Fibrinogen Polymorphisms *Taq I*, *Hae III* and *Bcl I* Are Not Associated with a Higher Risk of Deep Vein Thrombosis

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Key Words

Fibrinogen · Venous thrombosis · Polymorphism (genetics) · Aetiology of DVT

Abstract

High fibrinogen is recognised as a risk factor for atherosclerosis. It seems that high fibrinogen is also a risk factor for deep vein thrombosis (DVT). It has been shown that certain polymorphisms in fibrinogen genes can influence the fibrinogen level. In this study, fibrinogen levels and the frequency of the polymorphisms *Taq I*, *Hae III* and *Bcl I* were studied in 114 patients with DVT and 244 healthy subjects. In non-smokers, fibrinogen levels above 5 g/l were associated with an increased risk of DVT (odds ratio 3.3, 95% confidence interval 1.6–7.0). The frequencies of common alleles were similar in patients and healthy subjects for all polymorphisms. An association between fibrinogen levels and the polymorphisms *Taq I*, *Hae III* and *Bcl I* was found in healthy subjects, but not in the patients. It was concluded from these data that the polymorphisms *Taq I*, *Hae III* and *Bcl I* are not major risk factors for DVT.

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Introduction

Increased plasma fibrinogen has been repeatedly recognised as a risk factor for myocardial infarction and stroke [1–4]. For deep vein thrombosis (DVT), similar fibrinogen levels have been reported for patients and healthy subjects [5, 6]. However, in one study, a fibrinogen level above 5 g/l was associated with a four-fold risk of DVT [5].

Plasma fibrinogen is influenced by many factors, such as age, gender, smoking, physical activity, obesity, diabetes and hyperlipidaemia [7–9]. In addition, fibrinogen is an acute-phase protein [10]. However, these factors cannot explain fully the variation in fibrinogen levels among individuals. It has been shown that several polymorphisms in genes for fibrinogen polypeptide chains α , β and γ might play a role in the determination of the plasma fibrinogen concentration. Several restriction fragment length polymorphic sites have been found in the fibrinogen gene cluster [11]. The polymorphic *Taq I* and *Bcl I* sites have been mapped to the 3' end of the α - and β -fibrinogen genes, respectively [11, 12], and the polymorphic *Hae III* site to the 5' flanking region of the β -fibrinogen gene [13]. These polymorphisms have been shown to be associated with fibrinogen levels in healthy subjects in some [13–15], but not in other studies [16, 17]. In patients with coronary heart disease, these polymorphisms were

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Table 1. Polymorphism notations

Gene name	Gene symbol	Polymorphism site	SNP annotation
Fibrinogen, α chain	FGA	8884–8911del	<i>Taq</i> I
Fibrinogen, β chain	FGB	–455G>A	<i>Hae</i> III
Fibrinogen, β chain	FGB	3' untranslated region	<i>Bcl</i> I

SNP = Single nucleotide polymorphism.

significantly associated with fibrinogen concentration [18, 19]. In patients with DVT, only the influence of the *Hae* III polymorphism has been studied, and the results were conflicting; one study found a significant association between *Hae* III and fibrinogen levels [6], but in another study, no such association was observed [5]. If we speculate that certain genotypes elevate levels of fibrinogen and that high fibrinogen plays a causal role in the development of the disease, then these unfavourable genotypes should be more frequent in patients than in healthy subjects. In the Leiden Study of Myocardial Infarction [19], a similar prevalence of the three polymorphisms was observed among patients with myocardial infarction and healthy subjects, and so the hypothesis about the causal role of fibrinogen in the aetiology of myocardial infarction was rejected.

To establish whether the fibrinogen polymorphisms *Taq* I, *Hae* III and *Bcl* I represent risk factors for DVT, the frequency of these polymorphisms and their association with plasma fibrinogen levels were studied in patients with DVT and healthy subjects.

Patients and Methods

Patients and Healthy Subjects

Subjects participated in the study after they had given their full informed consent. All subjects originated from Central Europe and were not related to each other. None of the women included in the study was taking oral contraceptives or hormonal replacement therapy. The study was approved by the State Ethical Committee.

Subjects with a history of DVT were recruited from consecutive patients treated at the Department of Angiology (University Medical Centre) in Ljubljana, in the period from January 1992 to April 1996. One hundred and fourteen patients 19–60 years old (44 women and 70 men) were evaluated at least 3 months (on average 16 ± 9 months, mean $\pm$ standard deviation) after objectively confirmed acute DVT. The control group consisted of 244 apparently healthy unrelated volunteers (134 women and 110 men) with no history of DVT or pulmonary embolism. All subjects were asked about their smoking habits and then classified as current smokers or non-smokers. Ex-smokers were considered as non-smokers if they had stopped smoking at least 5 years previous to this study [9]. At the time of

sampling, patients and healthy subjects with an obvious inflammatory condition or a recent febrile episode were excluded.

Blood Collection and Determination of Fibrinogen and Lipids

From each patient and healthy subject, one blood sample collected between 7.00 and 9.00 a.m. after an overnight fast and 20 min of rest was obtained. Venous blood was drawn from the antecubital vein and collected into three Vacutainer® tubes (Becton Dickinson), the first without an anticoagulant, the second containing 0.11 mol/l sodium citrate and the third containing 0.17 mol/l K_3 -EDTA. Serum was harvested after 1 h and analysed the same day for total cholesterol and high-density lipoprotein cholesterol with an Ektachem 250 (Kodak) biochemical analyser. Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula [20]. Platelet-poor plasma was prepared from citrated blood by centrifugation at 2,000 *g* and 4 °C for 30 min, snap frozen and stored at –70 °C until analysis. In this plasma, fibrinogen levels were determined by a modified Clauss method using Multifibren® U (Dade Behring). From the blood collected into EDTA, genomic DNA was isolated using a commercially available genomic DNA purification kit (Wizard™, Promega, USA) or manually by a salting-out procedure [21].

Analysis of Genetic Polymorphisms

We identified the *Taq* I polymorphism (table 1) as a 28-bp deletion (BK001430) of a duplication of nucleotides 8884–8911 (GenBank accession number AF361104), which eliminated a *Taq* I recognition sequence. For the detection of this polymorphism, a new set of primers was designed: forward 5'-ATG AAA ATT AGG CCC C-HEX, and reverse 5'-ATG TCT CAG GTA CAT TTA GC. With these primers, a PCR fragment of 184 and 212 bp, depending on the presence or the absence of the deletion, was amplified using *Taq* polymerase (Promega). Genomic DNA was denatured at 95 °C for 5 min, and the 36 subsequent cycles were performed at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The amplicon size was determined by GeneScan (Abi Prism 310, Applied Biosystems). The common allele (184 bp) was coded as T1 and the rare allele (212 bp) as T2. The *Hae* III polymorphism (table 1) was detected by real-time PCR on a LightCycler (Roche) using hybridisation probes. The primers and hybridisation probes were designed by Tib Molbiol (Berlin, Germany) as follows: forward 5'-GAT GTG TAT TTT TCA TAG AAT AGG GTA, reverse 5'-ATT TGA CCT ACT CAC AAG GCA A, Sensor (A)mt 5'-CAT TAC TAT TGA TTT TAA TAG CCC CT and Anchor 5'-LC Red640-TGA AAT AGA ATT ATG TCA TTG TCA GAA AAC. The common allele was coded as H1 and the rare allele as H2. The *Bcl* I polymorphism (table 1) was genotyped after amplification of the relevant DNA region by PCR and digestion with the *Bcl* I endonuclease as described previously [11]. The common allele was coded as B1 and the rare allele as B2.

Statistical Analysis

For statistical analysis, Statistica 4.5 software (StatSoft Inc., Tulsa, Okla., USA) was utilised. Allele frequencies in patients and healthy subjects were compared by χ^2 analysis. A χ^2 test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. A two-by-two table and χ^2 test were used to calculate linkage disequilibrium, and Arlequin 2.000 (Genetics and Biometry Laboratory, Geneva, Switzerland) statistical software was utilised to calculate its coefficients D and D'. An odds ratio with 95% confidence interval (CI) was calcu-

lated as a measure of relative risk. Allele frequencies are presented with 95% CIs. Fibrinogen levels are given as means and standard deviations, and differences between groups were tested with ANOVA.

Results

One hundred and fourteen patients and 244 healthy subjects were included in the study. The data on patients and healthy subjects are given in table 2. Fibrinogen levels adjusted for age ($\beta = 0.06$), gender ($\beta = -0.01$) and LDL ($\beta = 0.14$, p for multiple regression <0.03) were similar in patients and healthy subjects (table 2). Fibrinogen levels above 5 g/l were associated with an increased risk of DVT only in non-smokers (odds ratio 3.3, 95% CI 1.6–7.0).

The frequencies of the common alleles T1 (*Taq* I polymorphism), H1 (*Hae* III polymorphism) and B1 (*Bcl* I polymorphism) were similar in patients and healthy subjects (table 3). The distribution of genotypes was as expected for a population in Hardy-Weinberg equilibrium. The *Hae* III polymorphism was in linkage disequilibrium with the *Bcl* I polymorphism (p < 0.001 , D = 0.16, D' = 0.95). Subjects with the rare T2 allele of the *Taq* I polymorphism, the H2 allele of the *Hae* III polymorphism or the B2 allele of the *Bcl* I polymorphism did not have a higher risk of DVT, with an odds ratio close to 1.

Table 2. Demographic data and risk factors of patients with DVT and healthy subjects

	Patients	Healthy subjects	p
Age, years	42 $\pm$ 10	47 $\pm$ 17	<0.01
Women/men	45/70	134/110	<0.01
Smokers/non-smokers	41/73	92/152	0.75
BMI, kg/m ²	26.8 $\pm$ 4.0	25.2 $\pm$ 3.9	0.001
Total cholesterol, mmol/l	6.2 $\pm$ 1.3	5.3 $\pm$ 1.1	<0.001
LDL cholesterol, mmol/l	4.1 $\pm$ 1.0	3.3 $\pm$ 1.0	<0.001
Fibrinogen ^a , g/l	2.87 $\pm$ 0.82	2.82 $\pm$ 0.81	0.74

BMI = Body mass index.

^a Fibrinogen levels adjusted for age, gender and LDL.

Table 3. Frequencies of the common alleles of the *Taq* I, *Hae* III and *Bcl* I polymorphisms among patients and healthy subjects and the risk of DVT for heterozygous and homozygous carriers of the rare alleles

Genotype	Patients	Healthy subjects	Odds ratio (95% CI)	Population odds ratio detectable at $\alpha = 0.05$ with 80% power
<i>Taq</i> I polymorphism				
T1T1	62 (54.4)	130 (53.3)		
T1T2	44 (38.6)	97 (39.7)	0.95 (0.6–1.5)	<0.50 or >1.94
T2T2	8 (7.0)	17 (7.0)	0.99 (0.4–2.4)	<0.09 or >2.85
Allele frequency T1 (95% CI)	0.74 (0.66–0.82)	0.73 (0.67–0.79)		
<i>Hae</i> III polymorphism				
H1H1	64 (56.1)	127 (52.0)		
H1H2	43 (37.7)	95 (39.0)	0.90 (0.6–1.4)	<0.50 or >1.94
H2H2	7 (6.1)	22 (9.0)	0.63 (0.3–1.6)	<0.17 or >2.64
Allele frequency H1 (95% CI)	0.75 (0.67–0.83)	0.72 (0.66–0.78)		
<i>Bcl</i> I polymorphism				
B1B1	71 (62.3)	146 (59.8)		
B1B2	35 (30.7)	82 (33.6)	0.88 (0.5–1.4)	<0.48 or >1.94
B2B2	8 (7.0)	16 (6.6)	1.03 (0.4–2.5)	<0.08 or >2.85
Allele frequency B1 (95% CI)	0.78 (0.70–0.86)	0.77 (0.72–0.82)		

Figures in parentheses represent percentages, except where otherwise indicated.

Among healthy subjects, an association between fibrinogen and the *Taq* I polymorphism was noted ($p = 0.06$), but no influence of the *Hae* III and *Bcl* I polymorphisms on fibrinogen was observed (table 4). If only non-smoking healthy subjects were included, a trend towards lower fibrinogen associated with the T2 allele was apparent, but was not statistically significant, while the difference in fibrinogen for the different genotypes of the *Hae* III and *Bcl* I polymorphisms became significant (table 4).

No influence of the different genotypes of the *Taq* I, *Hae* III and *Bcl* I polymorphisms on fibrinogen was observed in patients with DVT, although in non-smoking patients, a trend toward lower fibrinogen associated with the T2 allele of the *Taq* I polymorphism was noted (table 5).

Table 4. Fibrinogen levels in 244 healthy subjects according to genotypes of the different polymorphisms

Genotype	All healthy subjects (n = 244)			Non-smoking healthy subjects (n = 152)		
	number	fibrinogen level (g/l)	p	number	fibrinogen level (g/l)	p
<i>Taq</i> I polymorphism						
T1T1	130	2.88±0.84	0.06	74	2.82±0.72	0.27
T1T2	97	2.80±0.74		69	2.74±0.71	
T2T2	17	2.42±0.83		9	2.51±0.75	
<i>Hae</i> III polymorphism						
H1H1	127	2.77±0.82	0.17	84	2.70±0.68	0.04
H1H2	95	2.95±0.81		53	2.96±0.79	
H2H2	22	2.63±0.67		15	2.46±0.42	
<i>Bcl</i> I polymorphism						
B1B1	146	2.76±0.84	0.24	95	2.68±0.72	0.07
B1B2	82	2.96±0.77		48	2.98±0.75	
B2B2	16	2.69±0.73		9	2.48±0.30	

Fibrinogen levels are shown as mean ± standard deviation. The p values denote differences between groups (ANOVA).

Table 5. Fibrinogen levels in 114 patients with DVT according to genotypes of the different polymorphisms

Genotype	All patients (n = 114)			Non-smoking patients (n = 73)		
	number	fibrinogen level (g/l)	p	number	fibrinogen level (g/l)	p
<i>Taq</i> I polymorphism						
T1T1	59	2.84±0.79	0.30	74	2.91±0.91	0.10
T1T2	44	2.85±0.80		69	2.89±0.80	
T2T2	8	3.29±1.06		9	2.81±1.13	
<i>Hae</i> III polymorphism						
H1H1	64	2.91±0.93	0.84	84	2.92±0.98	0.94
H1H2	42	2.82±0.70		53	2.87±0.78	
H2H2	7	2.80±0.25		15	2.91±0.72	
<i>Bcl</i> I polymorphism						
B1B1	70	2.88±0.90	0.96	95	2.89±0.97	0.99
B1B2	34	2.88±0.73		47	2.91±0.77	
B2B2	8	2.83±0.34		10	2.92±0.74	

Fibrinogen levels are shown as mean ± standard deviation. The p values denote differences between groups (ANOVA).

Discussion

In this case-control study, the fibrinogen polymorphisms *Taq I* in α -fibrinogen and *Hae III* and *Bcl I* in β -fibrinogen did not represent major risk factors for DVT, although an influence of these polymorphisms on fibrinogen concentration was observed in healthy subjects.

The frequencies of the common alleles of the *Taq I*, *Hae III* and *Bcl I* polymorphisms in healthy subjects found in this study were similar to those previously reported [14, 22]. Furthermore, patients with DVT had similar allele frequencies to healthy subjects. Odds ratios for all genotypes were close to 1, and therefore, these polymorphisms were not associated with a higher risk of DVT. However, larger studies are needed that could detect smaller differences in odds ratios than was possible with our sample size.

Fibrinogen levels adjusted for age, gender and LDL cholesterol were similar in patients and in healthy subjects. Despite this, a statistically significant increase in thrombotic risk (odds ratio 3.3, 95% CI 1.6–7.0) was observed in non-smokers with fibrinogen levels above 5 g/l. If smokers were included, the significance was lost due to high fibrinogen levels in some healthy smokers. In the Leiden Thrombophilia Study, a four-fold increase in thrombotic risk was observed with fibrinogen levels above 5 g/l [5].

The *Taq I* polymorphism was associated with lower fibrinogen among healthy subjects. Although this association was not significant, a similar trend has been observed in previous studies in healthy subjects [15, 17] and in patients with cardiovascular disease [18, 19]. In our group of patients with DVT, this non-significant trend was observed only in non-smokers.

The *Hae III* polymorphism was associated with fibrinogen levels in healthy non-smokers, with the highest fibrinogen levels in heterozygotes, but no association was found in patients with DVT. For patients with DVT, our results confirmed the results of the Leiden Thrombophilia Study [5], in which fibrinogen was measured several months after the thromboembolic event, similarly to our study. In contrast, a significant association between the *Hae III* polymorphism and fibrinogen levels was reported by Renner et al. [6], but in their study, fibrinogen levels were measured during the acute phase of DVT. We have no clear explanation for why the mean fibrinogen level was the highest in heterozygote healthy non-smokers compared to homozygotes with or without the polymorphism.

A borderline association between the *Bcl I* polymorphism and fibrinogen levels was found in healthy non-

smokers, but not in patients with DVT. A significant influence of this polymorphism on fibrinogen level was described in patients with coronary artery disease [18, 23, 24], but to our knowledge, this association has not yet been investigated in patients with DVT. For healthy subjects, the absence of this association was reported in two studies [16, 17], but both studies included non-smokers as well as smokers.

It is quite likely that besides age, gender, smoking, physical activity, obesity, diabetes and hyperlipidaemia [7–9], other factors influence the level of fibrinogen. It has been shown that fibrinogen shows seasonal variation, being significantly increased in the cold months [25–27]. Therefore, sampling of blood in different seasons might increase the variation in fibrinogen and mask the effect of genetic polymorphisms. Moreover, the effect of genetic polymorphisms on fibrinogen levels as observed in the acute stage of DVT [6], but not in the chronic stage of the disease [5], might suggest that gene-environment interactions have different levels of importance in different subject groups.

In conclusion, the fibrinogen polymorphisms *Taq I*, *Hae III* and *Bcl I* were equally distributed among patients with DVT and healthy subjects. Although these polymorphisms to some extent modulated plasma fibrinogen levels in healthy subjects, no such observation was evident for patients with DVT. These data suggest that the fibrinogen polymorphisms *Taq I*, *Hae III* and *Bcl I* are not major risk factors for DVT; however, further studies on larger groups of patients with DVT are needed.

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