabolism. Finally, it is also worth noting that four out of five top hits for total PS levels were from the same genomic region (PPP2R3A). Although none of these SNPs rose to genome-wide significance levels ($p$-values between $10^{-06}$ and $10^{-05}$) they deserve special attention.

**Figure 1.** Quantile-quantile plots of theoretical (x-axis) vs. experimental (y-axis) $\chi^2$ statistics. Each plot represents one of the four plasma phenotypes (PC, fPS, funcPS and total PS). The red lines ($y = x$) correspond to equal theoretical and experimental distributions.
suggestive association with funcPS, underpinning the involvement of a significant SNP for fPS plasma levels (rs1413885) also showed significant procoagulant activity and resistance to activated PC [37]. Thus, a similar mechanism involving DNAJC6 and IPS is possible, although the validation of this hypothesis would require further investigation.

Even though we were successful in discovering a novel locus for fPS plasma levels (DNAJC6) and replicating previous data on PC plasma levels, we did not find any significant associations for funcPS and total PS plasma levels. An ad hoc query of the STRING database [http://string.embl.de] for pathways of possible biological relevance involving the top hits from each PS phenotype (as listed in Table 1) gave no evidence of protein–protein interactions.

In a previous linkage study based on the GAIT sample, we reported NQO1 as a candidate gene affecting variation in PC plasma levels [13]. Further analysis showed that one intronic SNP (rs1437135) from NQO1 was significantly associated with PC plasma levels [13]. Unfortunately, this SNP was not present in the Illumina chip that we used for our genome-wide association analysis, so no direct comparisons could be made. Nevertheless, another SNP from the NQO1 genomic region (rs1800566) was included in the Illumina chip. Although this SNP is in full LD with rs1437135, it did not show significant association with PC plasma levels on a genome-wide level (p = 0.061). Moreover, we have reported strong evidence of linkage between chromosomal region 1q32 and fPS plasma levels; interestingly, this region contains two genes of high biological relevance, C4BPA and C4BPR, coding for the principal binding protein of PS [14]. However, our genome-wide association study found no association between these two genes and any of the PS phenotypes on a genome-wide level.

This is the first time we have evidence that the PROCR gene is associated with PC plasma levels in the GAIT sample, as the linkage study performed previously [13] did not identify any linkage between the PROCR genomic region and this phenotype (logarithm of the odds (LOD) score = 0.670). In a similar manner, no linkage was previously found between the DNAJC6 genomic region and IPS plasma levels (LOD score = 0.089) [14]. Several issues arise from this apparent lack of concordance between genome-wide association and genome-wide linkage studies from the GAIT Project. It is important to note that GAIT is one of the few projects that allow us to perform this comparison.

From the methodological point of view, linkage differs from association in that it is based on the joint transmission of a marker and a functional site from parent to offspring (i.e. co-segregation), rather than on correlation due to LD. In this context, the association approach has difficulty in detecting rare variants through LD with common SNP markers, but such variants can be found by linkage. Thus, an explanation for the failure to detect the same loci in our analysis might be that linkage signals in GAIT might be due to rare variants at those loci, whereas association might be due to more common variants. This observation does not rule out the presence of other variants at those loci with small effect on PC or PS levels and our study does not have enough power to detect such small effects. It is important to note that if we cannot detect the effect of the other QTLs because it is small, this emphasizes our results that PROCR and DNAJC6 are major determinants of PC and PS levels in the Spanish population.

Taken together, the linkage and association analyses we carried out in the context of the GAIT Project are a good example of how rare and common variants underlying the genetic architecture of complex traits, such as PC and PS plasma levels. In addition, it is important to emphasize that no single method or model for studying genetic architecture can be adopted universally. No single method can answer all or even some of the questions without being used in concert with additional approaches.

In summary, our work provides evidence that the PROCR and DNAJC6 loci are involved in the genetic determination of the PC and IPS plasma levels respectively. However, these observations...
should be further validated by means of functional experiments, especially for the IPS plasma levels, as the function of the DNAJC6 gene is still unknown.

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Author Contributions

Conceived and designed the experiments: JCS MB JF. Performed the experiments: MB ML. Analyzed the data: AB LA GA JMS SL AMP. Contributed reagents/materials/analysis tools: JCS JMS MF ML LA. Wrote the paper: GA JMS.

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