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REGULAR ARTICLE

Cytokine gene variants and venous thrombotic risk in the BRATROS (*BRA*ZILIAN *TH*R*O*MBOSIS *S*TUDY)

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Abstract

Introduction: Venous thrombosis (VT) and inflammation are two closely related entities. In the present investigation we assessed whether there is a relation between genetic modifiers of the inflammatory response and the risk of VT.
Materials and methods: 420 consecutive and unrelated patients with an objective diagnosis of deep VT and 420 matched controls were investigated. The frequencies of the following gene polymorphisms were determined in all subjects: TNF-α−308 G/A, LT-α+252 A/G, IL-6-174 G/C, IL1-ra 86 bp VNTR, IL-10-1082 A/G and CD-31 125 C/G.
Results: Overall odds ratio (OR) for VT related to TNF-α−308 G/A, LT-α+252 A/G, IL-6-174 G/C, A1 allele (4 bp repeat) of the IL1-ra 86 bp VNTR, IL-10-1082 A/G and CD-31 125 C/G were respectively: 1.0 (CI95: 0.8–1.5), 1.3 (CI95: 1.0–1.7), 1.1 (CI95: 0.9–1.5), 1.6 (CI95: 1–2.5), 1.2 (CI95: 0.8–1.7) and 0.8 (CI95: 0.6–1.1). A possible interaction between polymorphisms was observed only for the co-inheritance of the mutant alleles of the LT-α+252 A/G and IL-10-1082 G/A polymorphisms (OR=2; CI95: 1.1–3.8). The risk of VT conferred by factor V Leiden and FII G20210A was not substantially altered by co-inheritance with any of the cytokine gene polymorphisms.
Conclusions: Cytokine gene polymorphisms here investigated did not significantly influence venous thrombotic risk.

Abbreviations: VT, venous thrombosis; OR, odds ratio; CI95, 95% confidence interval; FVL, factor V Leiden; BRATROS, Brazilian Thrombosis Study; TNF-α, tumor necrosis factor-alpha; LT-α, lymphotoxin-alpha; IL-6, interleukin-6; IL-1-ra, interleukin-1 receptor antagonist; IL-8, interleukin-8; IL-10, interleukin-10.

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Introduction

Venous thrombosis is a prevalent disease that is considered to be a "complex" or "multifactorial" trait, because several genetic and environmental factors contribute to its occurrence [1,2]. A list of well-established genetic risk factors of VT is now known; the main genetic abnormalities associated with increased risk of VT are the inherited deficiencies of antithrombin, protein C and protein S, and the point mutations known as factor V Leiden (FVL) and factor II (FII) G20210A. The association of other specific gene defects with thrombotic risk is less well-established or controversial.

Inflammation and VT are two closely related entities and the inflammatory process is associated with thrombogenesis through different mechanisms. One mechanism is the induction of pro-coagulant factors: tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8) have the potential to elicit tissue factor (TF) expression on endothelial cells and monocytes in vitro [3–5] and in vivo [6–10]. Another mechanism linking inflammation to thrombosis is inhibition of natural anticoagulants: TNF-α and IL-1 can induce internalization of thrombomodulin [11], inhibit transcription of the thrombomodulin gene [12] and inhibit the action of the heparin–antithrombin complex through glucosaminoglicans (GAGs) reduction on the vascular endothelial surface [13]. TNF-α can also inhibit fibrinolysis by increasing plasma levels of plasminogen activator inhibitor-1 (PAI-1) and PAI-2 [14], and IL-6 secretion is associated with platelet production [15]. Other lines of evidence linking inflammation to VT derive from animal studies dealing with the role of TNF-α, IL-6 and interleukin-10 (IL-10, an anti-inflammatory cytokine) in influencing the regulation of the inflammation process and thrombus formation [16,17], and from clinical, case-control studies in humans, such as the LETS (*Leiden Thrombophilia Study*), which has shown a mild but significant increase in risk to VT associated with high levels of TNF-α, IL-1β, IL-6 and IL-8 [18].

Given the above-mentioned data, it is conceivable that cytokines with known polymorphic gene sequences are potential risk factors for venous thrombotic disease insofar as the gene products seem to be involved in VT pathogenesis [19]. Several polymorphisms in cytokine-coding genes have been described and some of them will be briefly presented here. A G to A transition at nucleotide −308 in the TNF-α gene promoter (designated *TNF2* allele) [20] is a stronger transcription activator than the common *TNF1* allele [21,22] and is therefore a candidate risk factor for multifactorial diseases in which inflammation plays a role. Also of interest is a −252 A to G transition in the first intron of the lymphotoxin-alpha (LT-α) gene [23]; LT-α stimulates adhesion molecule production, thrombogenesis, smooth muscle proliferation, platelet activation and release of vasoactive agents [22], and the mutant (G) allele results in higher gene transcription and increased LT-α by in vitro stimulated mononuclear cells [22]. Other candidate risk factors for thrombosis (because of their relation with inflammation) are: a G to C transition at nucleotide −174 in the IL-6 gene (the G allele is a stronger transcription activator than the common C allele and is associated with higher plasma levels of IL-6) [24]; an 86-bp VNTR in intron 2 of the IL-1ra gene (that determines five alleles corresponding to 2, 3, 4, 5 and 6 copies of the 86 bp sequence) [25] — the number of repeats may influence gene transcription and protein production, and carriership of the A1 allele has been shown to be associated with higher IL-1ra secretion by mononuclear cells) [26]; a G to A transition at nucleotide −1082 in the promoter region of IL-10 gene (the A allele is correlated with lower production of IL-10 in T cells stimulated in vitro) [27]; a C to G substitution at codon 125 of platelet-endothelial-cell adhesion molecule 1 (PECAM-1, or CD-31) gene that is associated with a higher risk to GVHD in bone marrow transplantation [28]. CD-31 is a membrane glycoprotein that is constitutively expressed on vascular endothelial cells, platelets, most circulating leukocytes and bone marrow stem cells [29], and it is believed to have a role in interactions between leukocytes and endothelial cells, attracting leukocytes to inflammatory sites and possibly influencing angiogenesis [30].

Thus, the information available on the role of inflammatory mediators in VT encouraged us to conduct this investigation, in order to test the hypothesis that polymorphisms in the TNF-α, LT-α, IL-6, IL-1ra, IL-10 and CD-31 genes might be related to increased risk of VT. Each cytokine gene polymorphism was selected either because other studies suggested that it may influence the predisposition or outcome of multifactorial diseases, or because the variant was demonstrated to result in functional changes or altered expression of cytokines.

Subjects and methods

Patients and controls

The description of the *BRATROS* was published previously [31,32]. Included were four hundred thirty-four patients, consecutively admitted between October 1996 and August 2001, with an objectively

Table 1 *BRATROS*: general characteristics of patients with VT and controls

Variable	Patients ($n=420$)	Controls ($n=420$)
Median age (range)	42 (2–70)	42 (2–70)
Male/Female ratio	0.7	0.7
Ethnicity		
Whites	84.3%	84.3%
Blacks and Mulattos	15.6%	15.6%
Asians	0.1%	0.1%
Family history of VT	22%	–[#]
Spontaneous VT	53.4%	–[#]
Acquired risk factors for VT*	46.6%	–[#]
Genetic factors for VT		
Factor V Leiden	10.5%	2.4%
Factor II G20210A	6.6%	1.2%
Inherited inhibitor deficiency [&]	9.5%	–[#]

*Surgery/trauma/immobilisation, pregnancy/puerperium, and hormone use.
[&]Antithrombin, protein C and protein S deficiencies taken together.
[#]Information not available for all subjects.

verified episode of VT in a deep site (351 patients with lower limb VT, 23 patients with upper limb VT, 4 patients with intra-abdominal thrombosis, 40 with central VT, and 7 with thrombosis in other sites. Nine patients had VT in more than one site). Subjects aged more than 70 years or with malignant disease were excluded. Cases enrolled came from three Brazilian University Hospitals: School of Medicine of Ribeirão Preto (University of São Paulo, USP), School of Medicine of Botucatu (State University of São Paulo, UNESP) and Federal University of São Paulo (UNIFESP). Data on exposure to acquired risk factors for VT, *i.e.* immobilisation, surgery, trauma, use of oral contraceptives and hormone replacement therapy, were collected. Four hundred thirty-four controls (unrelated healthy blood donor candidates without a personal history of thrombosis) were matched with the patients for gender, age (±4 years), race, and time of recruitment. For the purposes of the present investigation, samples from 420 patients (out of the 434 cases originally included), and from the 420 respective controls, were available to be analysed. Table 1 lists general characteristics of the patient study group. The Institutional Ethical Committee approved this study and informed consent was obtained from the participants.

Mutation analysis

With the use of public databases, we selected cytokine gene polymorphisms that were suggested to influence predisposition and/or outcome of multifactorial diseases, or that were demonstrated to result in functional changes or altered expression of cytokines. Peripheral blood was collected and genomic DNA extracted from mononuclear leukocytes by the salting-out method [33]. Genotyping

Table 2 Summary of methodological information on detection of cytokine gene polymorphisms investigated

	TNF-α	LT-α	IL-6	IL-1ra	IL-10	CD-31
Polymorphism	G/A	A/G	G/C	86-bp VNTR	G/A	C/G
Polymorphism location	−308 (promoter region)	+252 (intron 1)	−174 (5′ UT region)	intron 2	−1082 (promoter region)	codon 125
Primers						
Upstream	5′-AGGCAATAGG TTTTGAGGGCCAT-3′	5′-CTCCTGCACCTG CTGCCTGGATC-3′	5′-GTGGTTCTG CTTCTTAGC-3′	5′-TCCTGGTC TGCAGGTAA-3′	5′-CTCCAGCACA TAGAATGAAA-3′	5′-AGAGGGTGAT GGGTGGAGAG-3′
Downstream	5′-TCCTCCCTGC TCCGATTCCG-3′	5′-GAAGAGACG TTCAGGTGTCAT-3′	5′-CTGATTGGAA ACCTTATTAAG-3′	5′-CTCAGCAAC ACTCCTAT-3′	5′-AATCCAAGAC AACACTACTA-3′	5′-ACGGTGCAAA ATGGGAAGAA-3′
PCR conditions						
Denaturation	94°C, 1 min	94°C, 1 min	94°C, 40 secs	94°C, 1 min	94°C, 30 sec	4°C, 30 ss
Annealing	60°C, 1 min	65°C, 1 min	59°C, 58 sec	59°C, 1 min	94°C, 30 sec	55°C, 30 s
Extension	72°C, 1 min	72°C, 2 min	72°C, 1 min	70°C, 2 min	X 72°C, 1 min	72°C, 1 minute
N. of cycles	40	35	35	36	35	40
Restriction enzyme	*Nco*I	*Nco*I	*Nla*III	Direct detection	*Mnl*I	*Mnl*I
Allele (bp)	−308G: 20+87	+252A: 368	−174G: 119	I: 410 IV: 325	−1082G:134+33+25+23+12	−125C: 237+78+49
	−308A: 107	+252G: 235+133	−174C: 70+49	II: 240 V: 595	−1082A:134+33+48+12	−125G: 315+49
				III: 500 VI: 120		

for each gene variation was performed by employing PCR and restriction–digestion protocols published previously [20,24,25,27,28], whose main characteristics are summarized in Table 2.

Statistical analysis

Allele frequencies were calculated by gene counting from observed genotypes. Odds ratios (OR) were calculated as an estimate of the relative risk of VT in an exposed category of subjects in relation to a reference category for which the OR is arbitrarily 1.0. 95% confidence intervals (CI95) were calculated by standard methods [34]. To search for interaction between cytokine gene polymorphisms and between these gene variations and prothrombotic mutations (FVL and FII G20210A), stratified analyses were performed. Thus, interaction between the cytokine polymorphisms was searched for by performing two-by-two stratified analyses, due to the rarity of co-inheritance of more than two polymorphisms (which would yield OR with too wide confidence intervals). By testing the combination of the six gene variations two by two, 15 different scenarios of co-inheritance were eventually analysed to look for gene–gene interactions. When testing these scenarios, the genotypes were combined according to their expected pro-inflammatory effect.

Results

TNF-α-308 G/A, LT-α+252 A/G, IL-6-174G/C, VNTR IL-1ra, IL-10-1082G/A and CD-31 125 C/G polymorphisms and the risk of VT

The mutant TNF-α allele was found in 102/420 controls (allele frequency 13%, carrier frequency 24%) and in 108/420 patients with VT (allele frequency 14%, carrier frequency 26%). These data yielded an overall OR for VT related to the TNF-α polymorphism of 1 (CI95: 0.8–1.5). The OR for homozygotes was 0.7 (CI95: 0.2–1.9), and for heterozygotes the OR was 1.1 (CI95: 0.8–1.5). The mutant LT-α allele was found in 222/420 controls (allele frequency 32%, carrier frequency 53%) and in 250/420 patients with VT (allele frequency 35%, carrier frequency 60%). These data yielded an overall OR for VT related to the LT-α polymorphism of 1.3 (CI95: 1–1.7). The OR for homozygotes was 1.3 (CI95: 0.8–2), and for heterozygotes the OR was 1.3 (CI95: 1–1.7) (Table 3).

The mutant (C) IL-6 allele was found in 188/420 controls (allele frequency 27%, carrier frequency 45%) and in 203/420 patients with VT (allele frequency 28%, carrier frequency 48%), yielding an overall OR for VT of 1.1 (CI95: 0.9–1.5). The OR for homozygotes was 0.9 (CI95: 0.5–1.6), and for heterozygotes the OR was 1.2 (CI95: 0.9–1.6).

The A1 allele of the VNTR IL-1ra was found in 374/420 controls (allele frequency 71%, carrier frequency 89%) and in 390/420 patients (allele frequency 72.5%, carrier frequency 93%). Overall OR for VT related to the A1 allele was 1.6 (CI95: 1–2.5).

The mutant IL-10 allele was found in 354/420 controls (allele frequency 64%, carrier frequency 84%) and in 363/420 patients with VT (allele frequency 62%, carrier frequency 86%). These data yielded an overall OR for VT related to the IL-10 polymorphism of 1.2 (CI95: 0.8–1.7). The OR for homozygotes was 1 (CI95: 0.7–1.6), and for heterozygotes the OR was 1.3 (CI95: 0.9–2).

The mutant CD-31 allele was found in 325 out of 420 controls (allele frequency 52%, carrier frequency 77%) and in 311 out 420 patients with VT (allele frequency 49%, carrier frequency 74%). These data

Table 3 Prevalence of TNF-α −308 G/A, LT-α +252 A/G, IL-1ra 86 bp VNTR, IL-6-174G/C, IL-10-1082G/A and CD-31 125 C/G in controls and patients with VT

Polymorphism	Controls (*n* = 420)	Patients (*n* = 420)	OR (CI95)
TNF-α−308 G/A			
GG	318 (76%)	312 (74%)	1[a]
GA	92 (22%)	101 (24%)	1.1 (0.8–1.5)
AA	10 (2%)	7 (2%)	0.7 (0.2–1.9)
GA+AA	102 (24%)	108 (26%)	1 (0.8–1.5)
LT-α+252 A/G			
AA	198 (47%)	170 (40%)	1[a]
AG	179 (43%)	203 (48%)	1.3 (1.0–1.7)
GG	43 (10%)	47 (11%)	1.3 (0.8–2)
AG+GG	222 (53%)	250 (60%)	1.3 (1–1.7)
IL-6 -174 G/C			
GG	232 (55%)	217 (52%)	1[a]
GC	153 (36%)	172 (41%)	1.2 (0.9–1.6)
CC	35 (9%)	31 (7%)	0.9 (0.5–1.6)
GC+CC	188 (45%)	203 (48%)	1.1 (0.9–1.5)
IL-1ra 86 bp VNTR			
Allele A1 (−)	46 (11%)	30 (7%)	1[a]
Allele A1 (+)	374 (89%)	390 (93%)	1.6 (1–2.5)
IL-10 -1082 G/A			
GG	66 (16%)	57 (13%)	1[a]
GA	176 (42%)	201 (48%)	1.3 (0.9–2)
AA	178 (42%)	162 (39%)	1 (0.7–1.6)
GA+AA	354 (84%)	363 (86%)	1.2 (0.8–1.7)
CD-31 125C/G			
CC	95 (23%)	109 (26%)	1[a]
CG	212 (50%)	205 (49%)	0.8 (0.6–1.2)
GG	113 (27%)	106 (25%)	0.8 (0.5–1.1)
CG+GG	325 (77%)	311 (74%)	0.8 (0.6–1.1)

[a] Reference category.

Table 4 TNF-α−308 G/A, LT-α+252 A/G, IL-6-174G/C, IL-1ra 86bp VNTR, IL-10-1082G/A and CD-31 125 C/G polymorphisms: combined inheritance and thrombotic risk

−308 TNF-α	+252 LT-α	Controls (*n*=420)	Patients (*n*=420)	OR (CI95)
GG	AA	187 (44%)	168 (40%)	1[a]
GA+AA	AG+GG	91 (22%)	106 (25%)	1.3 (0.9–1.8)
−308 TNF-α	−174 IL-6			
GG	GC+CC	225 (53.5%)	188 (44.7%)	1[a]
GA+AA	GG	185 (44%)	188 (44.7%)	1.2 (0.9–1.6)
−308 TNF-α	VNTR IL-1ra			
GG	A1 (−)	37 (31%)	25 (35%)	1[a]
GA+AA	A1 (+)	93 (13%)	103 (17%)	1.6 (0.9–2.9)
−308 TNF-α	−1082 IL-10			
GG	GG	47 (11%)	35 (8%)	1[a]
GA+AA	GA+AA	84 (20%)	86 (20%)	1.3 (0.8–2.3)
−308 TNF-α	125 CD-31			
GG	CC	66 (16%)	84 (20%)	1[a]
GA+AA	CG+GG	73 (17%)	83 (20%)	0.9 (0.6–1.4)
+252 LT-α	−174 IL-6			
AA	GC+CC	92 (22%)	93 (22%)	1[a]
AG+GG	GG	126 (30%)	140 (33%)	1 (0.7–1.6)
+252 LT-α	VNTR IL-1ra			
AA	A1 (−)	22 (5%)	18 (4%)	1[a]
AG+GG	A1 (+)	198 (47%)	238 (56%)	1.4 (0.7–2.8)
+252 LT-α	−1082 IL-10			
AA	GG	34 (8%)	18 (4%)	1[a]
AG+GG	GA+AA	191 (45%)	211 (50%)	2 (1.1–3.8)
+252 LT-α	125 CD-31			
AA	CC	40 (9%)	51 (12%)	1[a]
AG+GG	CG+GG	167 (39%)	192 (45%)	0.9 (0.6–1.4)
−174 IL-6	VNTR IL-1ra			
GC+CC	A1 (−)	25 (5%)	16 (3%)	1[a]
GG	A1 (+)	211 (50%)	203 (48%)	1.5 (0.8–2.9)
−174 IL-6	−1082 IL-10			
GC+CC	GG	34 (8%)	23 (5%)	1[a]
GG	GA+AA	202 (48%)	183 (43%)	1.3 (0.7–2.3)
−174 IL-6	125 CD-31			
GC+CC	CC	43 (10%)	57 (20%)	1[a]
GG	CG+GG	180 (42%)	165 (39%)	0.7 (0.4–1.0)
VNTR IL-1ra	−1082 IL-10			
A1 (−)	GG	5 (1%)	3 (0,7%)	1[a]
A1 (+)	GA+AA	314 (75%)	336 (80%)	1.8 (0.4–7.5)
VNTR IL-1ra	125 CD-31			
A1 (−)	CC	13 (3%)	5 (1%)	1[a]
A1 (+)	CG+GG	292 (69%)	286 (68%)	0.4 (0.1–1.1)
−1082 IL-10	125 CD-31			
GG	CC	12 (10%)	5 (20%)	1[a]
GA+AA	CG+GG	272 (42%)	273 (39%)	2.4 (0.8–7.0)

[a] Reference category.

yielded an overall OR for VT related to the CD-31 polymorphism of 0.8 (CI95: 0.6–1.1) (Table 3). The OR for homozygotes was 0.8 (CI95: 0.5–1.1), and for heterozygotes the OR was 0.8 (CI95: 0.6–1.2).

Interaction between TNF-α−308 G/A, LT-α+252 A/G, IL-6-174G/C, IL-1ra 86 bp VNTR, IL-10-1082G/A and CD-31 125 C/G

The frequencies of the co-inherited genotypes, and the correspondent OR and the CI95, are shown in Table 4. Significant interaction between polymorphisms was observed only for the co-inheritance of the mutant alleles (AG+ GG genotypes) of the LT-α+252 A/G polymorphism and the GA+ AA genotypes of the IL-10-1082 G/A polymorphism. The wild LT-α genotype co-inherited with the wild IL-10 alleles was found in 34/420 controls (carrier frequency 8%) and in 18/420 patients with VT (carrier frequency 4%), and the co-inheritance of the mutant LT-α with the mutant IL-10 alleles was found in 191/420 controls (carrier frequency 45%) and in 211/420 patients with VT (carrier frequency 50%). These data yielded an

Table 5 TNF-α−308 G/A, LT-α+252 A/G, IL-6-174G/C, IL-1ra 86bp VNTR, IL-10-1082G/A, CD-31 125 C/G: co-inheritance with FV Leiden and thrombotic risk

−308 TNF-α	FVL	Controls (*n*=420)	Patients (*n*=420)	OR (CI95)
GG	−	311 (74%)	281 (67%)	1[a]
GA+AA	+	3 (0.7%)	13 (3%)	4.8 (1.3–17)
+252 LT-α	FVL			
AA	−	194 (46%)	151 (36%)	1[a]
AG+GG	+	6 (1.4%)	25 (6%)	5 (2–13)
−174 IL-6	FVL			
GC+CC	−	185 (44%)	188 (44%)	1[a]
GG	+	7 (2%)	29 (7%)	4 (1.7–9.5)
VNTR IL-1ra	FVL			
A1 (−)	−	46 (11%)	27 (6%)	1[a]
A1 (+)	+	10 (2.4%)	39 (9%)	6.6 (2.9–15.4)
−1082 IL-10	FVL			
GG	−	65 (15%)	53 (13%)	1[a]
GA+AA	+	10 (2%)	40 (9.5%)	5 (2.2–11)
125 CD-31	FVL			
CC	−	93 (15%)	92 (13%)	1[a]
CG+GG	+	8 (2%)	27 (9.5%)	3.4 (1.5–8)

[a] Reference category.

overall OR for VT of 2 (CI95: 1.1–3.8) related to the wild LT-α plus wild IL-10 alleles (Table 4).

Interaction between cytokine gene polymorphisms, factor V Leiden and factor II G20210A

We also examined the possibility that each cytokine gene polymorphism tested in this study might interact with FVL and FII G20210A. The overall OR for VT associated with FVL was 4.7 (CI95: 2.3–9.4), and for FII G20210A the OR was 7.1 (CI95: 2.5–20.6) (prevalence of both mutations in patients and controls are shown in Table 1). Similar frequencies of all studied polymorphisms were found in patients and controls carrying or not carrying FVL or FII G20210A. The OR and respective CI95 calculated for each scenario of co-inheritance of FVL/FII G20210A with a given cytokine gene variant show an overlap with the OR and CI95 for FVL and FII G20210A, indicating that neither of the two prothrombotic mutations interacts with the cytokine polymorphisms investigated (Tables 5 and 6).

Discussion

We tested the hypothesis that specific polymorphisms in cytokine genes are risk factors for VT. Additionally,

Table 6 TNF-α−308 G/A, LT-α+252 A/G, IL-6-174G/C, IL-1ra 86bp VNTR, IL-10-1082G/A, CD-31 125 C/G: co-inheritance with FII G20210A and thrombotic risk

−308 TNF-α	FIIG20210A	Controls (*n*=420)	Patients (*n*=420)	OR (CI95)
GG	−	316 (75%)	289 (69%)	1[a]
GA+AA	+	3 (0,7%)	5 (1%)	1.8 (0.4–7.7)
+252 LT-α	FIIG20210A			
AA	−	196 (47%)	156 (37%)	1[a]
AG+GG	+	3 (0,7%)	14 (3%)	5.9 (1.6–21)
−174 IL-6	FIIG20210A			
GC+CC	−	185 (44%)	188 (44%)	1[a]
GG	+	2 (0,5%)	13 (3%)	6.4 (1.4–29)
VNTR IL-1ra	FIIG20210A			
A1 (−)	−	45 (11%)	28 (7%)	1[a]
A1 (+)	+	4 (1%)	26 (6%)	10.4 (3.3–33)
−1082 IL-10	FIIG20210A			
GG	−	65 (44%)	54 (13%)	1[a]
GA+AA	+	5 (0,5%)	25 (6%)	6 (2–17)
125 CD-31	FIIG20210A			
CC	−	93 (44%)	101 (13%)	1[a]
CG+GG	+	2 (0,5%)	20 (6%)	9 (2–40)

[a] Reference category.

we searched for interactions between the cytokine polymorphisms and between these gene variants and the prothrombotic mutations FVL and FII G20210A.

The prevalence of TNF-α−308 G/A, LT-α+252 A/G, IL-6-174 G/C, IL-1ra 86 bp VNTR, IL-10-1082G/A and CD-31 125 C/G polymorphisms did not differ significantly between patients with VT and controls, isolated or combined two by two. One exception might be the co-inheritance of mutant alleles for LT-α+252 A/G and IL-10-1082 G/A, which was found to be associated with a two-fold increase in thrombotic risk. However, this finding requires confirmation. The risk of VT conferred by FVL and FII G20210A was not substantially altered by co-inheritance with any of the cytokine gene polymorphisms tested.

A previous study has examined the TNF α 308 G/A polymorphism as a candidate risk factor for VT [35]. The work enrolled 575 patients with VT and 511 controls, and did not detect a significant risk for VT in relation to TNF α 308 G/A polymorphism, isolated or in combination with FVL. Our findings agree with those results, and taken together the data from both studies do not point to a relevant role of this gene variation as risk factor in VT.

In spite of several evidences from literature pointing to a link between *in vitro* levels of TNF α, IL 1 and IL 6 and hypercoagulability [3–5,11–15], and between plasma levels of TNF α and IL 6 and increased thrombotic risk in animals [36] and humans [6,8–10,18], the negative findings from the present investigation may lead us to speculate that increased cytokine secretion generated by isolated mutants alleles is insufficient to trigger a prothrombotic state with clinical repercussion. In agreement with such possibility are the findings of a work showing that administration of maximal doses of TNF α to humans does not cause VT [37].

The literature associates less intensity of thrombosis, in murine models, with high levels of IL 10 [16,38,39], and other data suggest a tendency to a lower risk of VT in patients with high plasma levels of this cytokine [18]. Here again, we might suggest that the lower levels of IL 10, associated with carriership of the A allele of the IL 10-1082 G/A polymorphism, are not sufficient to trigger VT. Still with regards to the IL-10 polymorphism, it should be mentioned that the evidence for an association between low IL-10 levels and VT derive mainly from animal studies, so that it cannot be ruled out that the absence of a thrombotic risk associated to the A allele in the incidence of VT is due to a variable response of the cytokine in relation to the species studied. Such a possibility was also reported for TNF-α [40].

The LT-α+252A/G polymorphism was characterised as a risk factor for myocardial infarction (MI) in two studies [41,42] and the CD-31 125C/G was found to be a risk factor for GVHD in bone marrow transplantation in humans [28] and also risk factor for MI [43]. Despite these data suggesting a participation of these polymorphisms in the risk of diseases in which inflammation plays a role, these variants did not influence the risk of venous thrombotic disease in the present study.

With regards to the two-fold increase in venous thrombotic risk associated to the co-inheritance of the LT-α+252 A/G and IL-10-1082G/A, it may be suggested that the existence of a pro-inflammatory state in this scenario gives rise to a higher production of LT-α and low production of IL-10, and this specific combination may be capable of inducing a procoagulant state and of triggering VT. However, there is a clear need for additional studies to explore such a hypothesis as well as to confirm the suggestion of the association yielded by our investigation.

To sum, despite evidences that VT and inflammation are two closely related entities, and that several cytokine gene polymorphisms influence cytokine production and may be causally related to some complex traits, the results from our investigation indicate that (either isolated or combined) the cytokine genetic variations here investigated are unlikely to contribute to the risk of VT.

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