

7-15-2012

## Nitrated Fibrinogen is A Biomarker of Oxidative Stress in Venous Thromboembolism

Marissa Martinez  
*University of Pennsylvania*

Adam Cuker  
*University of Pennsylvania*

Angela Mills  
*University of Pennsylvania*

Richard Lightfoot  
*University of Pennsylvania*

Yiyang Fan  
*Cleveland State University, Y.FAN67@csuohio.edu*

*See next page for additional authors*

Follow this and additional works at: [https://engagedscholarship.csuohio.edu/scimath\\_facpub](https://engagedscholarship.csuohio.edu/scimath_facpub)

 Part of the [Mathematics Commons](#)

[How does access to this work benefit you? Let us know!](#)

---

### Repository Citation

Martinez, Marissa; Cuker, Adam; Mills, Angela; Lightfoot, Richard; Fan, Yiyang; Tang, W.H. Wilson; Hazen, Stanley L.; and Ischiropoulos, Harry, "Nitrated Fibrinogen is A Biomarker of Oxidative Stress in Venous Thromboembolism" (2012). *Mathematics Faculty Publications*. 208.  
[https://engagedscholarship.csuohio.edu/scimath\\_facpub/208](https://engagedscholarship.csuohio.edu/scimath_facpub/208)

This Article is brought to you for free and open access by the Mathematics Department at EngagedScholarship@CSU. It has been accepted for inclusion in Mathematics Faculty Publications by an authorized administrator of EngagedScholarship@CSU. For more information, please contact [library.es@csuohio.edu](mailto:library.es@csuohio.edu).

---

**Authors**

Marissa Martinez, Adam Cuker, Angela Mills, Richard Lightfoot, Yiyang Fan, W.H. Wilson Tang, Stanley L. Hazen, and Harry Ischiropoulos

Original Contribution

## Nitrated fibrinogen is a biomarker of oxidative stress in venous thromboembolism

Marissa Martinez, Adam Cuker, Angela Mills, Richard Lightfoot, Yiyang Fan, W.H. Wilson Tang, Stanley L. Hazen, Harry Ischiropoulos

### Introduction

Venous thromboembolism (VTE) is a common thrombotic disease that encompasses both deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE affects around 1 per 1000 people per year within the United States, with increasing incidence for congenital and acquired thrombophilic risk factors [1–3]. VTE is a chronic disease with 30% of patients experiencing a recurrent event over 10 years [4,5] and is estimated to account for between 100,000 and 180,000 deaths each year [6]. Thus, VTE represents a significant health problem that requires considerable attention.

The precise molecular and biochemical mechanisms of thrombus initiation in VTE remain unclear. Virchow proposed that changes in blood rheology, induction of a hypercoagulable state, or endothelial injury are conditions required for venous thrombosis [7]. More recent studies indicate that inflammation plays an important role in VTE. However, the interplay between inflammation and Virchow's triad is still unresolved. One possibility is that components of the triad activate or damage endothelial cells within the vein wall resulting in upregulation of receptors for inflammatory and procoagulant molecules. Electron micrographs of early thrombus formation show leukocyte adhesion to the vein wall [8], presumably through the binding of P and E selectins that are expressed on activated endothelial cells [9,10]. Leukocytes shed tissue factor bearing microparticles, which induce coagulation and the production of a fibrin clot [11]. Neutrophils and monocytes, two of the most prevalent leukocytes in venous thrombi, are also able to generate nitrating intermediates, capable of lipid peroxidation and nitration of proteins [12,13].

One protein known to be modified by tyrosine nitration is the coagulant protein fibrinogen. Upon activation of the coagulation cascade, circulating plasma fibrinogen is cleaved by thrombin to fibrin monomers, which polymerize to form a fibrin clot. Recent studies demonstrate an emerging role for nitrated fibrinogen that links inflammation and oxidant production to coagulation.

Nitrated fibrinogen is elevated in the plasma of patients with coronary artery disease (CAD) and in smokers [14,15]. Additionally, in humans injected with small amounts of lipopolysaccharide, plasma levels of nitrated fibrinogen increase and remain elevated for at least 72 h postinjection [16]. This increase follows an elevation of circulating myeloperoxidase, an enzyme localized to the azurophilic granules of neutrophils, which is released during neutrophil activation and is capable of generating nitrating intermediates [12]. Although these data suggest that nitrated fibrinogen plays a key role linking inflammation, oxidant production, and arterial thrombotic diseases, its role in VTE remains undefined.

Moreover, nitrated fibrinogen is not simply a marker of inflammation and oxidative stress, but also has functional effects on fibrinogen and fibrin clotting. Fibrinogen nitration was shown to increase the rate of fibrin clot formation, diminish clot lysis rate, and alter fibrin clot structural and viscoelastic properties [14-16]. These functional effects suggest that nitrated fibrinogen may represent a risk factor for increased thrombotic tendency during inflammation and oxidant stress. However, the levels of nitrated fibrinogen in VTE have not been ascertained. Herein we quantified the levels of nitrated fibrinogen in patients presenting to the emergency department with suspected VTE and evaluated if nitrated fibrinogen is a biochemical risk factor that could explain associations of oxidative stress and inflammation with thrombotic complications in VTE.

## Materials and methods

### *Patient population*

We performed a prospective cohort study from January 2010 to March 2011 of consecutive subjects 18 years of age or older presenting to the Hospital of the University of Pennsylvania emergency department with suspected acute lower extremity DVT or PE. Exclusion criteria included history of VTE within the prior 4 weeks, unavailable for 90 day follow up, and inability to provide informed consent. DVT was diagnosed by compression ultrasonography and PE by computed tomographic pulmonary angiography. Patients with negative imaging were contacted by telephone 90 days after enrollment to confirm that they did not develop clinically apparent VTE. Those who reported development of VTE over this interval despite negative imaging were also considered positive for DVT and/or PE. Secondary variables were also collected, including demographic information, thrombotic risk factors, comorbidities, and medications. Before initiation of anticoagulant therapy in the emergency department, blood was collected from patients in 3.2% sodium citrate (BD Vacutainer; Franklin Lakes, NJ, USA). Blood was centrifuged at 150g for 15 min, and the supernatant, platelet rich plasma, was transferred to a new tube. Platelet rich plasma was then centrifuged at 10,000g for 10 min to obtain platelet poor plasma, followed by storage at 80 °C for future analysis. Study design was approved by the Institutional Review Board Involving Human Subjects at the University of Pennsylvania and written informed consent was obtained from all study participants.

### *Plasma nitrated fibrinogen levels*

Nitrated fibrinogen levels were measured in plasma using an ELISA described previously [16]. Briefly, 96 well plates were coated with 10 µg/ml anti nitrotyrosine antibodies generated and described elsewhere [17] in 50 mM carbonate buffer, pH 9, and incubated at 4 °C under constant rocking overnight. The next day, the plates were washed with 50 mM Tris, 150 mM NaCl,

0.05% Tween 20 (TBS T). The plates were blocked with 3% bovine serum albumin (BSA; Roche Diagnostics, Mannheim, Germany) (wt/vol) in TBS T for 2 h at 37 °C, followed by incubation with 1% BSA in TBS T for 1 h at 37 °C. A standard curve was created from purified fibrinogen (American Diagnostica, Stamford, CT, USA) that had been chemically nitrated and the levels of nitration were independently determined [16]. Plasma samples were diluted (1:10, 1:20) in 1% BSA in TBS T, added to the plate in duplicate, and incubated 2 h at room temperature under constant rocking. Plates were washed with TBS T and coated with 0.3 µg/ml horse radish peroxidase (HRP) conjugated rabbit anti human fibrinogen polyclonal antibody (DakoCytomation, Glostrup, Denmark) in 1% BSA in TBS T. After 2 h incubation at room temperature under gentle rocking, the plates were washed with TBS T and developed with TMB substrate (KPL, Gaithersburg, MD, USA) followed by quenching of the reaction with 2 M sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA). The absorbance was measured at 405 nm (Molecular Devices, Sunnyvale, CA, USA). Nitrated fibrinogen values were normalized to quality control plasma run with each plate. Each patient sample was analyzed a minimum of three times and samples that exceeded 15% coefficient of variation were excluded from analysis. The normalized mean intra assay and interassay variations were 8.23 and 9.32%.

### *Plasma fibrinogen level*

Fibrinogen levels were measured in plasma using an ELISA previously described [16]. Ninety six well plates (Maxisorb; Nunc, Rochester, NY, USA) were coated with 10 µg/ml mouse anti fibrinogen monoclonal antibody [16] in carbonate buffer, pH 9, and incubated at 4 °C, rocking overnight. The next day, the plates were washed with TBS T and blocked with 3% BSA in TBS T for 2 h at 37 °C. A standard curve was created from purified fibrinogen (American Diagnostica) in TBS T. Plasma was diluted (1:5000, 1:10,000), added to the plate in triplicate, and incubated for 1 h at 37 °C. The plates were washed with TBS T, then coated with 0.3 µg/ml HRP conjugated rabbit anti human fibrinogen polyclonal antibody (DakoCytomation) in 1% BSA in TBS T, and incubated 1 h at room temperature. The plate was developed with the TMB peroxidase substrate as described above. Fibrinogen values were normalized to quality control plasma run with each plate. The normalized mean intra assay and interassay variations were 11.23 and 14.0%.

Plasma fibrinogen levels were measured by the Clauss method [18] using a Destiny Max coagulation analyzer according to the manufacturer's protocol (Trinity Biotech, County Wicklow, Ireland).

### *D dimer*

Plasma D dimer levels were measured at the University of Pennsylvania Hospital Coagulation Laboratory using the Vidas D dimer exclusion assay (bioMérieux, Lyon, France). This assay measures the fibrin degradation product (FDP) D dimer by an enzyme linked fluorescence assay. This sandwich ELISA based method uses two anti FDP monoclonal antibodies (P10B5E12C9 and P2C5A10) coupled with fluorescence detection [19,20]. In accordance with the manufacturer's instructions, a D dimer level of < 0.5 µg/ml was considered negative.

The laboratory personnel and technologists performing all assays were blinded to patient outcome. Laboratory results obtained on the archived plasma samples were not made available to clinicians or patients.

## Statistics

Gaussian distribution was tested for all continuous variables using a D'Agostino and Pearson omnibus normality test, which indicated nonparametric distributions for all variables within our data. Thus, comparisons between groups were made using the Mann-Whitney *U* test. Nitrated fibrinogen, D dimer, and fibrinogen are given as the mean and the 95% confidence interval (CI). The adjusted model for nitrated fibrinogen levels was created using the generalized linear model, which accounted for demographics, comorbidities, and VTE risk factors. Odds ratios were compared by Fisher's exact test. Trends in nitrated fibrinogen levels by quartile were compared by  $\chi^2$  analysis. A *P* value < 0.05 was considered statistically significant. All statistical calculations were generated and analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) or R Statistical package (<http://www.r-project.org/>).

## Results

### Patient characteristics

A total of 251 patients were enrolled in this study. Of the 83 subjects with suspected DVT, 18 had a positive ultrasound and an additional 3 were determined to be positive on 90 day follow up. Among the 168 subjects with suspected PE, 28 had a positive computed tomographic pulmonary angiography and 1 additional patient was found to have new PE at 90 days. Patient demographics, disease history, risk factors, and medications are shown in Table 1. Three well accepted risk factors for VTE were more prevalent in the VTE positive subset: advanced age, active cancer, and surgery within the past 6 weeks. VTE positive patients were significantly older compared with negative patients and were more likely to have active cancer or recent surgery. Thrombophilia testing was not performed as part of the study. Nevertheless, 3 patients reported a history of hereditary thrombophilia, including 1 with protein C and 2 with protein S deficiency. Additionally, 2 patients reported having antiphospholipid syndrome. No other measured demographic variables, comorbidities, risk factors, or medication use differed between VTE positive and VTE negative patients (Table 1).

### Fibrinogen levels in VTE patients

Fibrinogen levels were quantified by ELISA, which measures fibrinogen antigen, and the Clauss assay, which measures fibrin clotting time. The mean levels of fibrinogen compared by Mann-Whitney *U* test were not different between subjects with and without VTE by either Clauss assay or by ELISA (Fig. 1A). Fibrinogen levels were also similar between DVT and PE patients irrespective of the assay employed (Clauss: DVT, *n*=17, 3.8 mg/ml, 95% CI 3.0–4.6; PE, *n*=27, 4.5 mg/ml, 95% CI 3.6–5.4. ELISA: DVT, *n*=21, 5.5 mg/ml, 95% CI 4.4–6.5; PE, *n*=28, 5.6 mg/ml, 95% CI 4.7–6.5; Fig. 1A). ELISA determined fibrinogen levels were higher than those reported by Clauss assay, which is consistent with findings in previous studies [21].

### Nitrated fibrinogen levels in VTE patients

Nitrated fibrinogen was measured by an ELISA as described previously [16]. The levels of nitrated fibrinogen were interpolated from a standard curve created with purified fibrinogen that was chemically nitrated, and levels of nitration were independently determined [16]. To ensure the integrity of each assay, every 96 well plate included a quality control plasma standard, and intra- and interassay variation did not exceed 15%. The mean

**Table 1**

Characteristics of VTE patient population.

	Non-VTE ( <i>n</i> 201)	VTE ( <i>n</i> 50)	<i>P</i> value
Age (years)	47.6 ± 16.7	53.3 ± 16.4	0.0206 <sup>a</sup>
Gender (% male)	63 (31.3%)	23 (46.0%)	
BMI (kg/m <sup>2</sup> )	31.4 ± 9.2	28.9 ± 6.7	
Race and ethnicity			
Caucasian	75 (37.3%)	26 (52.0%)	
African American	121 (60.2%)	22 (44.0%)	
Other	5 (2.5%)	2 (4.0%)	
Hispanic	6 (3.0%)	2 (4.0%)	
Disease history and risk factors			
H/O DVT	35 (17.4%)	9 (18.0%)	
H/O PE	28 (13.9%)	7 (14.0%)	
H/O CAD/MI/angina	15 (8.2%)	5 (6.8%)	
H/O stroke	15 (7.5%)	4 (8.0%)	
Liver disease	9 (4.5%)	2 (4.0%)	
Lupus	8 (4.0%)	0 (0.0%)	
Rheumatoid arthritis	4 (2.0%)	1 (2.0%)	
Peripheral artery disease	5 (2.5%)	4 (8.0%)	
Active cancer	38 (18.9%)	19 (38.0%)	0.0075 <sup>b</sup>
Recent surgery <sup>c</sup>	26 (12.9%)	15 (30.0%)	0.0089 <sup>b</sup>
Immobilization > 6 weeks	30 (14.9%)	11 (22.0%)	
Air travel <sup>c</sup>	4 (2.0%)	2 (4.0%)	
Tobacco use	77 (38.3%)	17 (34.0%)	
Pregnancy	5 (2.5%)	4 (8.0%)	
Trauma	11 (5.5%)	3 (6.0%)	
Infection	19 (9.5%)	5 (10.0%)	
Medication			
Antiplatelet	32 (15.9%)	8 (16.0%)	
Anticoagulant	25 (12.4%)	8 (16.0%)	
Statins	36 (17.9%)	11 (22.0%)	
Hormonal contraception	9 (4.5%)	4 (8.0%)	
Hormone therapy	1 (0.5%)	1 (2.0%)	
Antioxidants	6 (3.0%)	3 (6.0%)	
Immunosuppressants	29 (14.4%)	4 (8.0%)	

Values shown are means ± SD or the number and percentage of the sample group. VTE composition: DVT, *n* 21; PE, *n* 29.

<sup>a</sup> Age and BMI were compared by Mann-Whitney *U* test.

<sup>b</sup> All other variables were compared by Fischer's exact test.

<sup>c</sup> Within past 6 weeks.

levels of nitrated fibrinogen were significantly higher in VTE positive compared to VTE negative patients (Fig. 1B). Nitrated fibrinogen levels were not different between DVT and PE patients (DVT, *n*=17, 60.9 nM, 95% CI 52.3–69.6; PE, *n*=27, 63.9 nM, 95% CI 55.2–72.5).

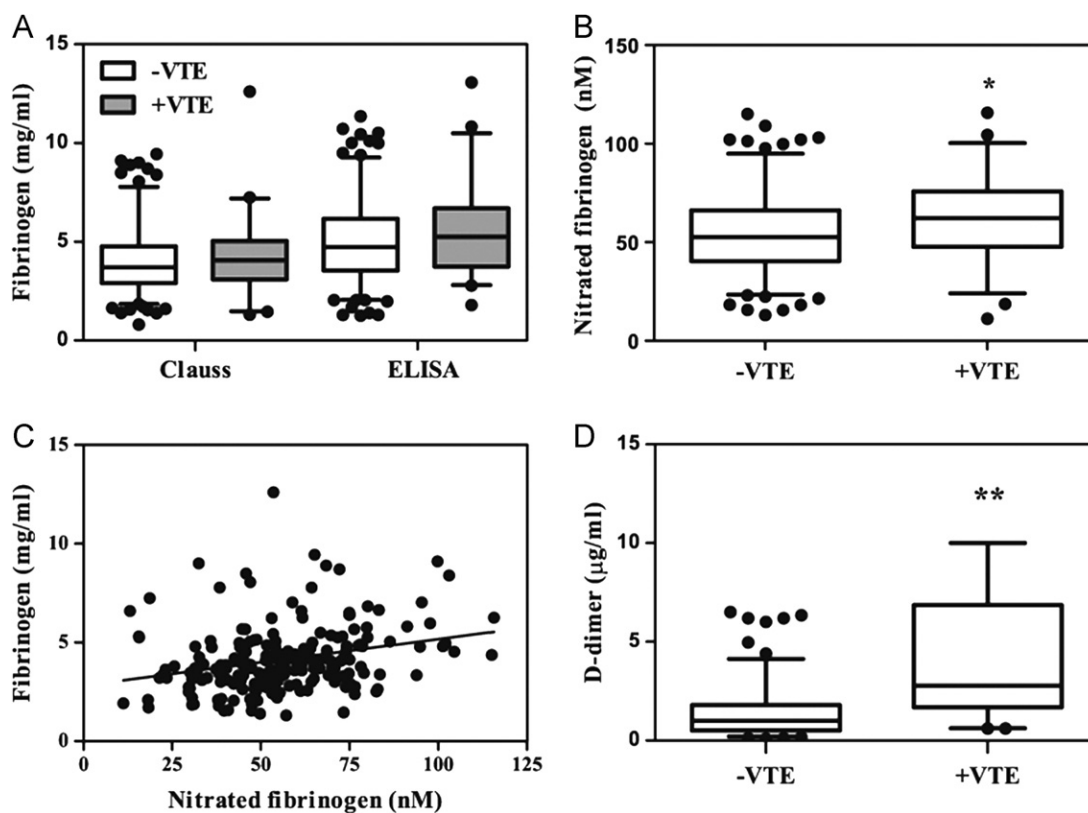
Previous data indicated that one functional effect of fibrinogen tyrosine nitration was increased rate of fibrin formation. Because the Clauss assay relies on fibrin clotting time in plasma to assess fibrinogen levels, Spearman correlation analysis was used to evaluate the relationship between nitrated fibrinogen and fibrinogen levels (Fig. 1C). Nitrated fibrinogen correlated linearly with Clauss measured fibrinogen levels (*n*=207, *r*=0.31, 95% CI 0.18–0.40; *P*<0.0001).

### D dimer levels in VTE patients

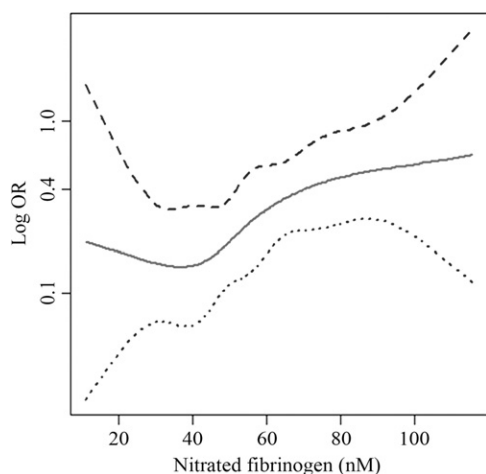
D dimers are fibrin degradation products that are produced in response to clot lysis by plasmin and are used as a diagnostic marker for VTE [19,22–25]. D dimer was measured with the Vidas D dimer exclusion assay according to the manufacturer's instructions. As expected, mean D dimer levels were significantly higher in VTE positive versus VTE negative patients (Fig. 1D).

### Odds ratio for venous thromboembolism across increasing nitrated fibrinogen levels

Cubic spline curves with 95% CI (*n*=226) were constructed to illustrate the relationship between nitrated fibrinogen levels and



**Fig. 1.** Fibrinogen, nitrated fibrinogen, and D-dimer levels in VTE-negative and VTE-positive patients. (A) Plasma fibrinogen levels were measured by two methods: ELISA, which measures fibrinogen antigen (VTE positive (+VTE),  $n = 49$ , 5.5 mg/ml, 95% CI 4.9–6.2; VTE negative (–VTE),  $n = 194$ , 5.0 mg/ml, 95% CI 4.7–5.3), and Clauss assay, which measures fibrin clotting time (+VTE,  $n = 44$ , 4.3 mg/ml, 95% CI 3.6–4.9; –VTE,  $n = 185$ , 4.0 mg/ml, 95% CI 3.8–4.2). Mean fibrinogen levels for each assay were compared by Mann–Whitney  $U$  test,  $P$  was nonsignificant for both comparisons. (B) Nitrated fibrinogen levels measured by ELISA were elevated in +VTE ( $n = 48$ ) compared to –VTE ( $n = 178$ ) patients (62.7 nM, 95% CI 56.6–68.8 vs 54.2 nM, 95% CI 51.4–57.1). Nitrated fibrinogen levels were compared by Mann–Whitney  $U$  test,  $P < 0.01$ . (C) Spearman’s rank correlation analysis was used to evaluate the relationship between nitrated fibrinogen and Clauss-measured fibrinogen levels ( $n = 207$ ,  $r = 0.31$ , 95% CI 0.18–0.40;  $P < 0.0001$ ). (D) D-dimer levels measured by Vidas D-dimer exclusion assay were higher in +VTE ( $n = 42$ ) versus –VTE ( $n = 157$ ) patients (4.22  $\mu\text{g/ml}$ , 95% CI 3.23–5.20 vs 1.42  $\mu\text{g/ml}$ , 95% CI 1.20–1.63). D-dimer levels were compared by Mann–Whitney  $U$  test,  $**P < 0.001$ . Data for (A), (B), and (D) are represented by box and whisker plots in which the midline in the box is the median, the bottom and top of the box represent the 25th and 75th percentiles, respectively, and lower and upper bars represent the 5th and 95th percentiles, respectively.



**Fig. 2.** Relationship between the odds ratio (OR) of acute VTE and nitrated fibrinogen levels. A cubic spline regression model of the odds ratio of acute VTE as a function of nitrated fibrinogen after adjustment for comorbidity, demographics, and risk factors is shown ( $n = 226$ ). Dotted lines represent the 95% CI.  $P < 0.005$ .

the odds ratio of clinically documented VTE (Fig. 2). This analysis shows a strong association between nitrated fibrinogen levels and increased risk for VTE ( $P < 0.005$ ). A similar relationship was found for rates of VTE across increasing nitrated fibrinogen

quartiles ( $\chi^2$  for trend 7.69;  $P < 0.01$ ; Table 2). Patients in the highest quartile were compared with those in the lowest quartile and were found to have an increased risk of VTE (Table 2). This risk was maintained after univariate adjustment for the risk factors found to be elevated in our VTE positive patient population: advanced age, active cancer, and recent surgery (Table 2, Models 1, 2, and 3). However, multivariate analysis with these risk factors did not reach statistical significance (Table 2, Model 4). This may reflect the small size of our VTE positive patient population, in that additional adjustments reduce the final sample size and eliminate statistical significance.

## Discussion

Previous studies have implied that inflammation and oxidative processes may be mechanistically linked to the pathogenesis of VTE [12 16,26 31]. Nitration of proteins and, specifically, nitrated fibrinogen have been quantified in the plasma of subjects who experience inflammatory changes and oxidative burden [14 16]. In this study the data indicate that oxidants, specifically nitrating intermediates produced in response to inflammation in VTE, result in nitration of fibrinogen. The levels of nitrated fibrinogen were significantly elevated in patients with acute VTE compared to those negative for the disease. This increase was not dependent on changes in fibrinogen, as mean levels of fibrinogen were similar between the two groups. Based on

**Table 2**  
Odds ratio of VTE by quartiles of nitrated fibrinogen.

	Quartile			
	1	2	3	4
Nitrated fibrinogen	< 43.5	43.5–54.1	54.1–69.6	≥ 69.7
–VTE	50	47	42	39
+VTE	7	9	14	18
Event rate	12.3%	16.1%	25.0%	31.6%
Unadjusted OR	1	1.37 (0.47–3.97)	2.38 (0.88–6.44)	3.30 (1.25–8.68)*
Adjusted OR (1)	1	1.30 (0.44–3.81)	2.22 (0.81–6.08)	3.25 (1.23–8.64)*
Adjusted OR (2)	1	1.37 (0.47–4.02)	2.42 (0.88–6.62)	2.84 (1.06–7.62)*
Adjusted OR (3)	1	1.26 (0.43–3.71)	2.23 (0.82–6.11)	2.84 (1.06–7.62)*
Adjusted OR (4)	1	1.21 (0.40–3.62)	2.17 (0.78–6.03)	2.55 (0.92–7.01)

Values listed as OR (95% CI). Models: 1, adjusted for age; 2, adjusted for cancer; 3, adjusted for surgery; 4, adjusted for age, cancer, surgery. Odds ratios were compared by Fisher's exact test.

\*  $P < 0.05$ .

previous studies, the levels of nitrated fibrinogen in VTE negative patients are higher than would be expected in healthy nonsmoking controls [14,15]. This is probably due to the presence of inflammatory and oxidative conditions in the VTE negative population, including CAD and smoking, which are associated with increased plasma concentration of nitrated fibrinogen [14 16]. Elevated levels of nitrated fibrinogen in VTE, CAD, and smokers suggest that inflammation and the production of nitric oxide derived oxidants are present in thrombotic disorders as well as in those at risk for the disease. Previous research on the functional effects of nitration on fibrinogen indicates that this modification contributes to a prothrombotic phenotype [14 16]. Thus, nitrated fibrinogen may serve as a comprehensive marker for activation of inflammatory pathways during thrombosis. Increasing nitrated fibrinogen levels were positively associated with risk of VTE. Compared with patients in the lowest quartile of nitrated fibrinogen, those in the highest quartile had a significantly increased risk of VTE. This relationship persisted after adjusting individually for the risk factors that were elevated in the VTE positive group: advanced age, active cancer, and recent surgery. However, this risk was not maintained after adjusting for the combined factors. The relatively small VTE positive patient group may account for this difference, whereby adjustment reduces the positive group sample size and eliminates statistical significance. A larger study would be needed to address this. The loss of significance in our multivariate regression analysis may also suggest that patients with more than one of these risk factors also have elevated nitrated fibrinogen levels independent of VTE. Earlier work has shown that nitrated proteins, including nitrated fibrinogen, were significantly elevated in lung cancer patients [31]. Further studies investigating nitrated fibrinogen in advanced age, various cancer types, and surgical patients would be necessary to evaluate these effects. Advanced age, malignancy, and the postoperative state are well accepted risk factors for VTE [1,32] and were more prevalent among VTE positive than among VTE negative subjects in our study. Other established risk factors were present in similar proportions among the two groups, possibly owing to the relatively small size of our study and the small number of patients harboring such risk factors.

In this study, nitrated fibrinogen was found to be elevated in the setting of acute VTE. It remains to be determined whether this elevation reflects an underlying mediator of VTE pathology or is merely a transient epiphenomenon of acute thrombosis. Studies in which nitrated fibrinogen levels are measured in patients at risk for VTE, as well as in the weeks and months following an event, are needed to address this question and may provide valuable information regarding VTE recurrence.

Previous work has revealed that fibrinogen is targeted for oxidative modifications in vivo and in vitro [14 16,31,33 35]. Tyrosine nitration even in the presence of other oxidized amino acids was shown to significantly accelerate clot formation and factor XIII cross linking [14,15,34,35]. Incorporation of nitrated fibrinogen molecules into fibrin clots was documented by immunoelectron microscopy and was found to alter clot architecture in a dose dependent manner [14,15]. Changes in structure were accompanied by changes in viscoelastic properties and a decrease in dissolution by the fibrinolytic systems [14,15]. Removal of the nitrated fibrinogen molecules restored the kinetics of fibrin clot formation and architecture [15]. These profound effects on fibrin clot structure were produced by a relatively small fraction of fibrinogen molecules modified by nitration. Based on previous data and data reported herein, the yield of nitration represents 1 6% of fibrinogen molecules [15]. Kinetic analysis of fibrin formation revealed that insertion of nitrated fibrinogen molecules accelerated lateral aggregation, an event that follows the initial nucleation and the formation of half staggered, double stranded protofibrils [36 38]. This acceleration is probably due to increased "B knob:b hole" interactions, which are facilitated by the site specific nitration of two tyrosine residues near the b hole of the  $\beta$  chain [15]. Overall, the concept that 1 nitrated molecule per 100 normal fibrinogen molecules can initiate the catalytic increase in lateral aggregation is intriguing and consistent with observations of other proteins that require nucleation events to form protein fibers [39]. These previous studies suggest that the elevated levels of nitrated fibrinogen in VTE positive patients would result in an increase in the kinetics of clot formation and subsequent changes in clot architecture. Additional studies are necessary to address this hypothesis.

In addition to nitrated fibrinogen, D dimer levels were significantly elevated in VTE positive patients consistent with previous studies [19,22 25]. Sensitivity and specificity for D dimer at a 0.5  $\mu\text{g/ml}$  cutoff in this patient population were 100% (95% CI 91.6 100) and 22.3% (95% CI 16.1 29.6), respectively, and are similar to what has been previously reported for the Vidas D dimer exclusion assay [19,25,40].

Fibrinogen is an independent risk factor and predictor of CAD and associated morbidity and mortality [41,42]. However, its role in VTE remains unclear. The Leiden Thrombophilia Study (LETS) showed elevated fibrinogen levels measured by Clauss assay in DVT patients compared with healthy controls [43]. However, in later investigations within LETS, increased risk of DVT with increasing fibrinogen levels was found only in patients > 45 years of age. In patients < 45 years, fibrinogen levels in the 95th percentile did not confer increased risk of DVT [44]. Furthermore,

in two separate studies in patients with suspected PE, the levels of fibrinogen were significantly lower in patients diagnosed as positive versus negative for the disease [45,46]. In this study, fibrinogen levels were similar between VTE positive and VTE negative groups, and there were no differences in DVT versus PE patients. Patient population or sample size may account for the differences between this study and LETS, in which DVT patients were compared to a large number of healthy age and sex matched controls. Likewise, patient population may also explain the dissimilarity between this study and the two previous studies investigating fibrinogen in PE [45,46].

Overall, fibrinogen nitration in VTE patients certainly reflects active inflammatory and oxidative states that could favor a procoagulant environment resulting in fibrin structures with altered biochemical and biophysical properties. These alterations in fibrin clots may profoundly influence the pathology of VTE.

## Acknowledgments

The work was supported by grants from the National Institutes of Health (HL54926, HL103918, and ES013508, NIEHS Center of Excellence in Environmental Toxicology to H.I.; HL098055 and HL076491 to S.L.H.; and HL103931 to W.H.T.). M.R.M. is supported by Hemostasis and Thrombosis Training Grant T32 HL07971. H.I. is the Gisela and Dennis Alter Research Professor of Pediatric Neonatology at the Children's Hospital of Philadelphia Research Institute.

## References

- Anderson Jr F. A.; Wheeler, H. B.; Goldberg, R. J.; Hosmer, D. W.; Patwardhan, N. A.; Jovanovic, B.; Forcier, A.; Dalen, J. E. A population-based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism: the Worcester DVT study. *Arch. Intern. Med.* **151**:933–938; 1991.
- Salomon, O.; Steinberg, D. M.; Zivelin, A.; Gitel, S.; Dardik, R.; Rosenberg, N.; Berliner, S.; Inbal, A.; Many, A.; Lubetsky, A.; Varon, D.; Martinowitz, U.; Seligsohn, U. Single and combined prothrombotic factors in patients with idiopathic venous thromboembolism: prevalence and risk assessment. *Arterioscler. Thromb. Vasc. Biol.* **19**:511–518; 1999.
- Pabinger, I.; Brucker, S.; Kyrle, P. A.; Schneider, B.; Korninger, H. C.; Niessner, H.; Lechner, K. Hereditary deficiency of antithrombin III, protein C and protein S: prevalence in patients with a history of venous thrombosis and criteria for rational patient screening. *Blood Coagul. Fibrinolysis* **3**:547–553; 1992.
- Heit, J. A.; Mohr, D. N.; Silverstein, M. D.; Petterson, T. M.; O'Fallon, W. M.; Melton 3rd L. J. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. *Arch. Intern. Med.* **160**:761–768; 2000.
- Schulman, S.; Lindmarker, P.; Holmstrom, M.; Larfars, G.; Carlsson, A.; Nicol, P.; Svensson, E.; Ljungberg, B.; Viering, S.; Nordlander, S.; Leijd, B.; Jahed, K.; Hjorth, M.; Linder, O.; Beckman, M. Post-thrombotic syndrome, recurrence, and death 10 years after the first episode of venous thromboembolism treated with warfarin for 6 weeks or 6 months. *J. Thromb. Haemostasis* **4**:734–742; 2006.
- U.S. Department of Health and Human Services Surgeon General's call to action to prevent deep vein thrombosis and pulmonary embolism. Washington, DC: DHHS; 2008.
- Virchow, R. *Thrombose und Embolie: Gefässentzündung und septische Infektion*. Frankfurt am Main: Von Meidinger & Sohn; 1856 pp. 219–732.
- Schaub, R. G.; Simmons, C. A.; Koets, M. H.; Romano 2nd P. J.; Stewart, G. J. Early events in the formation of a venous thrombus following local trauma and stasis. *Lab. Invest* **51**:218–224; 1984.
- Myers Jr D.; Farris, D.; Hawley, A.; Wroblewski, S.; Chapman, A.; Stoolman, L.; Knibbs, R.; Strieter, R.; Wakefield, T. Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis. *J. Surg. Res* **108**:212–221; 2002.
- Geng, J. G.; MP, Bevilacqua; Moore, K. L.; McIntyre, T. M.; Prescott, S. M.; Kim, J. M.; Bliss, G. A.; Zimmerman, G. A.; McEver, R. P. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* **343**:757–760; 1990.
- Hrachovinova, I.; Cambien, B.; Hafezi-Moghadam, A.; Kappelmayer, J.; Camphausen, R. T.; Widom, A.; Xia, L.; Kazazian Jr H. H.; Schaub, R. G.; McEver, R. P.; Wagner, D. D. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat. Med* **9**:1020–1025; 2003.
- Brennan, M. L.; Wu, W.; Fu, X.; Shen, Z.; Song, W.; Frost, H.; Vadseth, C.; Narine, L.; Lenkiewicz, E.; Borchers, M. T.; Lusic, A. J.; Lee, J. J.; Lee, N. A.; Abu-Soud, H. M.; Ischiropoulos, H.; Hazen, S. L. A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem* **277**:17415–17427; 2002.
- Hazen, S. L.; Zhang, R.; Shen, Z.; Wu, W.; Podrez, E. A.; MacPherson, J. C.; Schmitt, D.; Mitra, S. N.; Mukhopadhyay, C.; Chen, Y.; Cohen, P. A.; Hoff, H. F.; Abu-Soud, H. M. Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation in vivo. *Circ. Res* **85**:950–958; 1999.
- Vadseth, C.; Souza, J. M.; Thomson, L.; Seagraves, A.; Nagaswami, C.; Scheiner, T.; Torbet, J.; Vilaire, G.; Bennett, J. S.; Murciano, J. C.; Muzykantov, V.; Penn, M. S.; Hazen, S. L.; Weisel, J. W.; Ischiropoulos, H. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J. Biol. Chem* **279**:8820–8826; 2004.
- Parastatidis, I.; Thomson, L.; Burke, A.; Chernysh, I.; Nagaswami, C.; Visser, J.; Stamer, S.; Liebler, D. C.; Koliakos, G.; Heijnen, H. F.; Fitzgerald, G. A.; Weisel, J. W.; Ischiropoulos, H. Fibrinogen  $\beta$ -chain tyrosine nitration is a prothrombotic risk factor. *J. Biol. Chem.* **283**:33846–33853; 2008.
- Heffron, S. P.; Parastatidis, I.; Cuchel, M.; Wolfe, M. L.; Tadesse, M. G.; Mohler 3rd E. R.; Ischiropoulos, H.; Rader, D. J.; Reilly, M. P. Inflammation induces fibrinogen nitration in experimental human endotoxemia. *Free Radic. Biol. Med.* **47**:1140–1146; 2009.
- Heijnen, H. F.; van Donselaar, E.; Slot, J. W.; Fries, D. M.; Blachard-Fillion, B.; Hodara, R.; Lightfoot, R.; Polydoro, M.; Spielberg, D.; Thomson, L.; Regan, E. A.; Crapo, J.; Ischiropoulos, H. Subcellular localization of tyrosine-nitrated proteins is dictated by reactive oxygen species generating enzymes and by proximity to nitric oxide synthase. *Free Radic. Biol. Med.* **40**:1903–1913; 2006.
- Claus, A. Gerinnungsphysiologische schnellmethode zur bestimmung des fibrinogens Acta Haematol **17**: 237–246; 1957. <<http://dx.doi.org/10.1159/000205234>>.
- de Moerloose, P.; Desmarais, S.; Bounameaux, H.; Reber, G.; Perrier, A.; Dupuy, G.; Pittet, J. L. Contribution of a new, rapid, individual and quantitative automated D-dimer ELISA to exclude pulmonary embolism. *Thromb. Haemostasis* **75**:11–13; 1996.
- Perrier, A.; Desmarais, S.; Miron, M. J.; de Moerloose, P.; Lepage, R.; Slosman, D.; Didier, D.; Unger, P. F.; Patenaude, J. V.; Bounameaux, H. Non-invasive diagnosis of venous thromboembolism in outpatients. *Lancet* **353**:190–195; 1999.
- Kallner, A.; Egberg, N.; Yu, A.; Merzoug-Djilali, R.; Blomberg, I.; Jornekog, G.; Blomback, M. Are results of fibrinogen measurements transferable? *Clin. Chem. Lab. Med* **41**:804–808; 2003.
- Bounameaux, H.; Cirafici, P.; de Moerloose, P.; Schneider, P. A.; Slosman, D.; Reber, G.; Unger, P. F. Measurement of D-dimer in plasma as diagnostic aid in suspected pulmonary embolism. *Lancet* **337**:196–200; 1991.
- Bozic, M.; Blinc, A.; Stegnar, M. D-dimer, other markers of haemostasis activation and soluble adhesion molecules in patients with different clinical probabilities of deep vein thrombosis. *Thromb. Res* **108**:107–114; 2002.
- Declerck, P. J.; Mombaerts, P.; Holvoet, P.; De Mol, M.; Collen, D. Fibrinolytic response and fibrin fragment D-dimer levels in patients with deep vein thrombosis. *Thromb. Haemostasis* **58**:1024–1029; 1987.
- Di Nisio, M.; Squizzato, A.; Rutjes, A. W.; Buller, H. R.; Zwinderman, A. H.; Bossuyt, P. M. Diagnostic accuracy of D-dimer test for exclusion of venous thromboembolism: a systematic review. *J. Thromb. Haemostasis* **5**:296–304; 2007.
- Nordenholz, K. E.; Mitchell, A. M.; Kline, J. A. Direct comparison of the diagnostic accuracy of fifty protein biological markers of pulmonary embolism for use in the emergency department. *Acad. Emerg. Med* **15**:795–799; 2008.
- Wakefield, T. W.; Strieter, R. M.; Wilke, C. A.; Kadell, A. M.; Wroblewski, S. K.; Burdick, M. D.; Schmidt, R.; Kunkel, S. L.; Greenfield, L. J. Venous thrombosis-associated inflammation and attenuation with neutralizing antibodies to cytokines and adhesion molecules. *Arterioscler. Thromb. Vasc. Biol* **15**:258–268; 1995.
- Henke, P. K.; Varga, A.; De, S.; Deatrck, C. B.; Eliason, J.; Arenberg, D. A.; Sukheepod, P.; Thanaporn, P.; Kunkel, S. L.; Upchurch Jr G. R.; Wakefield, T. W. Deep vein thrombosis resolution is modulated by monocyte CXCR2-mediated activity in a mouse model. *Arterioscler. Thromb. Vasc. Biol* **24**:1130–1137; 2004.
- Lo, S. K.; Cheung, A.; Zheng, Q.; Silverstein, R. L. Induction of tissue factor on monocytes by adhesion to endothelial cells. *J. Immunol* **154**:4768–4777; 1995.
- Eiserich, J. P.; Hristova, M.; Cross, C. E.; Jones, A. D.; Freeman, B. A.; Halliwell, B.; van der Vliet, A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391**:393–397; 1998.
- Pignatelli, B.; Li, C. Q.; Boffetta, P.; Chen, Q.; Ahrens, W.; Nyberg, F.; Mukeria, A.; Bruske-Hohlfeld, I.; Fortes, C.; Constantinescu, V.; Ischiropoulos, H.; Ohshima, H. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res* **61**:778–784; 2001.
- Heit, J. A.; Silverstein, M. D.; Mohr, D. N.; Petterson, T. M.; O'Fallon, W. M.; Melton 3rd L. J. Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study. *Arch. Intern. Med* **160**:809–815; 2000.
- Parastatidis, I.; Thomson, L.; Fries, D. M.; Moore, R. E.; Tohyama, J.; Fu, X.; Hazen, S. L.; Heijnen, H. F.; Dennehy, M. K.; Liebler, D. C.; Rader, D. J.;



- Ischiropoulos, H. P. Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. *Circ. Res* **101**:368–376; 2007.
- [34] Inada, Y.; Hessel, B.; Blomback, B. Photooxidation of fibrinogen in the presence of methylene blue and its effect on polymerization. *Biochim. Biophys. Acta* **532**:161–170; 1978.
- [35] Shacter, E.; Williams, J. A.; Levine, R. L. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. *Free Radic. Biol. Med* **18**:815–821; 1995.
- [36] Doolittle, R. F.; Pandi, L. Binding of synthetic B knobs to fibrinogen changes the character of fibrin and inhibits its ability to activate tissue plasminogen activator and its destruction by plasmin. *Biochemistry* **45**:2657–2667; 2006.
- [37] Geer, C. B.; Tripathy, A.; Schoenfisch, M. H.; Lord, S. T.; Gorkun, O. V. Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. *J. Thromb. Haemostasis* **5**:2344–2351; 2007.
- [38] Litvinov, R. I.; Gorkun, O. V.; Galanakis, D. K.; Yakovlev, S.; Medved, L.; Shuman, H.; Weisel, J. W. Polymerization of fibrin: direct observation and quantification of individual B:b knob-hole interactions. *Blood* **109**:130–138; 2007.
- [39] Hodara, R.; Norris, E. H.; Giasson, B. I.; Mishizen-Eberz, A. J.; Lynch, D. R.; Lee, V. M.; Ischiropoulos, H. Functional consequences of  $\alpha$ -synuclein tyrosine nitration: diminished binding to lipid vesicles and increased fibril formation. *J. Biol. Chem* **279**:47746–47753; 2004.
- [40] Freyburger, G.; Trillaud, H.; Labrousse, S.; Gauthier, P.; Javorschi, S.; Bernard, P.; Grenier, N. D-dimer strategy in thrombosis exclusion—a gold standard study in 100 patients suspected of deep venous thrombosis or pulmonary embolism: 8 DD methods compared. *Thromb. Haemostasis* **79**:32–37; 1998.
- [41] Benderly, M.; Graff, E.; Reicher-Reiss, H.; Behar, S.; Brunner, D.; Goldbourt, U. Fibrinogen is a predictor of mortality in coronary heart disease patients: the Bezafibrate Infarction Prevention (BIP) Study Group. *Arterioscler. Thromb. Vasc. Biol* **16**:351–356; 1996.
- [42] Folsom, A. R.; Wu, K. K.; Rosamond, W. D.; Sharrett, A. R.; Chambless, L. E. Prospective study of hemostatic factors and incidence of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* **96**:1102–1108; 1997.
- [43] Koster, T.; Rosendaal, F. R.; Reitsma, P. H.; van der Velden, P. A.; Briet, E.; Vandenbroucke, J. P. Factor VII and fibrinogen levels as risk factors for venous thrombosis: a case-control study of plasma levels and DNA polymorphisms—the Leiden Thrombophilia Study (LETS). *Thromb. Haemostasis* **71**:719–722; 1994.
- [44] van Hylckama Vlieg, A.; Rosendaal, F. R. High levels of fibrinogen are associated with the risk of deep venous thrombosis mainly in the elderly. *J. Thromb. Haemostasis* **1**:2677–2678; 2003.
- [45] Kucher, N.; Kohler, H. P.; Dornhofer, T.; Wallmann, D.; Lammle, B. Accuracy of D-dimer/fibrinogen ratio to predict pulmonary embolism: a prospective diagnostic study. *J. Thromb. Haemostasis* **1**:708–713; 2003.
- [46] Kucher, N.; Schroeder, V.; Kohler, H. P. Role of blood coagulation factor XIII in patients with acute pulmonary embolism: correlation of factor XIII antigen levels with pulmonary occlusion rate, fibrinogen, D-dimer, and clot firmness. *Thromb. Haemostasis* **90**:434–438; 2003.