

# Activation of innate immunity in patients with venous thrombosis: the Leiden Thrombophilia Study

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**Summary.** *Background:* Previous studies have suggested that levels of inflammatory mediators are risk indicators for venous thrombotic disease. We have sought to confirm and extend these findings by measuring plasma tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-10 and IL-12p70 levels in a case-control study for venous thrombotic disease. *Methods:* The plasma levels of these inflammatory mediators were measured by flow cytometric analysis using a multiplexed bead assay. Patient and control samples came from the Leiden Thrombophilia Study (474 controls and 474 patients). *Results:* In a subset of patients and controls inflammatory mediators are detectable in plasma. The crude odds ratios (ORs) associated with the presence of detectable markers were 2.1 [TNF- $\alpha$ , 95% confidence interval (CI) 1.1, 3.9], 1.7 (IL-1 $\beta$ , 95% CI 1.1, 2.9), 2.4 (IL-6, 95% CI 1.9, 5.3), 2.8 (IL-8, 95% CI 1.8, 4.4), 0.8 (IL-10, 95% CI 0.3, 1.8), and 1.3 (IL-12p70, 95% CI 0.9, 2.0). Adjustment for putative confounders did not influence the risk estimates. *Conclusion:* TNF- $\alpha$ , IL-6, and IL-8 levels are risk determinants for venous thrombosis. Individuals with detectable levels of either of these mediators in plasma have an OR of about 2. In line with these findings, the odds for the anti-inflammatory cytokine IL-10 tend to be < 1. These results add further evidence for the contention that there is an inflammatory component to venous thrombotic disease and may explain why anti-inflammatory agents such as aspirin may be effective for prevention.

**Keywords:** chemokine, cytokine, inflammation, risk factor, venous thrombosis.

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## Introduction

Venous thrombosis is a common disorder with an annual incidence of 1–3 per 1000 [1,2]. It manifests itself mainly as deep-vein thrombosis of the leg and pulmonary embolism. Classical risk factors are immobilization (plaster casts, surgery, air travel) and cancer [3]. In recent decades, much progress has been made in the identification of hypercoagulability as a risk factor for thrombosis. These include abnormalities in the coagulation system (deficiencies of protein C, protein S, antithrombin, factor V Leiden, prothrombin 20210A, high levels of clotting factor (F)VIII, factor IX, factor XI) as well as the use of female hormones (oral contraceptives, postmenopausal replacement therapy) [4,5].

Recently, several studies have suggested a role for inflammatory markers in the etiology of venous thrombosis, following similar reports in arterial disease [6,7]. Even though venous thrombosis is not characterized by a chronic systemic inflammatory disease such as atherosclerosis in arterial disease, we hypothesized that an inflammatory response, regardless of its origin, could lead to hypercoagulability and hence increase the risk of venous thrombosis. Previously we have shown increased levels of interleukin (IL)-8 in patients with recurrent thrombosis and in patients with a first event of venous thrombosis compared with healthy controls [7]. We also found elevated levels of C-reactive protein (CRP) in patients who had suffered thrombosis [8]. We have sought to confirm and extend these findings by measuring plasma tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, and heterodimeric IL-12 (IL-12p70) levels in a case-control study for venous thrombotic disease.

## Methods

### *Patients and controls*

Patients were participants in the Leiden Thrombophilia Study, and were consecutive patients diagnosed by objective methods with a first deep vein thrombosis between 1988 and 1992, recruited from three Anticoagulation Clinics [9]. Inclusion criteria were age 18–70 years and the absence of overt

malignancy. Controls were recruited via the patients and were either friends of patients or partners of (other) patients. Controls were matched for age ( $\pm 5$  years) and sex.

All patients and controls were interviewed in person with regard to putative risk factors for thrombosis, comorbid conditions and use of medicinal drugs. Patients were seen at least 6 months after the discontinuation of oral anticoagulant treatment for blood collection (unless this treatment was prescribed indefinitely and could not be interrupted). Blood was taken in Sarstedt Monovettes, as 106 mm citrated plasma and stored at  $-70^{\circ}\text{C}$ .

#### Cytokine assays

A commercially available multiplex cytometric bead assay was employed for the simultaneous measurement of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70, and TNF- $\alpha$  (BD Biosciences, Alphen aan den Rijn, the Netherlands). In brief, 10  $\mu\text{L}$  of thawed plasma were mixed with 10  $\mu\text{L}$  1 : 10 diluted capture bead suspension in a sample tube. Subsequently 10  $\mu\text{L}$  of PE-labeled detection reagent were added to the assay tubes followed by incubation in the dark for 3 h. Wash buffer was added (200  $\mu\text{L}$ ) and the tubes were centrifuged at  $200 \times g$  followed by careful aspiration of the supernatant and gentle resuspension of the bead pellet in 150  $\mu\text{L}$  of wash buffer. All reagents and buffers were supplied by the manufacturer. The finished bead suspensions were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Alphen aan den Rijn, the Netherlands) according to the instructions of the bead assay manufacturer. The detection limit of each of the cytokines was 2.5  $\text{pg mL}^{-1}$ . In total 932 samples were analyzed.

#### Statistical analysis

We calculated odds ratios (ORs) as measures of relative risk, by cross tabulation and logistic regression. Confidence intervals (95% CI) were calculated according to Woolf or derived from the model [10]. Various cut-off points for cytokine levels were used to delineate 'normal' from 'elevated', i.e. in a first analysis as 'not detectable' vs. 'detectable', and in a second analysis for more pronounced elevated levels, i.e.  $> 50 \text{ pg mL}^{-1}$  for IL-12p70,  $> 20 \text{ pg mL}^{-1}$  for IL-1 $\beta$  and IL-8, and  $> 10 \text{ pg mL}^{-1}$  for TNF- $\alpha$ , IL-10 and IL-6. The latter was roughly equivalent to levels in the highest 5% of the distribution in healthy individuals. We performed analyses adjusted for age and sex by logistic modeling. In addition, we examined correlation between levels of various markers with correlation analysis. The effect of time since event on marker levels was assessed by linear regression.

#### Results

We included 474 patients and 474 controls, for whom plasma was available for 470 in each group. In the majority of patients and controls, no inflammatory markers could be detected, while detectable levels were present in varying numbers of patients and controls (2–16% for the various markers). When inflammatory markers were detectable, they had a wide range of values (Table 1).

Detectable levels were found more often in patients than in controls for all markers except IL-10. Table 2 shows the ORs associated with the presence of detectable markers, which were elevated for all markers except IL-10 (and only marginally so

**Table 1** Detectable levels of inflammatory markers in cases and controls

Analyte	Cases ( $n = 470$ )			Controls ( $n = 470$ )		
	Detectable $n$ (%)	Range ( $\text{pg mL}^{-1}$ )	Median ( $\text{pg mL}^{-1}$ )	Detectable $n$ (%)	Range ( $\text{pg mL}^{-1}$ )	Median ( $\text{pg mL}^{-1}$ )
TNF- $\alpha$	30 (6.3)	0–212	15.9	15 (3.2)	0–95.6	12.4
IL-1 $\beta$	40 (8.5)	0–387	12.3	24 (5.1)	0–162	16.4
IL-6	21 (4.4)	0–232	20.5	9 (1.9)	0–192	29.4
IL-8	73 (15.5)	0–213	10.4	29 (6.2)	0–112	7.6
IL-10	10 (2.1)	0–54	10.5	13 (2.8)	0–186	15.3
IL-12p70	56 (11.9)	0–945	20.8	44 (9.4)	0–1384	21.6

For abbreviations see text.

**Table 2** Odds ratios (OR) for the presence of detectable inflammatory markers

Adjustment factors	TNF- $\alpha$ OR (95% CI)	IL-1 $\beta$ OR (95% CI)	IL-6 OR (95% CI)	IL-8 OR (95% CI)	IL-10 OR (95% CI)	IL-12p70 OR (95% CI)
None	2.1 (1.1–3.9)	1.7 (1.1–2.9)	2.4 (1.9–5.3)	2.8 (1.8–4.4)	0.8 (0.3–1.8)	1.3 (0.9–2.0)
Age, sex	2.1	1.7	2.4	2.8	0.8	1.3
Age, sex, CRP	2.0	1.7	2.0	2.7	0.7	1.3
Age, sex, CRP, FVIII	1.8	1.6	1.9	2.5	0.6	1.1

CRP, C-reactive protein; FVIII, coagulation factor VIII. For other abbreviations see text.

for IL-12). We subsequently sought to explain the association by adjustment for putative confounders (viz. sex, age, CRP, coagulation FVIII), which, however, did not affect the risk estimates. In patients, time between the event and blood collection ranged from 6 months to nearly 6 years. In linear regression analysis, there was no association between the time since the event and the level of inflammatory markers (regression coefficient ranging from  $-0.104$  (IL-10) to  $2.5$  (IL-12), i.e. a decrease or an increase per year of  $< 2.5$  pg mL<sup>-1</sup>, with all confidence intervals around the null value).

A second analysis aimed at high levels of IL-12p70 ( $> 50$  pg mL<sup>-1</sup>,  $n = 26$ ), IL-1 $\beta$  ( $> 20$  pg mL<sup>-1</sup>,  $n = 25$ ), IL-8 levels ( $> 20$  pg mL<sup>-1</sup>,  $n = 23$ ), TNF- $\alpha$  ( $> 10$  pg mL<sup>-1</sup>,  $n = 29$ ), IL-10 ( $> 10$  pg mL<sup>-1</sup>,  $n = 16$ ) and IL-6 ( $> 10$  pg mL<sup>-1</sup>,  $n = 22$ ), compared with those with lower or non-detectable levels, led to similar or more pronounced results. For IL-12p70 there was no association with venous thrombosis (OR = 1.0, 95% CI 0.5, 2.2), and for TNF- $\alpha$  (OR = 2.3, 95% CI 1.1, 5.1), IL-6 (OR = 2.2, 95% CI 0.9, 5.4), IL-1 $\beta$  (OR = 1.5, 95% CI 0.7, 3.4) risk estimates were similar to the previous analysis. For high IL-10 levels the association with (the absence of) thrombosis became stronger (OR = 0.4, 95% CI 0.2, 1.3), as was the case for high levels of IL-8 (OR = 4.9, 95% CI 1.7, 15.4).

In summary, TNF- $\alpha$ , IL-6, and IL-8 levels are risk determinants for venous thrombosis. Overall, individuals that have detectable levels of either of these mediators in their plasma have a 2-fold increased risk. For IL-8 in particular, this risk seemed to increase with the actual level observed. In line with these findings, the risk for the anti-inflammatory cytokine IL-10 tended to be decreased. No association was apparent for levels of IL-12, while the association was weak for IL-1 $\beta$  levels.

## Conclusion

The cytokine and chemokine family consists of a multitude of proteins with differing and partly overlapping functions in innate and acquired immunity. We measured the levels of six distinct cytokines and chemokines. We were guided in our choice by the availability of a commercial kit that enabled the simultaneous and rapid measurement of all six proteins in a very small volume of plasma (10  $\mu$ L). TNF- $\alpha$ , IL-6, IL-8, and IL-10 are key players in innate host defense. With the exception of IL-8, these proteins are generally not detectable in plasma of healthy individuals. In human models of controlled inflammation using endotoxemia administration, rapid rises are observed in the blood [11]. In such models the proinflammatory mediator TNF- $\alpha$  appears first, followed by increases in proinflammatory IL-6, IL-8, and the anti-inflammatory mediator IL-10 [11]. IL-1 $\beta$  responds with a minor increase in endotoxemia models, whereas levels of IL-12p70 do not respond to such an inflammatory challenge [12,13].

Remarkably, differences in plasma levels between patients and controls seem to agree well with plasma alterations in endotoxin models. In particular, the fact that the OR for

IL-12p70 is close to 1 agrees with the fact that this cytokine does not respond to endotoxin *in vivo*. These data therefore indicate that the inflammatory state of venous thrombotic individuals represents an activation of the innate immune response.

In this study, blood collection took place after the thrombotic event. Therefore, an inflammatory reaction as a consequence rather than a cause of venous thrombosis cannot be ruled out. The absence of any attenuation of elevated levels with a larger time between the thrombotic event and the date of the blood draw argues against a post-thrombotic effect, unless this would be persistent over protracted periods of time. Prospective studies will be required to elucidate this issue further. If inflammation is indeed a cause of venous thrombosis, this may explain the weak antithrombotic effect of aspirin [14], and identification of individuals with high levels of inflammatory markers could assist in targeting this therapy, which has a lower risk of hemorrhagic complications than standard anticoagulant treatment.

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