

Inflammation, TNF α and endothelial dysfunction link lenalidomide to venous thrombosis in chronic lymphocytic leukemia

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Patients receiving lenalidomide are at an increased risk for deep venous thrombosis (DVT). Here, we prospectively investigated the DVT risk in patients with relapsed chronic lymphocytic leukemia (CLL) treated with lenalidomide ($n = 32$). Five patients developed six incidents of DVT over 1 year for an annual incidence of 16%. Three of these were considered drug-related. Median time to DVT was 105 days (range 56–259 days). No pulmonary embolism was detected. Hypercoagulability screen before study entry was negative in all patients who subsequently developed DVTs. Compared to normal volunteers CLL patients had increased baseline levels of D-dimer, thrombin-antithrombin, soluble vascular endothelial adhesion molecule 1 (sVCAM-1), and thrombomodulin ($p < 0.001$). After 1 week on lenalidomide D-dimer, thrombomodulin, sVCAM-1, factor VIII, TNF α , and C-reactive protein were significantly increased while protein C was decreased ($p < 0.001$). In patients with lenalidomide-related DVTs, TNF α , and sVCAM-1 were more strongly upregulated than in all other patients ($p < 0.05$) and TNF α and sVCAM-1 levels were significantly correlated ($r = 0.65$, $p < 0.001$). These data link lenalidomide associated DVTs with TNF α upregulation and endothelial cell dysfunction and suggest that aspirin may have a role for DVT prophylaxis in these patients. *Am. J. Hematol.* 86:835–840, 2011. © 2011 Wiley-Liss, Inc.

Introduction

The immunomodulating drugs (IMiDs) thalidomide and lenalidomide have shown promising activity in hematologic malignancies [1]. However, there is an increased risk of venous thromboembolism (VTE) in patients on IMiDs. The risk with single agent therapy appears low; for lenalidomide the incidence of VTE in multiple myeloma (MM) is generally <5% [2], and in patients with myelodysplastic syndrome the incidence is <1% [3]. In patients enrolled on phase II trials of single agent lenalidomide for relapsed chronic lymphocytic leukemia (CLL), pulmonary embolism has been reported in 2 of 45 patients [4] and a deep venous thrombosis (DVT) occurred in 1 of 44 patients [5], suggesting that the incidence of VTE in CLL and MM may be comparable. Because of the relatively low risk, VTE prophylaxis has not been routinely used in patients treated with single agent IMiDs [6]. In contrast, patients treated with IMiD-based combination therapies are at high risk for VTE, which in some studies affected more than 50% of patients with MM [6]. Several regimens for VTE prophylaxis have been shown to be efficacious in patients treated with IMiD-based combination regimens [6–8]. Although the American College of Chest Physicians strongly recommends against the use of aspirin for VTE prophylaxis in high-risk cancer patients [9], two expert panels recently established guidelines for MM patients undergoing IMiD-based combination therapy that recommend aspirin (81–325 mg) for patients with no or one risk factor and LMWH 40 mg daily or warfarin (INR 2–3) for patients at high risk for DVT [6,10]. There is an increased risk of bleeding during VTE prophylaxis in patients with liver or renal failure, or thrombocytopenia [6]. Thrombocytopenia, in particular, is a common side effect of lenalidomide in CLL [4,5].

Recommendations for prophylaxis are complicated by the fact that the factors contributing to the increased risk of VTE during IMiD therapy are not well understood [11]. In MM, none of the classical coagulation parameters were associated with increased risk of VTE [12]. In MM, several disease-specific factors related to the presence of the monoclonal protein are thought to contribute to a hypercoagulable state including acquired protein C resistance [12] and impaired

fibrinolysis [13]. Other factors described in MM that shift the balance toward activation of coagulation include high levels of von Willebrand factor antigen, plasminogen activator inhibitor-1 (PAI-1), and changes in endothelial cell function [11,13]. Gieseler proposed the reduction of soluble TM and the development of acquired protein C resistance as the major factors inducing a prothrombotic state during the treatment of MM [11]. It has been pointed out that the absence of an increased risk of VTE in MM patients treated with single agent thalidomide or lenalidomide indicate that these drugs are not directly thrombogenic. Thus, combination effects with other agents or disease-specific factors appear to significantly contribute to the risk.

The mechanism of antitumor efficacy of the IMiDs is an area of intense study. Although downmodulation of TNF α expression and antiangiogenic properties have initially been hypothesized to play an important role, increasing evidence implicates activation of immune effector cells and modulation of cytokine levels as key mechanisms [1,14]. Some of

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TABLE I. Patient Characteristics at Baseline and by DVT History Prior to Starting Therapy

	All patients, n = 32	No DVT, n = 27	DVT, n = 5	P ^a
Continuous variables				
	Median	Median	Median	
Age in years	64	64	63	n.s.
Prior therapies	3	3	1	n.s.
TNF α (pg/ml)	28.2	26.2	28.2	n.s.
CRP (<1 mg/dl)	0.41	0.41	0.41	n.s.
Thrombomodulin (μ g/ml)	95	104	85	n.s.
VCAM (349–991 μ g/ml)	2390	2368	2562	n.s.
D-dimer(<0.4 μ g/ml)	0.43	0.42	0.5	n.s.
Categorical variables				
	n	n	n	
Rai stage III-IV	16	14	2	n.s.
Bulky disease (LN >5 cm)	23	21	2	n.s.
Obesity (BMI > 30)	7	5	2	n.s.
Venous catheter	1	1	0	n.s.
Cardiac disease	2	2	0	n.s.
Renal disease	2	2	0	n.s.
Positive lower extremity doppler	0	0	0	n.s.
Protein S low (55–134%)	5	5	0	n.s.
Protein C low (59–144%)	0	0	0	n.s.
Antithrombin low (57–137%)	3	3	0	n.s.
Factor V Leiden mutation (n = 23)	2	2	0	n.s.
Prothrombin gene mutation (n = 23)	1	1	0	n.s.

^a The two-sided p-value for comparing each variable between patients with or without DVT prior to therapy.

these effects may be disease-specific. In CLL in particular, lenalidomide leads to a clinically significant inflammatory syndrome characterized by lymph node swelling in the context of a systemic cytokine release syndrome [4,5,15,16]. We and others have shown that lenalidomide induces the release of many different proinflammatory cytokines in CLL patients including TNF α [15,16]. These inflammatory side effects are unique to CLL and are not observed in MDS and very rarely in patients with MM, or lymphoma.

Little is known about specific risk factors for VTE in CLL in general or during lenalidomide therapy in particular. We therefore incorporated a prospective, systematic analysis of coagulation parameters into a phase II trial of single agent lenalidomide for relapsed CLL. Because of the many uncertainties related to risk of VTE in CLL with single agent lenalidomide, the risk of thrombocytopenia in our heavily pre-treated patient population, and the controversies about the best agent for prophylaxis we did not use routine prophylaxis.

Methods

Patients and clinical samples. With informed consent in accordance with the Declaration of Helsinki and approval from the NHLBI institutional Review Board, 33 patients with relapsed CLL were enrolled on this investigator-initiated phase II single agent lenalidomide trial (ClinicalTrials.gov Identifier: NCT00465127) and 32 received the study drug. One patient chose not to start drug. The starting dose for the first 10 patients was 20 mg daily; the starting dose for subsequent patients was lowered to 10 mg daily because of toxicities observed in other trials using lenalidomide in CLL [17]. Lenalidomide was given for four cycles of 21 days on followed by 21 days off drug. Responding patients could receive additional four cycles. Aspirin or VTE prophylaxis was not mandated. Corticosteroids were permitted in cases with severe inflammatory side effects as reported previously [16]. A hypercoagulability screen including testing for deficiencies of proteins C, S, or antithrombin, and analysis of factor V Leiden and prothrombin gene 20210 G->A polymorphism was performed prior to therapy. Venous Doppler ultrasound studies were obtained at baseline, after completion of Cycle 4, and Cycle 8, and whenever a DVT was suspected. DVTs that had a temporal relationship to drug ingestion and could not be explained by immobilization or mechanical compression were considered drug-related.

Measurement of inflammatory and coagulation markers. Serum and plasma samples were collected prior to therapy, on Day 8, Day 22, and Day 42 of Cycle 1 and were stored at -80°C. Serum samples were evaluated for TNF α (R&D systems, Minneapolis, MN, detection range

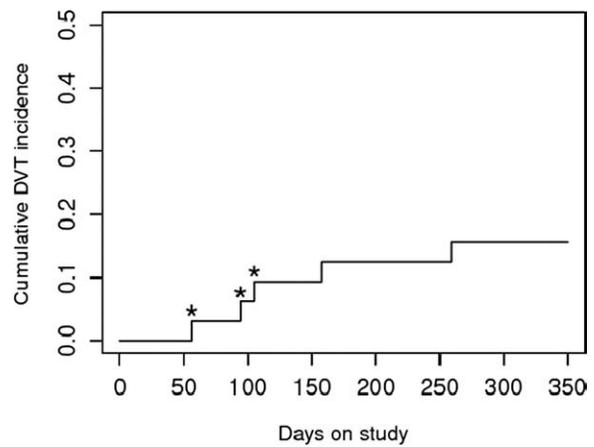


Figure 1. Lenalidomide exposure and cumulative DVT incidence over 1 year. The cumulative incidence of DVT is shown. The recurrent DVT in the second patient with that was noted on Day 314 on routine ultrasound is omitted from this graph. Lenalidomide-related DVTs are marked with an asterisk (*).

15.6–11218.7 pg/ml), thrombomodulin (TM, R&D systems, normal range 4–67 ng/ml), soluble vascular endothelial adhesion molecule 1 (sVCAM-1, R&D systems, range <0.6 ng/ml, normal range 349–991 μ g/ml) and C-reactive protein (CRP, Siemens Healthcare, Marburg, Germany, normal range <1 mg/dl). Plasma samples were evaluated for quantitative D-dimer (normal range <0.4 μ g/ml), protein C (normal range 59–144%), protein S (normal range 59–144%), factor VIII (normal range 41–184%), antithrombin (AT, normal range 57–134%) (all Diagnostica Stago, Parsippany, NJ), tissue factor (TF, Imubind tissue factor ELISA kit, American Diagnostica Stamford, CT, normal range <10 pg/ml) and thrombin-antithrombin (TAT) complexes (normal range 1.3–4 mg/l, Enzygnost TAT micro, Siemens Healthcare Diagnostics). All tests were carried out according to manufacturer’s guidelines.

Statistical analysis. Continuous and categorical variables for different subgroups were compared using the Wilcoxon rank-sum test and the Fisher’s exact test. Change of measurements of serum and plasma markers were examined by the Wilcoxon signed-rank test for paired data. To compare the baseline coagulation markers of CLL patients with normal volunteers, the Z-test was used to determine whether the percentage of patients exceeding the normal range is significantly greater than 10%. Pearson’s correlation coefficient was used to describe correlations. All tests were two-tailed and a p-value < 0.05 was used as the criterion for statistical significance. No adjustment for multiple comparisons has been made. Analyses were performed using the R statistical software (Version 2.10.1; www.r-project.org).

Results

Patient characteristics and incidence of DVTs

From May 2007 to February 2010, 32 patients were treated on this single center trial. Baseline characteristics of the study patients are summarized in Table I: median age was 64 years (38–78), median number of prior therapies 3, and 72% had bulky lymphadenopathy. Risk factors for VTE such as obesity, indwelling catheters, cardiac disease, or renal insufficiency were present in the minority of patients and all patients had a negative Doppler ultrasound prior to starting lenalidomide. Five patients had a history of DVT dating back 1–45 years prior to study entry. Three of these patients were on therapeutic anticoagulation. Two patients were found to have a factor V Leiden mutation in keeping with the incidence in the general population (18). These two patients were maintained on LMWH prophylaxis during the trial. One of these patients also carried the prothrombin gene 20210 G->A polymorphism. Five patients had a low protein S level and three patients had low antithrombin levels. Lower extremity venous Doppler studies performed prior to the start of treatment to rule out inclusion of patients with pre-existing DVTs were negative.

Five patients developed six incidents of DVT over a 1-year observation period for an annual incidence of 16%.

TABLE II. Patient Characteristics at Baseline by Incidence and Type of a New DVT Event During Lenalidomide Therapy

	All patients, n = 32	No DVT, n = 27	Unrelated DVT, n = 2	Related DVT, n = 3
Continuous variables	Median	Median	Median	
Age in years	64 (38–78)	64	59	77
Prior therapies	3 (1–8)	3	1	3
Categorical variables	n	n	n	n
Bulky disease (LN >5 cm)	23	19	2	2
Obesity (BMI > 30)	7	5	1	1
Venous catheter	1	1	0	0
Cardiac disease	2	2	0	0
Renal disease	2	2	0	0
History of DVT	5	2	1	2
Anticoagulation (Enoxa. 1 mg/kg bid)	3	1	0	2
Enoxaparin prophylaxis (40 mg)	2	2	0	0
Low dose aspirin (81 mg)	2	1	0	1
Protein S low (55–134%)	5	5	0	0
Protein C low (59–144%)	0	0	0	0
Antithrombin low (57–137%)	3	3	0	0
Factor V Leiden polymorph (n = 23)	2	2	0	0
Prothrombin gene polymorph (n = 23)	1	1	0	0

None of these was complicated by pulmonary embolism. The time to DVT after starting lenalidomide was variable (see Fig. 1). The earliest DVT occurred on Day 56 in a 77-year-old male with a history of provoked DVT who was maintained initially on LMWH anticoagulation and developed a DVT 2 weeks after starting oral anticoagulation. DVT on Day 94 occurred in a 78-year-old male who was maintained on low dose ASA. DVT at Day 105 occurred in a 63-year-old female with a history of unprovoked DVT who was on LMWH anticoagulation. These DVTs were classified as lenalidomide related. In contrast, three other DVTs could be explained by other mechanisms. The DVT at Day 158 occurred in a 53-year-old patient who developed a DVT in the setting of progressive disease with massive inguinal lymphadenopathy causing venous obstruction. The DVT at Day 259 occurred in a 63-year-old female who had a prior history of provoked DVT and was bed-ridden due to CMV colitis. DVT at Day 314 was a repeat DVT found on venous Doppler studies on routine restaging in the patient who had had a DVT at Day 94. This was the only subclinical DVT observed in the study. At the time he was off lenalidomide. He had been maintained on LWMH anticoagulation for 6 months after his first DVT, and resolution of the thrombus had been documented. Three patients suffered from proximal femoral vein thromboses, while one patient had an iliac vein thrombosis. In the majority of cases, the thromboses were extensive and three patients were treated with low-dose tPA delivered into the clot using a pulse-spray catheter.

The pretreatment hypercoagulability screen was negative in all patients who subsequently developed a DVT during the study period (Table II). Two of these patients were fully anticoagulated due to a preceding history of DVT, and two had a BMI > 30. Other risk factors for DVTs such as indwelling venous catheters, cardiac disease, or renal insufficiency were absent. The only baseline factor significantly different between patients who developed a DVT during the study period and patients with no DVT was a history of previous DVTs ($p = 0.02$).

CLL patients have abnormal coagulation parameters at baseline

There is a paucity of data on the state of the coagulation system in CLL patients [18]. Here, we measured CRP, a panel of coagulation factors, and endothelial cell markers in 32 relapsed CLL patients prior to starting lenalidomide (see Fig. 2). Four of these parameters were significantly increased in CLL patients compared to normal controls defined as >10% of patients having levels above the nor-

mal range ($p < 0.001$). The number of patients with values exceeding the normal range was 23 (72%) for TM, 29 (91%) for sVCAM-1, 17 (53%) for D-dimer, and 10 (31%) for TAT. The levels of CRP, F VIII, Protein S and C, and AT were within the normal range for >90% of the patients. These findings suggest ongoing activation of coagulation and altered endothelial cell function at baseline in a majority of these relapsed patients with extensive disease.

Lenalidomide-induced inflammation, TNF α upregulation, and activation of coagulation

Lenalidomide induces a prominent inflammatory state in CLL, especially during the first cycles [16]. CRP, as a sensitive marker of inflammation, significantly increased on lenalidomide therapy in all patients with a median upregulation of 4.5-fold on Day 8 and regressed to pretreatment levels by the end of the 3-week off drug period in the majority of patients (Fig. 3A). TNF α , a key inflammatory cytokine, [19] was also upregulated a median 3-fold by Day 8 (Fig. 3B) and regressed to almost baseline levels during the off drug period. Inflammation and in particular TNF α have been reported to cause endothelial dysfunction [20]. To estimate lenalidomide's effects on endothelial cells in vivo, we measured sVCAM-1 and sTM, whose serum levels increase in states of endothelial dysfunction [21]. Both markers were significantly increased in patients while on lenalidomide

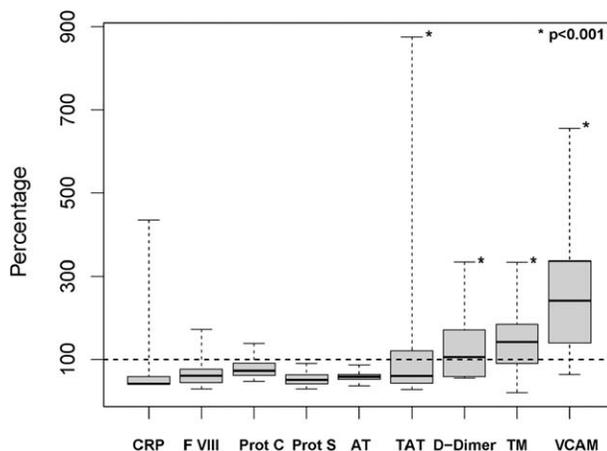


Figure 2. Abnormal coagulation parameters in CLL patients prior to initiating lenalidomide therapy. Parameters of inflammation, coagulation, and endothelial cell function at baseline (n = 32) are normalized to the corresponding upper limit of normal that is set as 100%. The Whisker plot shows the 25, 50, and 75th percentile as part of the box, the dashed line indicates the range.

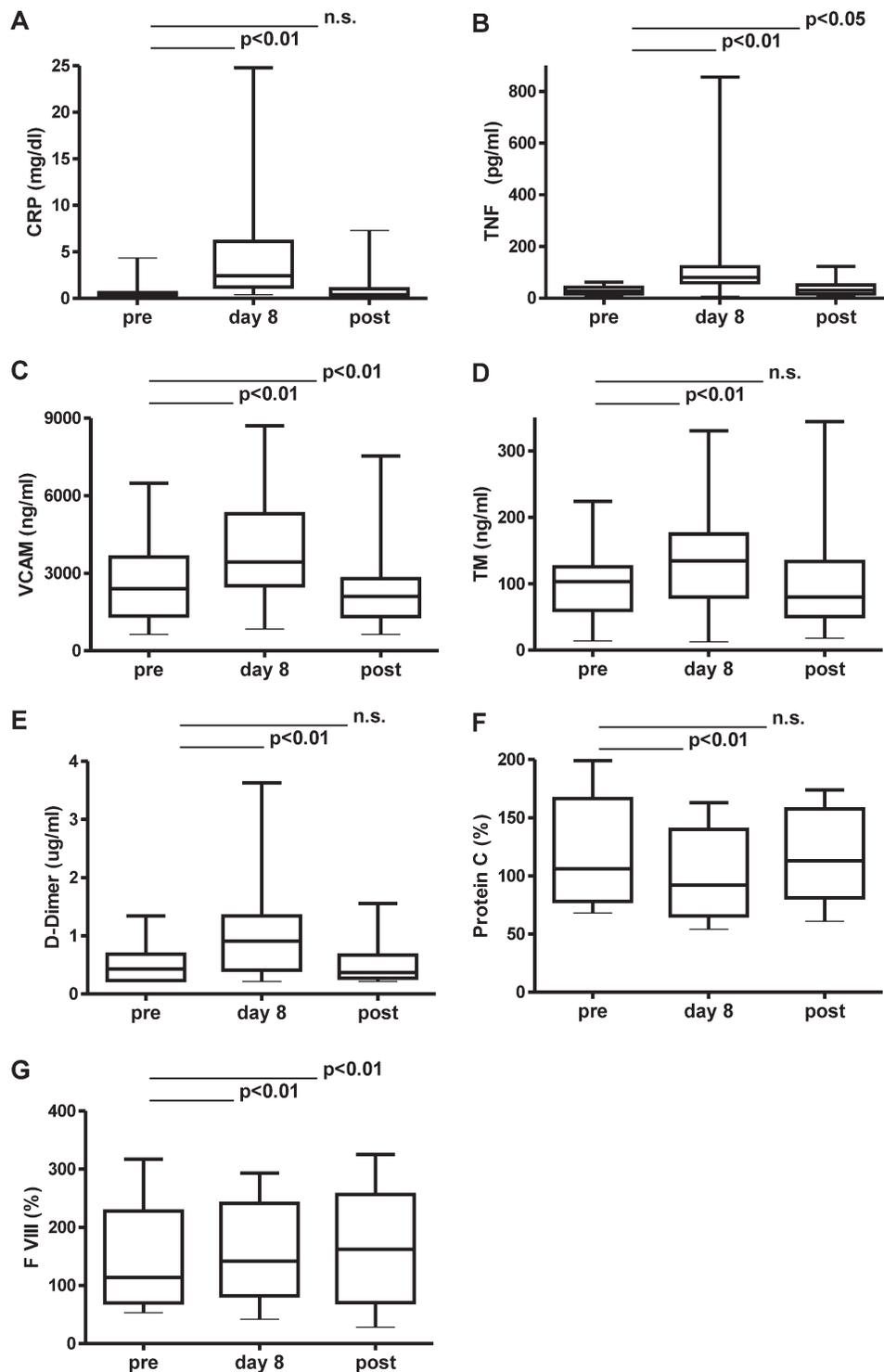


Figure 3. Effects of lenalidomide on markers of inflammation, and endothelial dysfunction and on coagulation factors. Measurements were obtained prior to therapy (pre), on Day 8 of lenalidomide and on Day 42 of Cycle 1, 3 weeks off lenalidomide (post). Only patients with all three measurements are shown. The 0, 25, 50, 75, and 100th percentiles are shown in box charts. (A) CRP ($n = 32$), (B) $TNF\alpha$ ($n = 31$), (C) sVCAM1 ($n = 30$), (D) sTM ($n = 27$), (E) D-dimer ($n = 31$), (F) Protein C ($n = 29$), (G) FVIII ($n = 24$).

therapy (Fig. 3C,D). D-dimer levels also increased by Day 8 of lenalidomide therapy, indicating increased fibrin turnover and returned to pretreatment values thereafter (Fig. 3E). Protein C levels rapidly decreased in the initial phase of lenalidomide therapy and returned to pretreatment values when the drug was stopped (Fig. 3F). Factor VIII levels increased on lenalidomide therapy and remained increased at Day 42 (Fig. 3G). On Day 8 of lenalidomide levels of

TAT, Protein S, and TF were unchanged (data not shown). Effects continued through 3 weeks of drug therapy as indicated by persistent elevation of CRP ($p < 0.01$, $n = 24$), $TNF\alpha$ ($p < 0.01$, $n = 25$), sVCAM1 ($p < 0.01$, $n = 25$), sTM ($p < 0.05$, $n = 20$), and factor VIII ($p < 0.01$, $n = 21$) on Day 22. In summary, lenalidomide induces an inflammatory state, leads to endothelial dysfunction, and activates the coagulation system.

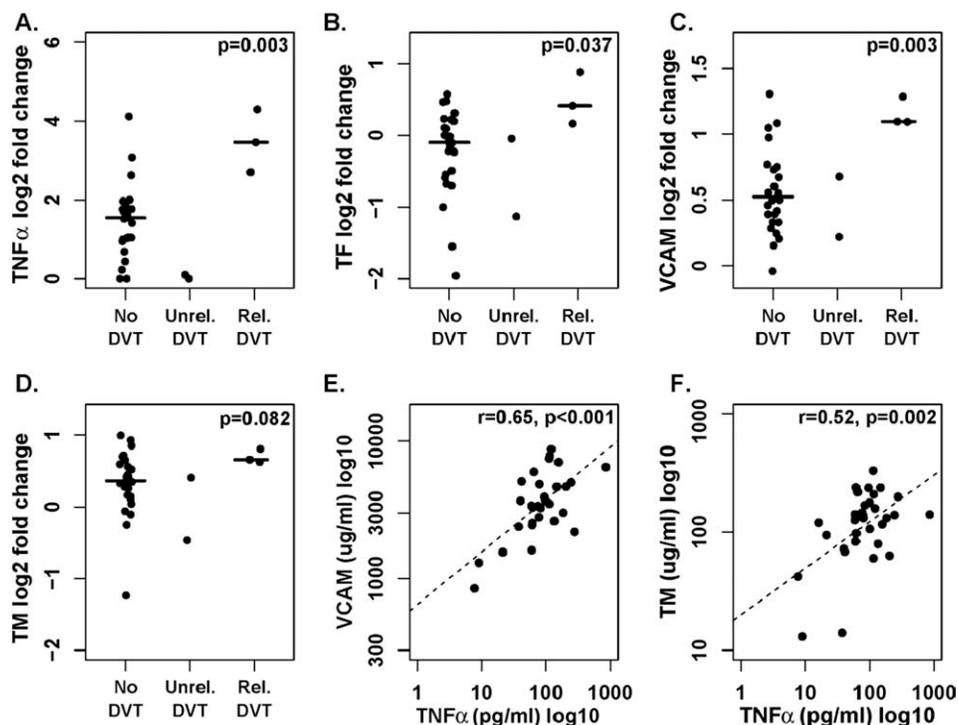


Figure 4. Correlation between TNF α , endothelial dysfunction, and lenalidomide-related DVTs. (A–D) Change of expression levels in patients without DVTs, DVTs unrelated to lenalidomide (Unrel. DVT) and related to lenalidomide (Rel. DVT), with exact *p*-value for comparison between patients with lenalidomide-related DVT and all other patients using the Wilcoxon rank-sum test: (A) TNF α (B) TF, (C) sVCAM1, and (D) sTM. (E, F) Pearson's correlation between TNF α on Day 8 of lenalidomide and (E) sVCAM1 (*n* = 30) and (F) sTM (*n* = 32).

TNF α and endothelial dysfunction as mediators of lenalidomide-related VTE

To explore whether lenalidomide-induced changes in the coagulation system contribute to an increased incidence of VTE, we compared patients with lenalidomide-related DVTs (*n* = 3) to all other patients (*n* = 29). Upregulation of TNF α , TF, and sVCAM1 was significantly higher in patients with lenalidomide-related DVTs compared to patients with no DVT or DVTs unrelated to lenalidomide (Fig. 4A,B,C). sTM was also relatively more increased in patients with lenalidomide-related DVTs, but this difference did not quite reach statistical significance (Fig. 4D). Next, we examined correlations between TNF α and markers of endothelial dysfunction. TNF α blood levels on Day 8 correlated highly with both sVCAM-1 and sTM (Fig. 4E,F).

Discussion

VTE is a frequent complication of cancer, and patients with hematologic malignancies are at highest risk. One study reported a 28-fold increase in the incidence of VTE in patients with hematologic malignancies as compared to controls [22]. The specific incidence of VTE in CLL patients is not well defined and little is known about disease-specific predisposing factors. In our cohort with a maximum observation period of 1 year, 5 of 32 CLL patients (16%) developed 6 incidents of DVT while receiving lenalidomide. This is a higher rate of VTE than has been reported for single agent lenalidomide treatment in MM [2], MDS [3], or in other phase II studies of lenalidomide in CLL where it was <5% [4,5]. Although five patients in our study had a history of prior VTE, this is also considerably higher than the expected annual recurrence rate of 5–6% in patients with prior proximal DVT or PE [23]. Thus, additional disease-specific and treatment-related factors appear to have contributed to a hypercoagulable state. Consistent with disease-related activation of coagulation and endothelial cell

dysfunction, a majority of our patients showed elevations of TAT complexes, D-dimer, sTM, and sVCAM1 prior to starting lenalidomide (see Fig. 2), a finding that has not been previously reported. However, these baseline characteristics failed to identify patients with subsequent DVTs. In two patients, DVTs could be attributed to immobility and local compression, respectively. In three patients, DVTs were related to lenalidomide and all of these occurred in the first 3 months of therapy during a period of inflammation.

Immune activation is thought to be a major contributor to the antitumor effects of lenalidomide [1,14]. We and others have shown that lenalidomide-induced immune activation frequently causes significant systemic symptoms, lymph node swelling, increased CRP, and cytokine release [4,5,15,16]. Although the association between chronic inflammation and cardiovascular disease has been firmly established [24], the relationship with VTE remains less clear [25]. Supporting such a link is the observation that acute infections in a community setting have been associated with a 2-fold increase in VTE rates in the subsequent weeks to months [26]. Increased levels of TNF α , IL-6, IL-8, and MCP-1 have been found in patients with a history of VTE. In these studies, blood collections took place after the thrombotic event was diagnosed. Thus, it is possible that the increase in these cytokines is reactive. In contrast, we collected plasma samples on all study participants prospectively and found lenalidomide-induced upregulation of CRP, TNF α , and FVIII consistent with an acute phase reaction. sVCAM1 and sTM were significantly elevated indicating worsening endothelial dysfunction on lenalidomide (see Fig. 3). In addition, protein C, a crucial anticoagulant, was significantly decreased (see Fig. 3). Nonspecific suppression of protein synthesis in the liver is unlikely to explain the effect on protein C given that protein S, AT, and albumin showed no significant changes. Although serum markers were measured in the first cycle, drug-related DVTs

manifested clinically over a period of 3 months from the start of treatment. It is noteworthy that patients with pronounced inflammatory reactions typically also had more pronounced reactions in subsequent cycles. It thus appears that changes we observed in Cycle 1 do not immediately cause DVTs, but may primarily contribute an additional risk factor.

Several factors could contribute to inflammation-induced VTE. However, in our series only lenalidomide-induced upregulation of TNF α , sVCAM1, and TF were significantly associated with subsequent thrombosis (see Fig. 4). To study the relative predictive value of a prior history of DVT and increases in TNF α and sVCAM1, we compared a number of logistic regression models with lenalidomide-associated DVTs as the primary outcome. In these models either the degree of increase in TNF α or sVCAM was more predictive than DVT history, or the best prediction for the development of a drug-related DVT was the combination of history of DVT with increase in TNF α ($p = 0.0013$; data not shown). Whether lenalidomide-induced upregulation of TNF α and sVCAM1 could be useful to identify patients at highest risk for treatment-related VTE awaits confirmation in a larger cohort of patients.

Our *in vivo* observations are consistent with *in vitro* findings that culturing HUVEC cells in the presence of IMiDs and TNF α lead to increased TF activity [27]. Thus, our data indicate a possible mechanism for lenalidomide-related VTE that centers on drug-induced upregulation of TNF α with consequent endothelial dysfunction and increased activation of TF. Endothelial cell damage has also been invoked to explain the increased incidence of DVT when antiangiogenesis drugs were combined with chemotherapy agents such as cisplatin or gemcitabine [28,29].

Effective strategies for DVT prevention in lenalidomide-treated patients may therefore profitably focus on protecting the endothelium by reducing the inflammatory syndrome and in particular TNF α upregulation. Although aspirin is not considered an effective DVT prophylaxis in typical medical or surgical settings, it has proven to be effective in MM patients and some ongoing trials of lenalidomide in CLL also incorporate aspirin prophylaxis. Interestingly, *in vitro* studies also indicated a mild inhibitory effect of aspirin on TF [27]. There has been no apparent dose-related difference between 81 and 325 mg aspirin for VTE prophylaxis in MM. In CLL, the pronounced inflammatory reaction to lenalidomide raises the question whether aspirin at a dose with an increased anti-inflammatory effect could be more effective. However, the dose of aspirin that would be optimal for downregulation of TNF α is not clear, and its effectiveness may also be influenced by regulation of other inflammatory cytokines [30]. Thus, the optimal prophylaxis for lenalidomide-related DVTs remains to be defined.

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