Venous and arterial thrombosis : associations and risk factors

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Inflammation markers, D-dimer and the risk of recurrent venous thrombosis

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Abstract

Several studies have suggested an association between inflammation and coagulation. Whether the risk of recurrent venous thrombosis is increased with high levels of cytokines is not fully elucidated. We investigated the associations of cytokine levels (TNF-α, IL-1β, IL-6, IL-8, IL-12p70 and IL-10), CRP and D-dimer with recurrent venous thrombosis in the Leiden Thrombophilia Study (LETS). We included 399 LETS participants who were followed for a mean of 8 years after cessation of treatment for their first venous thrombosis. We compared the recurrence risk for low or high cytokine levels (>10 pg/ml for TNF-α, IL-6 and IL-10, >20 pg/ml for IL-1β and IL-8 and >50 pg/ml for IL-12p70) versus undetectable cytokine levels, for CRP >3 versus ≤3 mg/L and D-dimer >250 versus ≤250 ng/ml. The blood draw took place 19 (range 6-68) months after the initial event. Sixty patients experienced a recurrence (19 per 1000 person years, 95% CI 14-24). Recurrence rates were not higher in individuals with low or high levels of cytokines compared to those with undetectable levels. The adjusted risk for age, sex and BMI was 2.2 (95% CI 1.3-3.8) for elevated CRP and 1.7 (0.9-3.4) for elevated D-dimer. Individuals with either elevated D-dimer or CRP and those with both elevated CRP and D-dimer had higher risk as compared to patients with low CRP and D-dimer levels (HR 1.9; 95% CI 1.1-3.5 and 3.1; 1.4-7.2 respectively).

In conclusion, high levels of CRP and D-dimer are associated with thrombosis recurrence while high levels of pro-inflammatory cytokines are not.

Introduction

Venous thrombosis is an important morbidity and mortality cause in Western society. Each year about 2 per 1000 individuals in Western populations develop venous thrombosis [1;2], 7% (range 3-13%) experience a recurrence in the first year and 12-25% recur over 5 years [3;4]. The risk profile for recurrent venous thrombosis appears to be different from that for a first event [5]. In contrast to the first events, age and most prothrombotic abnormalities do not appear to play an important role in provoking a recurrence in the general population [6;7] while male sex is a risk factor for recurrence but not for the first event [5]. Rare deficiencies of natural anticoagulants and antiphospholipid syndrome at most have a moderate effect on the risk of recurrent venous thrombosis [8-11]. In line with the first event, cancer, continued oestrogen use and obesity are known as determinants of recurrence risk [6;7;12-15]. Hemostatic activation, assessed by D-dimer level, appears to be an important determinant of the risk of recurrent venous thrombosis, although not all studies agree [16-20]. The effect of elevated levels of FVIII and FIX on the risk of recurrent event are either controversial or little discussed [7;21-23].

Several, mainly laboratory studies have suggested a link between coagulation and inflammation [24-26]. Studies evaluating the relation of the risk of first VTE with a promoter polymorphism (-174, G>C) of IL-6, known to influence IL-6 levels [27], or haplotypes of IL1RN failed to prove an association [28;29]. Further, a population-based study in which cytokine levels were measured prior to the first thrombosis showed no association between cytokine levels and the risk of first thrombosis [30]. These findings challenge earlier results that suggested an association between cytokines and the development of a first VTE [31;32]. Data on association of cytokine levels with risk of recurrent venous thrombosis are scarce. One study observed associations of IL-8, IL-6 and MCP-1 levels and recurrent events of venous thrombosis, however it is not mentioned whether the blood was drawn before or after the recurrences [33]. In contrast, the Prevention of Recurrent Venous Thromboembolism trial (PREVENT) study reported no association between genetic variants in 86 candidate genes involved in the inflammatory process and recurrent venous thrombosis [34]. Results of studies investigating the relation between CRP (C-reactive protein) and venous thrombosis are contradictory [35-41]. Altogether, the role of cytokines and CRP in the recurrence of venous thrombosis

is not yet clarified. In this study we aimed to evaluate the risk of recurrent venous thrombosis associated with plasma levels of the inflammatory markers, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, CRP and D-dimer.

Materials and methods

Study population

This study is comprised of all patients who participated as cases in a population-based case-control study; the Leiden Thrombophilia Study (LETS). Details of this study have been published previously [42]. Briefly, 474 patients younger than 70 years of age with a first deep vein thrombosis were recruited from anticoagulation clinics in Leiden, Amsterdam and Rotterdam (the Netherlands) between January 1988 and December 1992. Participants had no overt malignancy. Blood was drawn at least 3 months after discontinuation of oral anticoagulant treatment unless treatment was prescribed indefinitely and could not be stopped (n=48). The median time interval between the first venous thrombosis and the blood draw was 19 (range 6-68) months.

Patients were followed as described previously [7] from the end of initial anticoagulation treatment period until January 1st 2000. Information during follow-up on the occurrence of risk situations, use of anticoagulation treatment, and recurrent events was gathered by repeated mailed screening questionnaires. Patients were interviewed by telephone when they responded positively to any item of the questionnaire or when they did not return it. Information on recurrent events and risk situations was confirmed by contacting patients' physicians. Recurrent events were confirmed by objective diagnostic tests, i.e. ultrasound, venography or impedance plethysmography for deep vein thrombosis and positive perfusion lung scan (at least one segmental perfusion defect), ventilation-perfusion lung scan (intermediate or high probability) or computerized tomography scan for pulmonary thrombosis. Venous events occurring within 90 days of the first event were considered a progression of the initial event. A provoked event was defined as occurring during pregnanacy or puerperium (period of 6 weeks after delivery), during (or within 30 days after the cessation of) oral contraceptive use, during immobilization (period of less than 3 month being immobilized due

to hospitalization or due to being bedridden at home) or within a period of 30 days after major trauma or surgery. The initial and follow-up phases of the LETS were approved by the medical ethics committee of the Leiden University Medical Center and all patients signed an informed consent for the participation in the follow-up phase.

Cytokines, CRP and D-dimer measurement

Blood (0.9 vol) was collected into Sarstedt Monovette tubes containing 0.106 M of trisodium citrate (0.1 vol), prepared by centrifugation for 10 minutes at 2000x*g* and stored in aliquots at –70 °C until assayed. Plasma was available for 470 patients with a first venous thrombosis. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 were measured simultaneously using a commercially available multiplex cytometric bead assay (BD Biosciences, Alphen aan den Rijn, The Netherlands), as described earlier [31]. The detection limit of each of the cytokines was 2.5 pg/ml. CRP was measured by a sandwich enzyme immunoassay (Kordia, Leiden, The Netherlands) based on two polyclonal rabbit antibodies against CRP. D-dimer was measured using ELISA, as described earlier [43].

Statistical Analysis

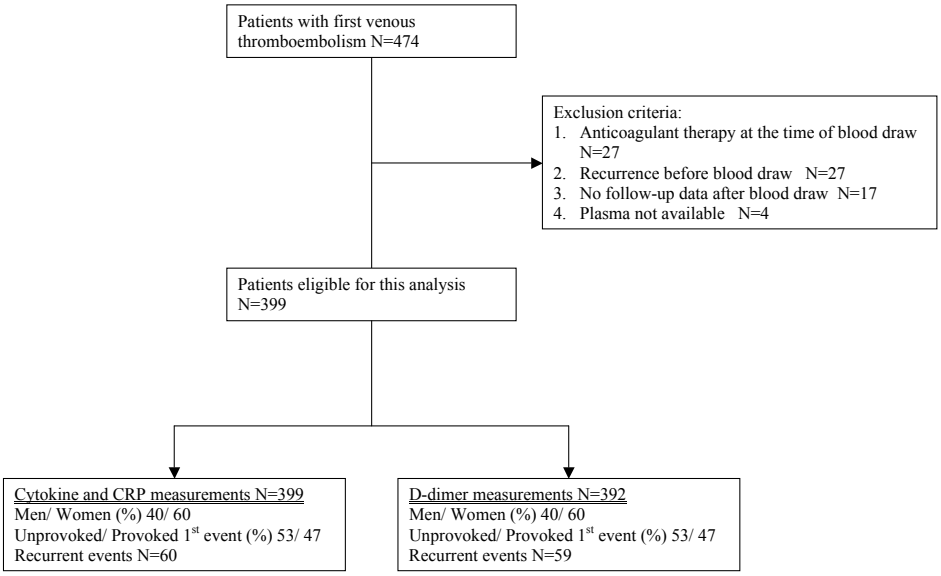
To evaluate the association between cytokine levels CRP, D-dimer and the risk of thrombosis recurrence we excluded the individuals who experienced recurrent venous thrombosis before the blood draw (n=27), who were taking oral anticoagulation therapy at the time of the blood draw (n=27) and those for whom we did not have follow-up information (n=17) from the analysis. (Figure 1)

We compared recurrence rates of thrombosis in patients with low (detectable but not high) or high levels of cytokines with patients with undetectable cytokine levels. We applied the same cut-off levels as used earlier in the LETS to discriminate low and high cytokine levels [31]. These levels are approximately equivalent to the 95th percentile in healthy individuals: above 10 pg/ml for TNF-α, IL-6 and IL-10, above 20 pg/ml for IL-1β and IL-8 and above 50 pg/ml for IL-12p70. The 95th percentile of D-dimer levels (250 ng/ml) in the LETS control population and the clinical cardiovascular risk cut-off level (3 mg/L) for CRP [44] were used as the cut-off points for high CRP and D-dimer levels. Follow-up time was defined from 90 days after the initial event until the recurrent event, death, or the last date

of follow-up, whichever occurred first. As cut-off points might be arbitrary, we also addressed other cut-off points for CRP and D-dimer; 50th, 67th, 80th and 90th percentiles of the control group distributions. Patients with CRP and D-dimer levels below the 50th percentile level in the LETS controls served as the reference group in this sensitivity analysis. Besides, we compared the risk of recurrence in patients with both elevated D-dimer and CRP or patients with either of them to patients whose D-dimer and CRP were below the cut-off.

Recurrence rates were calculated by dividing the number of recurrent events by the sum of the follow-up years. Ninety-five percent confidence intervals (95% CIs) were calculated according to Poisson distribution for the number of events [45]. Hazard ratios (HR), as estimation of the relative risk, were calculated using Cox regression. We adjusted hazard ratios for possible confounders like age, sex and body mass index (BMI; kg/m^2) and stratified the adjusted hazard ratios according to recurrence location (i.e. contralateral vs ipsilateral) and type of first or recurrent event (unprovoked vs provoked). To calculate the risk of unprovoked recurrences, we subtracted periods of exposure to risk factors (i.e. oral contraceptive use, pregnancy, trauma, surgery and immobilization) and the use of oral anticoagulants from the follow-up duration. Moreover, because of the broad range of time between the initial event and the blood draw, we computed the recurrence risks for high D-dimer or CRP stratifying by time elapsed between the first venous thrombosis and the blood draw (in tertiles)

Figure 1. Flow-chart of selection of patients for the analysis



Results

A total of 399 LETS patients were eligible for this analysis (figure 1). The mean age of the patients was 45 (range 14-69) years at the start of follow-up and 160 (40%) of them were men. Approximately half (n=210, 53%) of the first thromboses occurred in the absence of known risk factors for venous thrombosis. The mean follow-up duration was 8 years (range 1-12). In the 399 patients 60 (15%) recurrent events occurred with an incidence of 19 per 1000 person years (95% CI 14-24). The recurrences were located in the leg (n=47), arm (n=3), lung (n=7), leg and lung (n=2) and Budd-Chiari syndrome with extension into the vena cava (n=1).

Table 1 shows the mean, median and range of cytokines, CRP and D-dimer levels for individuals with detectable levels. Few patients had high levels of the cytokines (1 to 4% for the various cytokines). Elevated CRP (>3 mg/l) was seen in 107 patients (27%) and high D-dimer (>250 ng/ml) in 49 patients (13%).

The incidence rate of recurrent venous thrombosis was similar in individuals with high pro-inflammatory cytokines (i.e. all cytokines except IL-10) (n=38; 20 per 1000 person years; 95% CI 7-47) and those with undetectable pro-inflammatory levels (n=301; 22 per 1000 person years; 95% CI 16-30). The recurrence rate in individuals with detectable but not high levels (n=60) of at least one pro-inflammatory cytokine was 30 per 1000 person years (95% CI 15-54). The adjusted relative risks of recurrent venous thrombosis for age, sex and BMI in individuals with high and detectable (but not high) levels of at least one pro-inflammatory cytokine compared to those with undetectable levels were also not increased; hazard ratios were 1.0 (95% CI 0.6-1.5) for individuals with high levels and 1.3 (95% CI 0.7-2.4) for those with detectable but not high levels (Table 2). Similarly, no increased risks were found for each separate cytokine (Tables 3 and 4). None of the four individuals with high or low levels of the anti-inflammatory cytokine IL-10 had recurrent events.

The incidence of recurrent event in patients with elevated CRP (n=107) was 37 per 1000 person years (95% CI 24-55), which was higher than the incidence in patients with low CRP (n=292, 18 per 1000 person years; 95% CI 13-25). The corresponding hazard ratio for recurrence with elevated CRP, adjusted for age, sex and BMI, was 2.2 (95% CI 1.3-3.8). Elevated CRP was most predictive when

blood was drawn within 14 months after the index event (i.e. the first tertile of the period between the first venous thrombosis and blood draw) (HR 2.5, 95% CI 1.1-5.8). The HRs in de second (blood draw between 14 and 24 month of the first event) and third tertile (blood draw after 24 month of the first event) were 1.8 (95% CI 0.7-4.9) and 2.0 (95% CI 0.7-5.5).

The recurrence rate for levels of D-dimer above 250 ng/ml was 38 per 1000 person years (95% CI: 19-68), which was higher than the recurrence rate in patients with levels below 250 ng/ml (21 per 1000 person years; 95% CI 16-28). The adjusted hazard ratio was 1.7 (95% CI 0.9-3.4) for patients with high D-dimer levels compared to those with low levels. Similar to CRP, high levels of D-dimer were only associated with higher risk of recurrence when blood was drawn within 14 months after the index event (HR 5.8, 95% CI 2.1-16.2). The HRs in de second (blood draw between 14 and 24 month of the first event) and third tertile (blood draw after 24 month of the first event) were 1.2 (95% CI 0.3-4.1) and 0.7 (95% CI 0.1-3.1). Compared to patients with low levels of CRP and D-dimer, the recurrence risk was increased for individuals with either elevated D-dimer or CRP (HR 1.9; 95% CI 1.1-3.5) and for subjects with both elevated D-dimer and CRP (HR 3.1; 95% CI 1.4-7.2).

The adjusted hazard ratios did not substantially change when stratifying for the location of the recurrent event (contralateral versus ipsilateral), except for D-dimer (Table 4). Patients with elevated D-dimer showed a higher risk for recurrence in the contralateral leg (HR 3.1; 95% CI: 1.2-8.3) than in the ipsilateral leg (HR 0.9; 95% CI: 0.3-3.2).

Hazard ratios for the cytokines and CRP did not differ when stratifying for the type of recurrent event, i.e. unprovoked vs. provoked (data not shown). High D-dimer and CRP levels seemed to increase the risk of recurrence especially in those with provoked first events (HR 3.5; 95% CI 1.3-9.6, HR 3.6; 95% CI 1.5-8.7 respectively for D-dimer and CRP) compared to those with unprovoked first events (HR 1.2; 95% CI 0.5-2.9, HR 1.7; 95% CI 0.9-3.4). Evaluation of different cutpoints defining elevated CRP or D-dimer did not reveal a threshold that might perform better in identifying risk status. (Table 5)

Table 1. Mean, median and range of inflammatory markers and D-dimer levels in individuals with detectable levels

Analyte	N (detectable)	Mean	Median	Range	5th-95th percentile range
TNF-α*	26	28	14	5-212	5-160
IL-1β*	33	32	12	5-161	6-131
IL-6*	19	25	18	7-81	7-81
IL-8*	57	21	10	5-120	5-80
IL-10*	6	22	18	7-44	7-44
IL-12p70*	42	53	21	5-259	6-218
CRP#	399	3.8	1.4	0.0-142.3	0.2-15.8
D-dimer$	392	168	109	11-1946	35-671

Abbreviations: TNF=tumor necrosis factor, IL=interleukin, CRP=C-reactive protein.
Unites: * pg/ml, # mg/l, $ ng/ml

Table 2. Incidence and risk of recurrent thrombosis in individuals with undetectable, low and high levels of at least one pro-inflammatory cytokines

	Total N	Number of recurrences (%)	Incidence of recurrent thrombosis per 1000 person years (95% CI)	Adjusted HR** (95% CI)
Undetectable level of at least one pro-inflammatory cytokine	301	44 (15)	22 (16-30)	Ref.
Low level of at least one pro-inflammatory cytokine	60	11 (18)	30 (15-54)	1.3 (0.7-2.4)
High level of at least one pro-inflammatory cytokine	38	5 (13)	20 (7-47)	1.0 (0.6-1.5)

**Adjusted for age, sex and BMI

Table 3. Incidence of recurrent events associated with inflammatory markers and D-dimer

	N	Recurrent events N (%)	Incidence per 1000 person-year (95% CI)
TNF-α*			
Non-detectable	373	57 (15)	23 (18-30)
0-10	9	1 (11)	17 (0.4-95)
>10	17	2 (18)	18 (2-66)
IL-1β*			
Non-detectable	366	54 (15)	23 (17-29)
0-20	21	4 (19)	32 (9-81)
>20	12	2 (17)	25 (3-90)
IL-6*			
Non-detectable	380	56 (15)	23 (17-29)
0-10	6	1 (17)	23 (0.6-127)
>10	13	3 (23)	40 (8-116)
IL-8*			
Non-detectable	342	50 (15)	22 (16-29)
0-20	43	9 (21)	37 (17-70)
>20	14	1 (7)	10 (0.3-58)
IL-10*			
Non-detectable	393	59 (15)	23 (18-30)
0-10	2	1 (50)	108 (3-599)
>10	4	0 (0)	0 (0.0-132)
IL-12p70*			
Non-detectable	357	54 (15)	23 (17-30)
0-50	31	4 (13)	20 (5-50)
>50	11	2 (18)	30 (3-99)
CRP#			
≤ 3	292	36 (12)	18 (13-25)
> 3	107	24 (22)	37 (24-55)
D-dimer$			
≤ 250	343	48 (14)	21 (16-28)
> 250	49	11 (22)	38 (19-68)

Abbreviations: BD=blood draw, TNF=tumor necrosis factor, IL=interleukin, CRP=C-reactive protein. Unites: * pg/ml, # mg/l, $ ng/ml

Table 4. Hazard ratios of recurrent events associated with inflammatory markers and D-dimer level

Marker	Crude HR	Adjusted HR**				
		All recurrences N=60	Contralateral recurrences N=21	Ipsilateral recurrences N=27	Unprovoked 1st event N=210	Provoked 1st event N=189
TNF-α*						
0-10	0.8 (0.1-5.6)	0.8 (0.1-5.5)	0.8 (0.3-2.3)	1.0 (0.4-2.3)	0.9 (0.1-6.6)	***
>10	0.8 (0.2-3.4)	0.6 (0.2-2.6)	0.8 (0.1-5.7)	0.8 (0.1-5.7)	0.8 (0.2-3.3)	***
IL-1β*						
0-20	1.4 (0.5-4.0)	1.4 (0.5-3.7)	1.4 (0.6-3.1)	1.2 (0.5-2.7)	1.4 (0.4-4.7)	1.2 (0.2-8.8)
>20	1.1 (0.3-4.6)	1.0 (0.2-4.2)	1.4 (0.2-10.5)	1.2 (0.2-8.7)	0.7 (0.1-4.8)	3.2 (0.4-27.5)
IL-6*						
0-10	1.0 (0.1-7.0)	0.6 (0.1-4.6)	1.3 (0.5-3.2)	1.5 (0.7-3.3)	0.7 (0.1-5.3)	***
>10	1.9 (0.6-6.0)	1.8 (0.6-5.7)	1.6 (0.2-11.6)	3.2 (0.7-13.7)	1.5 (0.4-6.4)	3.1 (0.3-28)
IL-8*						
0-20	1.7 (0.9-3.6)	1.7 (0.8-3.5)	0.9 (0.4-2.2)	1.2 (0.5-2.5)	1.8 (0.8-3.9)	1.2 (0.2-9.1)
>20	0.5 (0.1-3.6)	0.5 (0.1-3.4)	***	1.1 (0.1-8.5)	0.6 (0.1-4.5)	***
IL-10*						
0-10	5.0 (0.7-36.0)	5.0 (0.7-37.1)	***	1.0 (0.2-4.0)	7.5 (0.9-58.1)	***
>10	***	***	***	***	***	***
IL-12p70*						
0-50	0.9 (0.3-2.4)	0.8 (0.3-2.3)	1.2 (0.5-2.8)	0.8 (0.3-2.1)	0.9 (0.3-3.1)	0.5 (0.1-4.1)
>50	1.2 (0.3-4.9)	1.0 (0.2-3.9)	1.4 (0.2-10.6)	1.0 (0.1-7.2)	0.6 (0.1-4.2)	5.4 (0.6-50.5)
CRP#						
> 3	2.0 (1.2-3.4)	2.2 (1.3-3.8)	2.6 (1.1-6.3)	2.2 (0.9-4.8)	1.7 (0.9-3.4)	3.6 (1.5-8.7)
D-dimer$						
> 250	1.8 (0.9-3.4)	1.7 (0.9-3.4)	3.1 (1.2-8.3)	0.9 (0.3-3.2)	1.2 (0.5-2.9)	3.5 (1.3-9.6)

The reference group was patients with non-detectable levels for cytokines, £3 mg/l for CRP and £250 ng/ml for D-dimer .***No recurrent events occurred

Table 5. Hazard ratios of recurrent events for different cut-off levels of CRP and D-dimer

	Cut-off level*#	N	Recurrent events N (%)	Crude HR	Adjusted HR**
CRP					
< 50 %		169	21 (14)	Ref	Ref
> 50 %	1.1	230	39 (20)	1.5 (0.9-2.5)	1.6 (0.9-2.8)
> 67 %	2.0	148	27 (22)	1.6 (0.9-2.8)	1.7 (1.0-3.2)
> 75 %	2.5	117	25 (27)	1.9 (1.1-3.4)	2.2 (1.2-4.0)
> 80 %	3.0	105	24 (30)	2.1 (1.2-3.7)	2.3 (1.2-4.3)
> 90 %	5.1	64	13 (26)	1.9 (0.9-3.7)	2.0 (1.0-4.0.0)
> 95 %	9.7	42	9 (27)	1.9 (0.9-4.2)	1.9 (0.8-4.5)
D-dimer					
< 50 %		128	17 (15)	Ref	Ref
> 50 %	74.0	264	42 (19)	1.3 (0.7-2.2)	1.4 (0.8-2.6)
> 67 %	103.0	204	31 (18)	1.2 (0.7-2.2)	1.4 (0.7-2.6)
> 75 %	121.4	171	27 (19)	1.3 (0.7-2.4)	1.5 (0.8-2.8)
> 80 %	136.3	145	24 (20)	1.4 (0.7-2.5)	1.6 (0.8-3.1)
> 90 %	185	91	16 (21)	1.5 (0.7-2.9)	1.7 (0.8-3.5)
> 95 %	250	49	11 (22)	1.8 (0.9-3.4)	1.7 (0.9-3.4)

* The cut-off levels are applied from the LET's controls.
# mg/l for CRP and ng/ml for D-dimer. **Adjusted for age, sex and BMI

Discussion

In this prospective study, we observed that "low detectable" or high levels of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70) do not increase the risk of a second thrombosis. Elevated levels of CRP (> 3 mg/d) were associated with an increased risk of recurrent venous thrombosis (adjusted HR 2.2; 95% CI 1.3-3.6), as well as D-dimer levels above 250 ng/ml (adjusted HR 1.7; 0.9-3.4).

To our best knowledge this is the first study evaluating the association of inflammatory markers and the risk of thrombosis recurrence. Several studies have

suggested that increased levels of inflammatory factors might be a result and not a cause of venous thrombosis. [46-48] This may explain why we could not demonstrate an association between cytokines and recurrent disease. It may also explain the small percentage of patients showing elevated levels of the various cytokines in our study (range 1-4%), as blood from all patients was drawn at least six months after the initial event. We did find a 2-fold increased risk of recurrence though in patients with elevated CRP levels. The clinical implication of this finding needs further investigation. The association we observed between high D-dimer levels and thrombosis recurrence (HR 1.7, 95% CI: 0.9-3.4), confirms previous studies, which found a 2 to 3-fold higher risk of recurrence associated with D-dimer levels more than 250 ng/ml [49-51].

A systemic increase in inflammatory markers in patients with venous thrombosis might reflect locally damaged veins and might therefore predict ipsilateral recurrent events. However, we did not find such an association, which is consistent with studies showing that residual vein thrombosis also correlates with contralateral recurrent events [50;52]. It can, however, not be excluded that plasma concentrations of inflammatory markers do not reflect the situation at the thrombus site. Interestingly, we have observed that increased recurrence risk for elevated CRP and D-dimer was present only in patients who had their blood drawn within 14 months after the initial event. However, these results should be interpreted with caution because few recurrent events happened in the second and third tertile of the time between the initial event and the blood draw especially for D-dimer risk estimation.

Our study has several limitations. Firstly, the measurements were only done once which precluded the evaluation of the association of persistent elevation of cytokines on the risk of thrombosis recurrence. Also, since the average time interval between the blood draw and the index thrombotic event was 19 months, we were unable to calculate the recurrence risk within the first year of thrombotic events when the absolute risk of recurrence is the highest.

In conclusion, elevated levels of CRP and D-dimer appear to increase the risk of venous thrombosis recurrence while high levels of pro-inflammatory cytokine do not. Furthermore, individuals with simultaneous elevated CRP and D-dimer have the most risk of recurrence compared to those with none of them elevated.

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