1-1-2012

The Role of Fibrin Clot Properties and Fibrinogen Nitration in the Pathology of Venous Thromboembolism.

Marissa Rose Martinez
University of Pennsylvania, marissarmartinez@gmail.com

The Role of Fibrin Clot Properties and Fibrinogen Nitration in the Pathology of Venous Thromboembolism.

Abstract
Deep vein thrombosis (DVT) and pulmonary embolism (PE) together comprise the disease state of venous thromboembolism (VTE). Thrombi in the veins of the lower extremities (DVT) can embolize, resulting in complete or partial occlusion of circulation through the pulmonary vasculature (PE). Despite a common etiology between DVT and PE, the cause of embolization remains mostly unknown. Research indicates that fibrin clot structure and functional properties are altered in VTE compared to healthy controls. Whether these properties differ between DVT and PE subjects remains to be determined, and may underscore possible mechanisms of embolization. Inflammation and oxidant production are involved in the pathogenesis of VTE. However, biomarkers documenting these pathways in humans are lacking. Elevated levels of nitrated fibrinogen have been documented during inflammatory and oxidative challenges and may serve as a functional biomarker in VTE. Differences in fibrin clot structure and functional properties between DVT and PE, as well as the role of nitrated fibrinogen as a biomarker of VTE were evaluated in the plasma of subjects presenting with symptoms of VTE to the emergency department at the Hospital of the University of Pennsylvania. Plasma fibrin clots derived from PE subjects showed increased establishment of viscoelastic properties and faster lysis time compared with DVT. The rate and extent of factor XIIIa crosslinking of fibrin were similar between DVT and PE. Clots from PE subjects showed decreased fiber density and no differences in fiber bundling. Together these data suggests that fibrin fibers are formed faster in PE subjects, possibly resulting in earlier lysis, which may lead to instability and embolization. Nitrated fibrinogen levels were elevated in VTE positive compared to VTE negative subjects, with no differences in fibrinogen concentration. Subjects in the highest quartile of nitrated fibrinogen had an increased risk of VTE compared with the lowest quartile. This risk persisted after univariate adjustment for advanced age, active cancer, and recent surgery, but not after multivariate adjustment for all variables. Thus, nitrated fibrinogen is an oxidative risk marker in VTE, providing a novel mechanistic link between oxidant production, inflammation, and VTE.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Pharmacology

First Advisor
Harry Ischiropoulos

Keywords
Deep vein thrombosis, Fibrinogen, Nitration, Pulmonary embolism, Venous thromboembolism

THE ROLE OF FIBRIN CLOT PROPERTIES AND FIBRINOGEN NITRATION IN THE PATHOLOGY OF VENOUS THROMBOEMBOLISM.

Marissa Rose Martinez

A DISSERTATION

in

Pharmacology

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2012

Supervisor of Dissertation

_____

Harry Ischiropoulos, Ph.D., Professor, Department of Pediatrics

Graduate Group Chairperson

_____

Julie Blendy, Ph.D., Professor, Department of Pharmacology

Dissertation Committee

Lawrence Brass (Chair), MD, Ph.D., Professor, Department of Medicine

Adam Cuker, MD, Assistant professor, Department of Medicine

Sriram Krishnaswamy, Ph.D., Professor, Department of Pediatrics

John Weisel, Ph.D., Professor, Department of Cell and Developmental Biology

THE ROLE OF FIBRIN CLOT PROPERTIES AND FIBRINOGEN NITRATION IN THE PATHOLOGY OF VENOUS THROMBOEMBOLISM.

DEDICATION

I would like to dedicate my thesis to my grandmother, Anna Puglisi, who highly valued education and was an inspiration as a working woman, mother, grandmother, and friend.

ACKNOWLEDGMENT

I would like to thank Harry, my advisor, who has stayed with me throughout this very long and sometimes turbulent road to graduation. Without his support I don't think I would be writing this. I truly appreciate everything that he has done for me including pushing me to be a better scientist. John Weisel has also acted as a second mentor to me, and I am very grateful for his guidance and insight not just on fibrinogen, but on science and life in general. I would like to thank all the members of the Ischiropoulos lab, especially Margarita for her mentoring, advice, and collaboration and Todd for all his knowledge on proteomics during those early years. Jenn, Kristen, Karthik, and Elpida have also been invaluable as friends, as well as lab mates, and have helped me succeed through emotional support. I would like to thank the members of the Weisel lab, Irina, Sekar, and Rustem for all their knowledge on fibrinogen and guidance through experiments. I would like to thank my family and friends, who have supported me throughout this process. Finally, I would like to thank my partner, Steven for always being my biggest cheerleader and helping me navigate a winding and often bumpy road toward graduation.

ABSTRACT

The role of fibrin clot properties and fibrinogen nitration in the pathology of venous thromboembolism.

Marissa Rose Martinez

Harry Ischiropoulos, Ph.D.

Deep vein thrombosis (DVT) and pulmonary embolism (PE) together comprise the disease state of venous thromboembolism (VTE). Thrombi in the veins of the lower extremities (DVT) can embolize, resulting in complete or partial occlusion of circulation through the pulmonary vasculature (PE). Despite a common etiology between DVT and PE, the cause of embolization remains mostly unknown. Research indicates that fibrin clot structure and functional properties are altered in VTE compared to healthy controls. Whether these properties differ between DVT and PE subjects remains to be determined, and may underscore possible mechanisms of embolization. Inflammation and oxidant production are involved in the pathogenesis of VTE. However, biomarkers documenting these pathways in humans are lacking. Elevated levels of nitrated fibrinogen have been documented during inflammatory and oxidative challenges and may serve as a functional biomarker in VTE. Differences in fibrin clot structure and functional properties between DVT and PE, as well as the role of nitrated fibrinogen as a biomarker of VTE were evaluated in the plasma of subjects presenting with symptoms of VTE to the emergency

department at the Hospital of the University of Pennsylvania. Plasma fibrin clots derived from PE subjects showed increased establishment of viscoelastic properties and faster lysis time compared with DVT. The rate and extent of factor XIIIa crosslinking of fibrin were similar between DVT and PE. Clots from PE subjects showed decreased fiber density and no differences in fiber bundling. Together these data suggests that fibrin fibers are formed faster in PE subjects, possibly resulting in earlier lysis, which may lead to instability and embolization. Nitrated fibrinogen levels were elevated in VTE positive compared to VTE negative subjects, with no differences in fibrinogen concentration. Subjects in the highest quartile of nitrated fibrinogen had an increased risk of VTE compared with the lowest quartile. This risk persisted after univariate adjustment for advanced age, active cancer, and recent surgery, but not after multivariate adjustment for all variables. Thus, nitrated fibrinogen is an oxidative risk marker in VTE, providing a novel mechanistic link between oxidant production, inflammation, and VTE.

TABLE OF CONTENTS

DEDICATION ..... **III**

ACKNOWLEDGMENT ..... **IV**

ABSTRACT ..... **V**

LIST OF TABLES ..... **IX**

LIST OF FIGURES ..... **X**

CHAPTER 1: INTRODUCTION **1**

1.1 Introduction to VTE **2**
1.1.1 Prevalence of VTE 2
1.1.2 Pathophysiology of VTE 3
1.1.3 VTE diagnosis 7
1.1.4 Antithrombotic therapy for VTE 9

1.2 Fibrinogen and clot formation **11**
1.2.1 Factor XIII and fibrin crosslinking 13
1.2.2 Fibrinolysis 15
1.2.3 Fibrin clot structure and functional properties in thrombotic disease 15
1.2.3.1 Fibrin clot properties in arterial disease 17
1.2.3.2 Fibrin clot properties in venous thromboembolic disease 18

1.3 Production of reactive oxygen and nitrogen intermediates in inflammation **18**
1.3.1 Modifications of tyrosine residues by nitrative species 20
1.3.2 Tyrosine nitration plays a role in inflammation 21
1.3.3 Tyrosine nitration in VTE 24

1.4 Specific Aims **26**

CHAPTER 2: INCREASED FIBRINOLYSIS AND ACQUISITION OF FIBRIN CLOT VISCOELASTIC PROPERTIES ARE ASSOCIATED WITH PULMONARY EMBOLISM **28**

2.1 Abstract **29**

2.2 Introduction **30**

2.3 Materials and Methods **31**

2.4 Results **36**

2.5 Discussion **44**

CHAPTER 3: NITRATED FIBRINOGEN IS A BIOMARKER OF OXIDATIVE STRESS IN VENOUS THROMBOEMBOLISM. **48**

3.1 Abstract **49**

3.2 Introduction **50**

3.3 Materials and Methods **52**

3.4 Results **57**

3.5 Discussion **61**

CHAPTER 4: SUMMARY AND DISCUSSION **67**

4.1 Fibrin clot properties and structure are differentially altered in VTE subjects **68**
4.1.1 Fibrinolysis may regulate embolization 68
4.1.2 Clots formed from PE subjects establish viscoelastic properties faster than DVT 70
4.1.3 FXIIIa crosslinking of fibrin does not contribute to differences in fibrin clot properties 71
4.1.4 Fibrinogen variants, fibrinogen post-translational modifications, and plasma constituents may contribute to differences between DVT and PE 72
4.1.5 Shear rates may affect embolization in proximal veins 75
4.1.6 Future directions 76

4.2 Nitrated fibrinogen is a biomarker for VTE **79**
4.2.1 Nitrated fibrinogen levels are elevated in VTE positive subjects 80
4.2.2 Nitrated fibrinogen is a risk factor for VTE 80
4.2.3 Nitrated fibrinogen may affect clot formation in VTE 81
4.2.4 Plasma fibrinogen levels are not associated with VTE 84
4.2.5 Future directions 85

4.3 Risk factors for VTE **87**

4.4 VTE, antioxidants, and pharmaceutics **89**

REFERENCES **92**

LIST OF TABLES

CHAPTER 2

Table 2-1 Study population demographics, history and medication use 37

Table 2-2 Clot formation and lysis time of fibrin clots in DVT and PE subjects 38

Table 2-3 Kinetic analysis of elastic and viscous properties of fibrin clots in DVT and PE subjects 40

CHAPTER 3

Table 3-1 Characteristics of VTE subject population 56

Table 3-2 Odds ratio of VTE by quartiles of nitrated fibrinogen 61

LIST OF FIGURES

CHAPTER 1

Figure 1-1 Schematic representation of the coagulation cascade 6

Figure 1-2 Crystal structure of human fibrinogen 12

Figure 1-3 Schematic representation of fibrin polymerization 14

Figure 1-4 Formation of nitrotyrosine residues 20

CHAPTER 2

Figure 2-1 Averaged clot formation and lysis time curves from DVT and PE subjects 39

Figure 2-2 Viscoelastic properties in DVT and PE subjects 41

Figure 2-3 Factor XIIIa crosslinking of fibrin fibers within plasma clots as a function of time in DVT and PE subjects 42

Figure 2-4 Scanning electron micrographs of clots formed from the plasma of DVT and PE subjects 44

CHAPTER 3

Figure 3-1 Fibrinogen, nitrated fibrinogen and D-dimer levels in VTE negative and VTE positive subjects 58

Figure 3-2 Relationship between the odds ratio of acute VTE and nitrated fibrinogen levels 60

CHAPTER 1

Introduction

1.1 Introduction to VTE

Venous thromboembolism (VTE) is a common thrombotic disease state that encompasses both deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT is the formation of one or more thrombi within the body's large veins, and is usually localized to the veins of the lower extremities. Production of these thrombi can result in partial or complete blockage of venous circulation. As such, patients often present with symptoms and signs of pain, swelling, discoloration, and tenderness in the affected area. One of the most serious complications of DVT is PE, which occurs when the thrombus embolizes and becomes lodged within the pulmonary vasculature, partially or completely blocking a pulmonary artery or branch. In this way, DVT and PE are two disease states related by a common origin, which subsequently diverge due to distinct mechanisms that have yet to be determined.

1.1.1 Prevalence of VTE

VTE affects around 1 per 1000 people per year within the United States, with increasing incidence for congenital and acquired thrombophilic risk factors (Anderson et al., 1991). Using computer modeling, it is estimated that over 900,000 events of incident or recurrent, fatal and non-fatal VTE occur annually within the U.S., more than either myocardial infarction or stroke (Heit et al., 2005). History of DVT is itself a risk factor for a second episode, as 30 % of patients experience a recurrent event within 10 years, with the greatest risk within 6-12 months after the initial event (Heit et al., 2000a, Schulman et al., 2006). Patients with recurrent VTE are also more likely to experience the same type of occurrence as the incident event (Schulman et al., 2006, Anonymous

1997). Thirty percent of VTE cases die within three months (Heit et al., 1999). Although DVT is not a significant cause of death, a longitudinal study investigating the etiology of VTE found that 9 % of cases of DVT resulted in death (Cushman et al., 2004), and modeling for both hospital and community based DVT estimate that over 2200 deaths per year are attributed to DVT (Heit et al., 2005). PE is the most significant cause for mortality within VTE, with epidemiological studies indicating that 23 % of patients die immediately from PE, and an additional 25 % die within 3.5 years (Anderson et al., 1991). Together, DVT and PE are estimated to account for between 100,000 and 180,000 deaths each year within the United States (Rathbun., 2009).

1.1.2 Pathophysiology of VTE

The formation of thrombi in either the arterial or venous systems is due to aberrant activation of the coagulation system, likely due to some combination of changes in blood flow, endothelial activation or disruption, and a hypercoagulable state, as Virchow described (Virchow., 1856). Unlike arterial thrombosis, venous thrombosis occurs in the absence of endothelial denudation or apparent damage (Schaub et al., 1984, von Bruhl et al., 2012), suggesting that the other initiators of thrombosis described above, as well as participation from inflammatory mediators play a more central role in VTE pathology (Schaub et al., 1984, Sevitt., 1974). DVT often occurs in areas of stasis or disturbed blood flow such as in valve pockets or sinuses (Sevitt., 1974). To maintain quiescent conditions, endothelial cells produce and secrete factors that prevent cell adhesion and activation of coagulation, including thrombomodulin, protein C, prostacyclin, and nitric oxide. Although the central cause of VTE remains unknown it is clear that during VTE endothelial cells become activated resulting in the surface expression of adhesion

proteins such as P-selectin and von Willibrand factor (von Bruhl et al., 2012, Myers et al., 2002, Palabrica et al., 1992, Geng et al., 1990, Wakefield et al., 1995). Neutrophils and monocytes express P-Selectin Glycoprotein-1, and are subsequently recruited to P-selectin expressing endothelial cells (von Bruhl et al., 2012, Myers et al., 2002, Palabrica et al., 1992, Geng et al., 1990, Wakefield et al., 1995). Upon binding to these proteins, neutrophils, and monocytes become activated and shed tissue factor bearing microparticles that contain anionic membranes capable of assembling the coagulation cascade complexes (Figure 1-1) (von Bruhl et al., 2012, von Bruhl et al., 2012, Hrachovinova et al., 2003, Biro et al., 2003, Jy et al., 1995). Ultimately, fibrin is formed in the last step of the coagulation cascade, producing a thrombus composed mainly of fibrin and leukocytes with some platelets and trapped erythrocytes (Schaub et al., 1984, von Bruhl et al., 2012, Sevitt., 1974, Palabrica et al., 1992, McGuinness et al., 2001). Alternatively in VTE, thrombi can also have two distinct cellular compositions. Thrombi near the site of initiation are red, composed mainly of erythrocytes and fibrin, and thrombi downstream of the initiation site are white, composed of mainly platelets and some fibrin (Sevitt., 1974). The thrombus can fully or partially abrogate blood flow through the vein resulting in pain, swelling, and discoloration in the affected area.

During and following thrombus formation, cellular factors work to resolve the thrombus, which resembles wound healing. The formation of fibrin, in either soluble or fiber form (Rakoczi et al., 1978, Garman and Smith., 1982, Thorsen et al., 1972), acts as a cofactor for tissue plasminogen activator (tPA) cleavage of the serine protease plasminogen, forming a ternary complex that produces plasmin and results in the degradation of the fibrin clot (Hoylaerts et al., 1982). Although leukocytes are capable of endothelial

damage during chemotaxis and subsequent diapedesis, they are also required for thrombus resolution (Stewart et al., 1974). Neutrophils are the first cells to invade the thrombus, and they function to degrade collagen and fibrin (Varma et al., 2003). Their depletion during thrombus resolution results in larger thrombi (Varma et al., 2003). Similar results are seen with macrophages where direct administration of peritoneal macrophages decreased thrombus size by more than fivefold and deletion of the monocyte attractant chemokine, CCR2, inhibited monocyte recruitment and thrombus resolution (Ali et al., 2006).

Upon embolization, the thrombus can become lodged in a pulmonary artery or branch, with smaller thrombi traveling farther through the vasculature (Pryce and Heard., 1956). Large emboli can completely abrogate blood flow, and may result in death due to insufficient blood flow to fill the left side of the heart and maintain systemic arterial pressure (Corrin and Nicholson., 2011). Large and small emboli can both result in complete or partial infarction, which is necrosis of the tissue distal to the site of embolic obstruction. Complete infarction is rare and thought to require congestion in the lungs due to conditions such as heart disease (Dalen et al., 1977). Complete infarction can be healed by repair mechanisms similar to those described for DVT accompanied by the production of scar tissue (Corrin and Nicholson., 2011). Partial infarction is much more common and is characterized by edema, followed by capillary rupture and a filling of the alveoli with blood (Corrin and Nicholson., 2011). These infarcts are resolved within a few days, and usually little to no production of scar tissue is evident (Corrin and Nicholson., 2011).

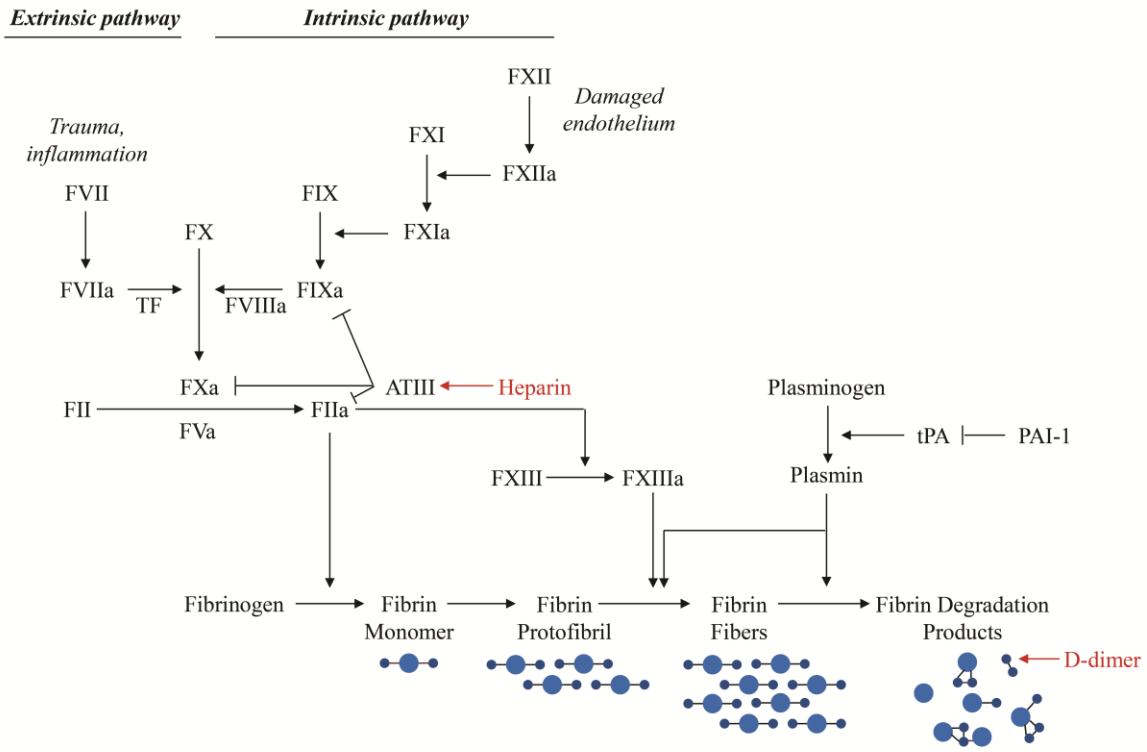

Figure 1-1. The coagulation cascade. Endothelial cell damage, trauma, or inflammation result in the activation of the coagulation cascade during times of both hemostasis and thrombosis. The coagulation cascade factors with the exception of TF, FV, FVIII, and FXIII circulate as zymogen proteases, requiring cleavage to become active serine proteases. The extrinsic pathway is considered the initiation pathway and the intrinsic, the propogation pathway. The extrinsic pathway involves only FVIIa and TF, produced either in circulation or by activated leukocytes. The intrinsic pathway induces the activation of FXIIa, FXIa, FIXa, and FVIIIa. Both the extrinsic and intrinsic pathways flow into the common pathway, resulting in the activation of FXa. FXa along with FVa and prothrombin (II) form the prothrombinase complex, converting prothrombin to thrombin (IIa). FpA and FpB on fibrinogen are cleaved by thrombin to create fibrin monomers. Monomers self-associate to form protofibrils, and then laterally aggregate to form fibrin fibers. Thrombin also activates FXIIIa, which crosslinks fibrin. Plasminogen together with tPA bind to fibrin, creating a ternary complex. Fibrin acts as a cofactor for the activation of plasminogen to plasmin, which subsequently lyses fibrin clots, creating fibrin degradation products. Calcium is a requirement of many of the reactions in the cascade including the production of FIXa, FXa, thrombin, fibrin, and FXIIIa crosslinking. In addition, anionic phospholipids are required for the production of FXa and thrombin. Of note, D-dimer is used as a non-invasive marker in the diagnosis of VTE, and Heparin, which activates ATIII, inhibiting FIXa, FXa, and thrombin, is used as a first line treatment for VTE.

Although the cause of embolization remains unknown, the location of the thrombus is associated with risk for PE. Deep veins of the lower extremities include the veins above the knee i.e. proximal and those veins below the knee or distal. Proximal veins include popliteal, femoral, or iliac and distal veins include tibial, peroneal, and the muscular calf veins, soleal and gemellar. Distal DVT is rarely associated with symptoms (Ciuti et al., 2012), while 88 % of patients with symptomatic DVT have thrombi localized to the

proximal veins (Cogo et al., 1993). Proximal, and not distal DVT, is frequently accompanied by PE (Moser and LeMoine., 1981). In one early study, PE occurred in 0 of 31 subjects with distal DVT, whereas in subjects with proximal vein thrombosis, PE occurred in 4 of 9 subjects (Kakkar et al., 1969). Furthermore, in symptomatic DVT subjects without chest symptoms of PE, 40 % had evidence of PE upon lung scanning (Moser et al., 1994). Nearly 100 % of subjects with proximal DVT also had evidence of distal DVT, indicating that thrombosis starts in the calf veins and propagates to the proximal veins, whereby it may embolize.

1.1.3 VTE diagnosis

DVT is diagnosed by several imaging techniques, but the reference standard is contrast venography. To view the entire deep venous system, iodinated contrast agent is injected into the dorsal foot vein and lack of venous filling is an indicator of DVT (Bates et al., 2012). Contrast venography is highly sensitive (95 %) and specific (92 %) (Killewich et al., 1989), but is expensive, invasive, and contraindicated in patients with renal disease and allergy to contrast media (Lensing et al., 1990). Thus, the utility of non-invasive diagnostic tests are preferentially used during diagnosis of DVT. Clinical probability scoring is used in the primary analysis of likely DVT and is recommended by the American College of Chest Physicians as the first assessment for diagnosis. Wells pretest probability scoring is the most widely used and takes into account signs, symptoms and risk factors for DVT and assigns a low, moderate or high probability (Wells et al., 1997). Following a low pretest probability, D-dimer testing is recommended. D-dimers are a crosslinked fibrin degradation product produced during the intravascular thrombosis and thrombolysis (Figure 1-1) (Ruckley et al., 1970). Several assays have been developed for

the rapid measurement of these products in both plasma and blood. These include qualitative assays such as whole blood agglutination (John et al., 1990) and quantitative assays such as latex agglutination (Elms et al., 1986), and enzyme-linked immunosorbent (ELISA) or immunofluorescence (ELFA) assays (Whitaker et al., 1985). Agglutination assays rely on monoclonal anti-D-dimer antibody linked red blood cells or latex beads that aggregate in the presence of antigen (D-dimer) (Elms et al., 1986). The ELISA and ELFA assays also utilize monoclonal anti-D-dimer antibodies in a multi-well format coupled with either chemiluminescence or fluorescence detection (de Moerloose et al., 1996, Bounameaux et al., 1991). Compared to latex (57-100 %) and whole blood agglutination (53-100 %), ELISA (71-100 %) and ELFA (88-100 %) have the highest sensitivity. Specificity remains low in all tests (Di Nisio et al., 2007), which is partly attributed to the high number of non-thrombotic conditions, including pregnancy, cancer, infection etc. (Francalanci et al., 1995, Hafter et al., 1985, Kuller et al., 2008) that also suffer from increased D-dimer levels. Because of this, D-dimer is used as an exclusionary test, with patients below a certain D-dimer level precluded from VTE. Recent efforts have been made to identify additional markers of VTE to aid in non-invasive diagnosis, including coagulation and inflammatory factors such as factor XI, c-reactive protein, and myeloperoxidase (MPO) (Cushman et al., 2009, Kucher et al., 2003b, Nordenholz et al., 2008, Mitchell et al., 2008, Martinez et al., 2012). Studies on MPO have shown that elevated MPO is not only is a risk factor for VTE, but moderately improves upon the specificity of D-dimer for VTE diagnosis (59 % vs. 73 %) (Nordenholz et al., 2008, Mitchell et al., 2008). Nonetheless, identification of additional markers of VTE may help

explain the underlying etiology or pathology of VTE, and may also prove to be useful in future diagnostic marker of the disease.

Due to its non-invasive nature and high accuracy, the most commonly used imaging modality for DVT diagnosis is ultrasound (US). Distal DVT is not usually associated with PE or other clinically adverse events, and thus compression US (CUS) is often used and focused primarily on the proximal veins such as femoral and popliteal. The inability to collapse the vein under pressure is indicative of DVT by CUS (Bates et al., 2012), which has a high sensitivity (97 %) and specificity (86 %) (Heijboer et al., 1992). For patients diagnosed with distal DVT, it is often recommended that the patient receive additional follow-up US 5-7 days following the initial US to ensure that thrombosis has not extended to the proximal veins (Bates et al., 2012).

Both clinical probability scoring and D-dimer levels are also used as initial non-invasive tests for PE diagnosis. A high or moderate likelihood of PE requires subsequent use of imaging, of which computed tomographic pulmonary angiography (CTPA) is the accepted standard of care. In CTPA the patient receives an iodinated contrast agent that, upon scanning will show filling, or in the case of embolism, a defect in filling of the pulmonary vessels. A recent meta-analysis indicates that sensitivity of CTPA alone is comparable to CTPA plus additional CUS in identifying PE (97.4 % with vs. 97.3 % without CUS) (Mos et al., 2009). This study also demonstrated high negative predictive value (98.8 %) for CTPA indicating that overall, CTPA alone is sufficient to accurately identify positive and negative PE patients (Mos et al., 2009).

1.1.4 Antithrombotic therapy for VTE

Treatment modalities are similar for DVT and PE patients. Therapy for VTE targets the coagulation cascade, supporting a main role for fibrin rather than platelets in VTE. Heparin is a standard first line treatment for acute VTE. It functions to bind and activate antithrombin III causing a conformational change, which results in the inhibition of thrombin, factor IXa, and factor Xa (Figure 1-1) (Chuang et al., 2001). Heparin is a heterogeneous mixture of highly sulfated glycosaminoglycans and comes in two main types, unfractionated heparin (UFH), which is composed of polysaccharides ranging in size from 5-40 kDa and low molecular weight heparin (LMWH), which contains only polysaccharides equal to or less than 8 kDa (Lane et al., 1979). Homogenous polysaccharides such as fondaparinux function in a similar manner to the other heparin derivatives, but are less frequently used due to cost. UFH and LMWH have both been shown to resolve acute thrombosis and prevent recurrent VTE to similar degrees (Kearon et al., 2006), but LMWH has decreased risk for osteoporosis and heparin-induced thrombocytopenia (Monreal et al., 1994, Martel et al., 2005), and thus is recommended as initial antithrombotic treatment in VTE (Kearon et al., 2012) . Initial therapy for VTE usually proceeds for 5-10 days followed by long-term therapy for 3-6 months. The goal of long-term therapy is the complete resolution of the thrombus during the initial episode of VTE and to prevent recurrence. During this phase of treatment, patients are usually switched to vitamin K antagonists such as warfarin. Warfarin inhibits the enzyme vitamin K epoxide reductase, preventing vitamin K production (Magnusson et al., 1974). The coagulation factors VII, IX, X, prothrombin, protein S, and protein C require vitamin K for the conversion of glutamic acid to γ-carboxyglutamic acid, which is essential for calcium binding of phospholipid membranes (Furie et al., 1979). Continuation of therapy

past 6 months may be required in those where the risk of VTE remains either unknown as in idiopathic VTE, or ongoing as in cancer (Kearon et al., 2012). Patients with proximal DVT or PE are recommended for treatment as described above. However, patients with distal DVT without indication of risk factors for extension to proximal DVT are not recommended for treatment due to the high risk of bleeding outweighing benefits of anticoagulant therapy (Kearon et al., 2012).

1.2 Fibrinogen and clot formation

Fibrinogen is a 340 kDa protein involved in the end steps of the coagulation cascade (Figure 1-2). It is produced in liver hepatocytes and secreted into the blood (Whipple and Hurwitz., 1911), where it circulates with a half-life of about 3 days (Stein et al., 1978). Fibrinogen is composed of two pairs of three non-identical chains termed Aα, Bβ, and γ. Each chain is encoded by a different gene, all of which reside on chromosome 4 (Kant et al., 1983, Crabtree and Kant., 1982, Olaisen et al., 1982, Humphries et al., 1984). Together the chains comprise a symmetrical molecule composed of one globular E region flanked on each side by globular D regions (D-E-D) (Figure 1-2) (Hall and Slayter., 1959, Kollman et al., 2009, Yang et al., 2001, Brown et al., 2000). The central E region is connected to each of the D regions by triple helical coiled-coils comprised of the Aα, Bβ, and γ chains (Figure 1-2) (Kollman et al., 2009, Yang et al., 2001, Brown et al., 2000). The chains are held together by 5 symmetrical disulfide bonds and an asymmetrical disulfide ring (Blomback et al., 1976, Huang et al., 1993). The N-termini of the Aα, Bβ, and γ chains are all located in the E region and comprise the central nodule (Blomback et al., 1976, Zhang and Redman., 1992). The D regions are comprised of β and γ nodules formed from the carboxy terminal portions of the β and γ chains, respectively (Figure 1-

2) (Yee et al., 1997). The carboxy terminal portion of the α chain contains an independently folded compact domain called the αC domain (Burton et al., 2007) that is tethered amino terminally to a flexible region termed the αC connector (Tsurupa et al., 2002, Medved et al., 1983). The αC region and connector are hypothesized to be highly flexible and unable to be crystalized. Thus, they are not represented in Figure 1-2. The N-termini of Aα and Bβ chains contain fibrinopeptides A (FpA) and B (FpB) (Figure 1-3) (Kollman et al., 2009, Yang et al., 2001, Brown et al., 2000). Thrombin cleavage of these peptides exposes 'A' and 'B' knobs, resulting in the formation of fibrin monomers (Lorand., 1951, Blomback et al., 1978, Blomback et al., 1972, Bettelheim., 1956, Budzynski et al., 1983). 'A' and 'B' knobs are positively charged and have complementary negatively charged binding sites within the γ and β nodules in the D region of adjoining monomers termed 'a' and 'b' holes (Figure 1-3) (Pratt et al., 1997, Spraggon et al., 1997). Knob-hole associations each result in the formation of half-staggered, double stranded protofibrils (Krakow et al., 1972, Fowler et al., 1981), which

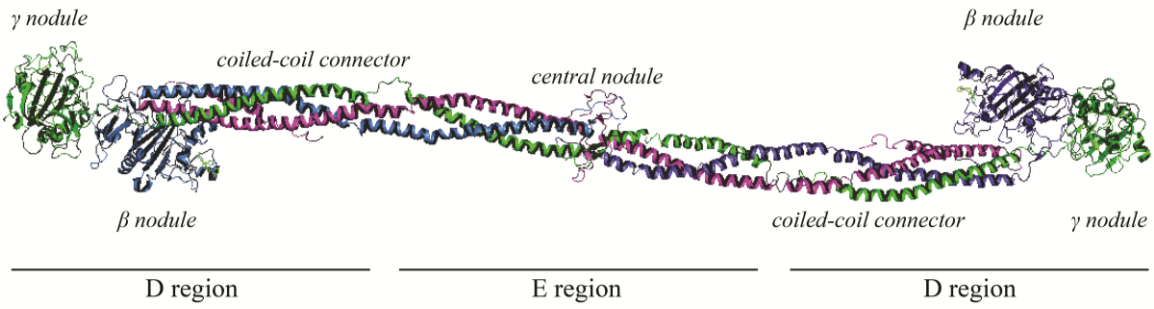

Figure 1-2. The crystal structure of human fibrinogen. Fibrinogen is a symmetrical molecule comprised of two sets of three non-identical polypeptide chains: Aα (red), Bß (blue), and γ (green). All three chains constitute the E region, which contains the central nodule. The carboxy termini of the β and γ chains create β and γ nodules, and together constitue the D-region. The E region is flanked on each side by a D region, which are connected by triple helical coiled-coils comprised of all three chains of fibrinogen.

then associate laterally to form fibrin fibers (Krakow et al., 1972, Hewat et al., 1983) and a branching network (Hantgan and Hermans., 1979). The number of protofibrils as well as the number of aggregated fibers together determine fiber diameter (Weisel., 1986, Weisel and Nagaswami., 1992). FpA release occurs at a faster rate than FpB (Blomback et al., 1976, Nossel et al., 1974) and thus, A:a interactions largely dictate fibrin polymerization rates. FpB release is not required for fibrin polymerization, but B:b interactions do function to align D-regions during polymerization, contributing to lateral aggregation and fiber formation, and also preventing the dissociation of protofibrils (Blomback et al., 1978, Shainoff and Dardik., 1983). As a consequence, in the absence of FpA release, B:b interactions alone result in thicker fibers and a more porous structure (Mosesson et al., 1987).

1.2.1 Factor XIII and fibrin crosslinking

Factor XIII (FXIII) is a transglutaminase that crosslinks fibrin subunits, providing stability and elasticity to the clot (Figure 1-1) (Gerth et al., 1974, Brown et al., 2009, Lorand and Jacobsen., 1962, Collet et al., 2000). FXIII is a heterotetrameric protein composed of two sets of A and B homodimeric domains (Schwartz et al., 1971). The A domains function as carriers of the B domains (Chung et al., 1974), which are activated by thrombin through cleavage of the N-terminal activation peptide (Nakamura et al., 1975). Activated FXIII crosslinks fibrin via the formation of ε-(γ-glutamyl) lysine bonds between γ-Gln^{398} and γ-Lys^{406} on each of the γ chains (Wang., 2011), creating γ-dimers. α-polymer crosslinks are formed primarily between α-Gln^{328}, α-Gln^{366} and α-Lys^{556} and α-Lys^{580} (Sobel and Gawinowicz., 1996, Cottrell et al., 1979). α-γ heteropolymers are also formed with γ-Lys^{406} and α-Gln^{328} and/or α-Gln^{366} (Sobel and Gawinowicz., 1996).

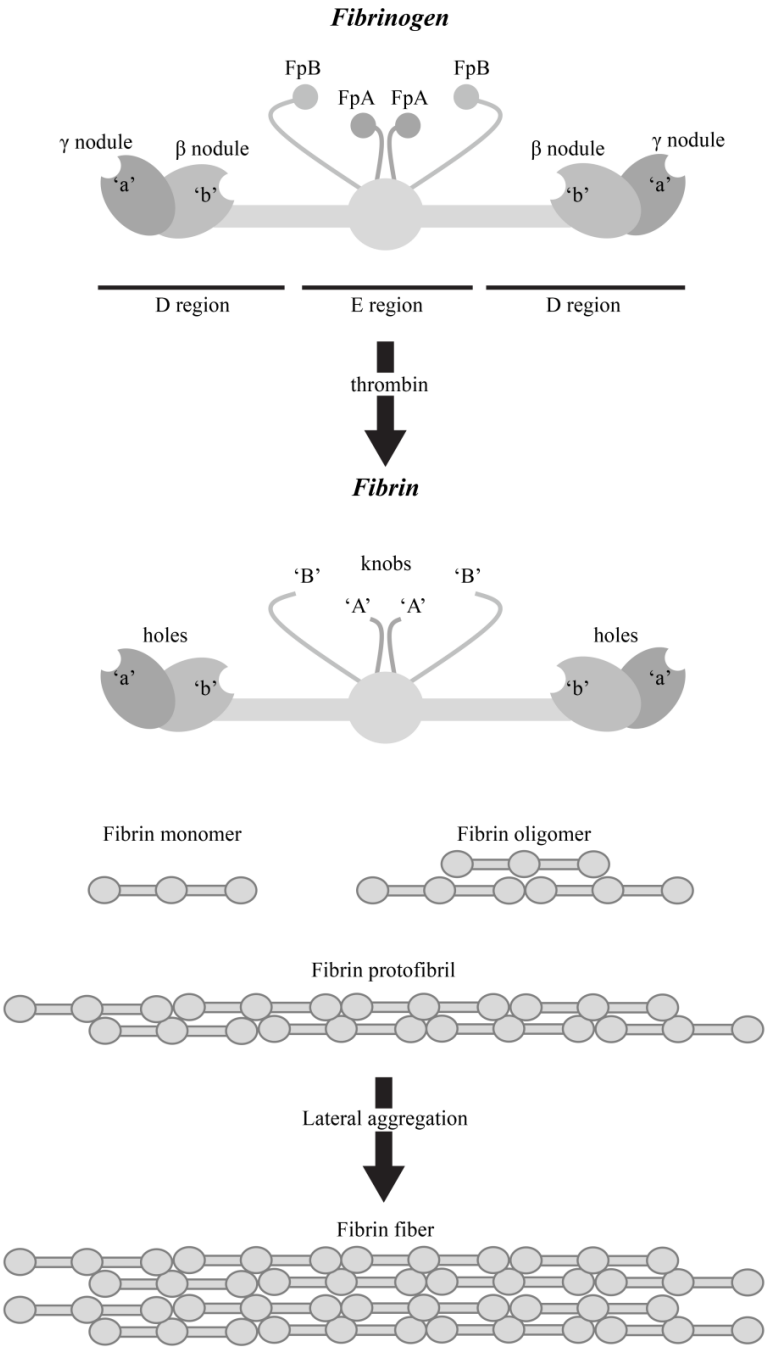

Figure 1-3. Fibrin polymerization. Fibrinogen is a circulating plasma protein involved in hemostasis and thrombosis. It has a trinodular structure, one E region flanked on each side by two D regions. Each D region is composed of a β nodule and γ nodule. The E region contains fibrinopeptides A (FpA) and B (FpB), which are cleaved by thrombin to yield 'A' and 'B' knobs. These knobs fit into 'a' and 'b' holes localized within the D region of adjacent fibrin molecules. The initial association of fibrin monomers produces small fibrin oligomers that elongate to form protofibrils. Subsequently, the protofibrils laterally aggregate to form fibrin fibers, which go on to form a complex branching network (not shown). Adapted from L. Medved and J. Weisel. Journal of Thrombosis and Haemostasis. 7: 355-359.

1.2.2 Fibrinolysis

tPA is a serine protease involved in fibrinolysis. The main function of tPA in hemostasis is to activate the zymogen precursor, plasminogen by cleavage of Arg^{561}-Val^{562}, generating the active serine protease, plasmin (Robbins et al., 1967). This occurs when tPA binds to fibrin at sites $A\alpha^{148-160}$ and $\gamma^{312-324}$, which are exposed during fibrin polymerization (Schielen et al., 1991a, Schielen et al., 1991b). Subsequently, plasminogen is recruited and forms a ternary complex with tPA and fibrin, which increases the catalytic efficiency of tPA activation of plasminogen to plasmin (Hoylaerts et al., 1982). Both tPA and plasminogen have preferential binding to fibrin over fibrinogen (Hoylaerts et al., 1982) preventing unwarranted degradation of fibrinogen in circulation. Plasmin binds and cleaves fibrin at high-affinity lysines located in the αC region (Bok and Mangel., 1985, Tsurupa and Medved., 2001), subsequently exposing additional lysine residues that plasmin further cleaves into smaller fragments (Bok and Mangel., 1985). Plasmin cleavage produces fibrin degradation products of various sizes and compositions, most notably X, Y, D, D-dimer, and E (KOPEC et al., 1973, Gaffney et al., 1980). Endogenous inhibitors of fibrinolysis target both tPA and plasmin, and include plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin (α_2-PI), and thrombin-activatable fibrinolysis inhibitor (TAFI). FXIII, while not an actual inhibitor of fibrinolysis, does reduce fibrinolytic potential by crosslinking of fibrin fibers as well as crosslinking α_2-PI to fibrin fibers (Lorand and Jacobsen., 1962, Gormsen et al., 1967).

1.2.3 Fibrin clot structure and functional properties in thrombotic disease

The role of the clot and its components has been evolutionarily optimized to prevent bleeding and maintain hemostasis. However, many environmental and genetic factors can predispose the clotting process towards a prothrombotic phenotype (Uitte de Willige et al., 2005, Parastatidis et al., 2008). Furthermore, many studies have suggested that changes due to factors such as plasma constituents, fibrinogen variants and post-translational modifications affect fibrin clot structure and functional properties and present as thrombotic risk factors (Parastatidis et al., 2008, Konings et al., 2011, Gersh et al., 2009). In general, fibrin clot structures with increased fiber density, decreased fiber diameter, and decreased porosity have generally been associated with diminished lysis (Collet et al., 2000) and are considered as a risk factor for thrombosis (Collet et al., 2006). Although individual thin fibers are lysed faster than thicker fibers, clots that are composed of thin fibers with increased fiber density lyse more slowly than clots composed of thick fibers of decreased fiber density (Collet et al., 2000). This is likely due to the lateral transection of plasmin across fibers rather than uniform digestion from the outside in of a fiber (Collet et al., 2000). Also, tPA is a limiting constituent during lysis, and clots with decreased fiber density have a higher tPA to fiber ratio, resulting in increased lysis (Bannish et al., Accepted b, Bannish et al., Accepted a). Denser clots also tend to have increased elastic (G') and viscous (G") properties (Collet et al., 2006, Roberts et al., 1974, Ryan et al., 1999), which are measures of stiffness and deformability, respectively. These two properties may be especially important with regards to embolization. Within the vessel, blood flow exerts shear stress on the thrombus. Clots that are less capable of withstanding those shear forces may be more amenable to embolization. It is unclear whether clots that are stiffer would be more or

less likely to embolize, as increased stiffness might impart stability or strain-hardening and brittleness. Similarly, clots that are more viscous may deform better under shear or might be very unstable and friable.

1.2.3.1 Fibrin clot properties in arterial disease

Previous studies investigating the effects of fibrin clot structure and functional properties have mainly focused on arterial thrombotic diseases. Compared to healthy age-matched controls, clots derived *ex vivo* from the plasma of subjects with premature CAD with a past history of acute coronary syndrome, exhibited increased fiber density, decreased porosity, fiber diameter and length. Plasma clots from these subjects also exhibited increased final clot stiffness and diminished clot lysis (Collet et al., 2006). Fibrin clot stiffness and length were independently correlated with both lysis and CAD, providing further evidence that clot structure is related to both functional properties and disease risk. In addition, recent studies have shown that plasma fibrin clot structure and functional properties are related to *in vivo* thrombus composition. In 45 ST-segment elevation myocardial infarction subjects both thrombus structure, including fibrin, cholesterol, and cellular components, as well as plasma fibrin clot properties were measured (Silvain et al., 2011). The structural components measured from *in vivo*-excised thrombi correlated with clot properties derived from *ex vivo* created plasma clots, including the elastic component and lysis time. In addition, fibrin was a main component of the thrombus (60 %) and was positively correlated with ischemic time. These data support the relevance of *ex vivo* plasma clots structure and functional properties with relation to thrombotic pathology.

1.2.3.2 Fibrin clot properties in venous thromboembolic disease

Fibrin clot properties have begun to be evaluated in VTE. In a recent study, VTE subjects showed diminished lysis and decreased clot permeability compared to healthy controls (Lisman et al., 2005, Undas et al., 2009), similar to what has been shown in CAD (Collet et al., 2006). In a sub-analysis of the data, the authors also show that PE subjects have enhanced clot lysis and increased permeability compared to DVT subjects (Undas et al., 2009). These data suggest that fibrin clot structure and functional properties may be different between DVT and PE patients. Because DVT and PE share a common pathogenesis with divergent outcomes, identifying differential clot properties may underscore the mechanisms that regulate embolization.

1.3 Production of reactive oxygen and nitrogen intermediates in inflammation

Reactive oxygen and nitrogen intermediates are produced during inflammatory processes. This includes the production of superoxide ($O_2 \cdot^-$) by the enzymes NADPH oxidase and xanthine oxidase, located in neutrophils and endothelial cells, respectively (McCord and Fridovich., 1968, Babior et al., 1976). Superoxide can undergo dismutation reaction by superoxide dismutase, generating hydrogen peroxide (H_2O_2) and water (McCord and Fridovich., 1969, Klug et al., 1972). Hydrogen peroxide can be reduced to the more potent oxidant, hydroxyl radical ($\cdot OH$) and hydroxide ion (OH^-) by Fe (II) through Fenton chemistry (Fenton., 1894), or oxidized to hypochlorous (HOCl) or hypobromous (HOBr) acids by peroxidase enzymes located in leukocytes (van Dalen et al., 1997, Klebanoff and Shepard., 1984).

The small, free radical, gaseous molecule, nitric oxide (·NO) is produced constitutively by many cell types, but also at heightened levels in response to inflammation (Furchgott and Zawadzki., 1980, Furchgott and Bhadrakom., 1953, Ignarro et al., 1987, Palmer et al., 1987). Nitric oxide can react with other free radicals including superoxide to produce the potent oxidant, peroxynitrite ($ONOO^-$) and its conjugate acid, peroxynitrous acid (ONOOH) (Reaction 1) (Gryglewski et al., 1986, Beckman et al., 1990). Peroxynitrous acid can undergo homolytic fission to generate hydroxyl radical and nitrogen dioxide ($\cdot NO_2$) (Reaction 2) (Beckman et al., 1990), which are capable of oxidation and nitration of proteins and lipids (Prutz et al., 1985). Despite this, it is more likely that under physiological conditions (Beckman et al., 1990) peroxynitrite reacts with the high levels of dissolved carbon dioxide (1-5 mM) to form the intermediate nitrosoperoxycarbonate ($ONOOCO_2^-$) (Reaction 3) (Gow et al., 1996, Lymar and Hurst., Denicola et al., 1996). The homolytic cleavage of this intermediate produces nitrogen dioxide and carbonate radical ($CO_3\cdot^-$) (Reaction 4) (Goldstein and Czapski., 1999), which are also capable of nitration and oxidation reactions. Peroxidases such as MPO and eosinophil peroxidase are also able to oxidize nitrite, producing the nitrative intermediate, nitrogen dioxide (van der Vliet et al., 1997).

1) $\cdot NO + O_2\cdot^- \longrightarrow ONOO^-$

2) $ONOOH \longrightarrow \cdot NO_2^- + \cdot OH^-$

3) $ONOO^- + CO_2 \longrightarrow ONOOCO_2^-$

4) $ONOOCO_2^- \longrightarrow \cdot NO_2^- + \cdot CO_3^-$

1.3.1 Modifications of tyrosine residues by nitrative species

The peroxidase/nitrite pathway or nitrosoperoxycarbonate decomposition result in the formation of nitrogen dioxide as described above. In one proposed mechanism of nitrotyrosine formation, tyrosyl radical is produced by oxidation of tyrosine by either nitrogen dioxide, hydroxyl radical, or carbonate radical, in the case of nitrosoperoxycarbonate decomposition (Santos et al., 2000). Subsequently, radical-radical recombination by tyrosyl radical and nitrogen dioxide produce nitrotyrosine (Figure 1-4) (Prutz et al., 1985).

Not all tyrosines are capable of becoming nitrated. The secondary protein structure is an important determinant of which tyrosines become modified. Surface exposure of the tyrosine residue, location of the tyrosine within a loop structure, proximity to negative charges, and proximity to metal centers aid in the specificity of tyrosine nitration (Souza et al., 1999). The physiologic and pathologic yields of nitration are low (10-100 μmol nitrotyrosine/ mol tyrosine), corresponding to 1 to 10 tyrosines nitrated for every 100,000

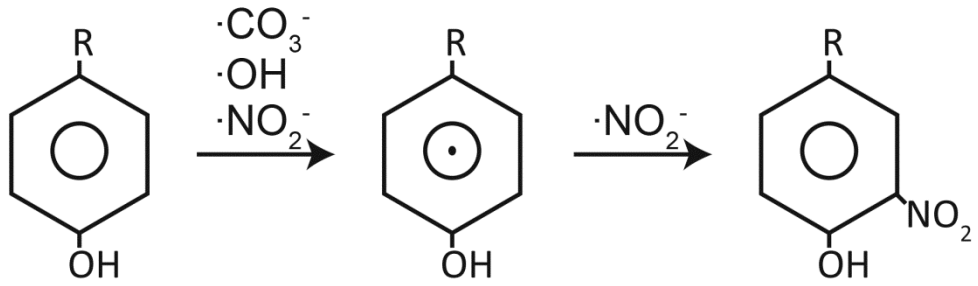

Figure 1-4. Formation of nitrotyrosine residues. Tyrosine is oxidized to tyrosyl radical by oxidants such as carbonate radical, hydroxyl radical, or nitrogen dioxide. Subsequently, radical-radical recombination by tyrosyl radical and nitrogen dioxide produce nitrotyrosine.

(Parastatidis et al., 2008, Brennan et al., 2002, Shishehbor et al., 2003). Because of this low yield, the biological significance of tyrosine nitration has been questioned. Often, post-translational modification of a protein that results in a loss of function requires a significant percentage of protein molecules to be modified. However, several proteins exhibit gain of function effects upon nitration, including actin and fibrinogen (Parastatidis et al., 2008, Aslan et al., 2003, Vadseth et al., 2004). Interestingly, both proteins are involved in polymerization reactions. Here, the addition of a few modified protein molecules enhances protein-protein interactions, and greatly affects the rate of polymerization (Parastatidis et al., 2008, Aslan et al., 2003, Vadseth et al., 2004). In fibrinogen, Tyr^{292} and Tyr^{422} were found to be nitrated in the plasma of smokers. These residues are located in the carboxyl termini of the β chains of fibrinogen near holes 'b' and nitration may function to enhanced lateral aggregation (Parastatidis et al., 2008). Elevated nitrated fibrinogen levels increased the rate of fibrin polymerization and were positively correlated with the clot viscoelastic properties, elastic and viscous moduli, and inversely correlated with fibrinolysis rate (Parastatidis et al., 2008). These clots also exhibited increased fiber density and fiber clustering. Thus, nitration of a few protein molecules can indeed affect protein function and may have significant biological consequences.

1.3.2 Tyrosine nitration plays a role in inflammation

Leukocytes are key mediators of the immune response against foreign pathogens, but also play a role in chronic disease states such as atherosclerosis and VTE. Neutrophils, monocytes and macrophages all contain the enzyme MPO. In neutrophils, MPO is localized to azurophilic granules, and in monocytes and macrophages it is localized to

lysosomal granules (Nichols et al., 1971, Bos et al., 1978). Upon activation, these leukocytes degranulate and release MPO into the extracellular space (Leffell and Spitznagel., 1974). MPO catalyzes the reduction of hydrogen peroxide, followed by the oxidation of halides (Cl^-, Br^-) to produce hypochlorous and hypobromous acids (van Dalen et al., 1997, Klebanoff and Shepard., 1984). These acids function as oxidants aimed at killing foreign pathogens. In addition, these cells produce nitric oxide through the both the constitutive and inducible forms of nitric oxide synthase (Wallerath et al., 1997), and are capable of producing the oxidative post-translational modification, nitrotyrosine through MPO oxidation of nitrite (NO_2^-). Nitrotyrosine has classically been a hallmark of peroxynitrite formation. However, more recent studies also indicate MPO as a key producer of nitrotyrosine. In an inflammatory challenge model of C. albicans administration, nitrotyrosine formation was markedly increased in wild-type, but not $MPO^{-/-}$ mice (Brennan et al., 2002). Neutrophils activated with 12-D-tetradecanoylphorbol-13-acetate released MPO from their granules, which subsequently transcytosed to the subendothelial matrix, colocalizing with and nitrating fibronectin (Baldus et al., 2001). This effect was absent in enoxaparin treated mice, which prevented MPO transcytosis. Thus, the release of MPO into the extracellular space may serve as a mechanism by which circulating proteins become nitrated.

The production of reactive oxygen and nitrogen species in inflammation has been extensively studied, and nitrotyrosine has been identified as both a marker and a functional modifier of proteins during inflammatory processes. In one study, lipopolysaccharide was administered to healthy human subjects and resulted in increased production of protein mediators of inflammation TGF-β, c-reactive protein, and MPO

(Heffron et al., 2009). Nitrated fibrinogen was also increased in this challenge model, peaking at 72-hrs post-injection, much later than the other inflammatory mediators. In addition, nitration of fibrinogen post-LPS injection resulted in increased rate of clot formation. This study implies that 1) nitration results as a direct consequence of inflammatory challenge, 2) nitrating intermediates are produced for prolonged periods of time following insult, and 3) tyrosine nitration effects protein function, specifically fibrinogen, which provides further evidence for the role of inflammation in thrombotic disorders.

Peroxynitrite has been extensively studied and found to play a role in inflammatory processes, including, but not limited to the nitration of protein tyrosine residues. In response to cellular activation, peroxynitrite is produced by endogenous nitric oxide formation in macrophages (Ischiropoulos et al., 1992). Peroxynitrite can inhibit cellular respiration in macrophages, producing DNA double-strand breaks, resulting in cytotoxicity associated with macrophage infiltration during inflammation (Szabo and Salzman., 1995). Peroxynitrite also activates Erk through the MAP kinase pathway, resulting in increased expression of $\alpha_M\beta_2$ on neutrophils and induces integrin dependent adhesion of neutrophils to endothelial cells (Zouki et al., 2001). Some studies have suggested that nitric oxide released from endothelial cells into the vascular lumen can interact with superoxide produced by activated neutrophils, producing peroxynitrite (Sohn et al., 2003). This could result in the production of nitrated and oxidized proteins within vascular circulation or in close proximity to the site of neutrophils or possibly macrophages.

Monoclonal and polyclonal antibodies to nitrated proteins, peptides and nitrotyrosine have been produced in mice and rabbits (Heijnen et al., 2006). This suggests that *in vivo* nitrotyrosine may be a target for endogenously produced anti-nitrotyrosine antibodies. Indeed, antibodies have been detected that recognize nitrated epitopes in acute lung injury, as well as in atherosclerotic lesions and the circulation of CAD patients (Thomson et al., 2007). Furthermore, in lymphoid tissues, antigen presenting cells displayed nitrated peptides by MHC class II molecule I-A, eliciting a CD4 T-cell response (Herzog et al., 2005). This indicates that the production of nitrotyrosine elicits an immune response in acute lung injury and CAD, and may have indications in other diseases where nitrated proteins have been identified, such as VTE.

1.3.3 Tyrosine nitration in VTE

VTE is a thrombotic disease mediated by inflammation. Neutrophils and monocytes home to activated endothelial cells and are primary constituents of the thrombus in VTE (von Bruhl et al., 2012, Myers et al., 2002, Palabrica et al., 1992, Geng et al., 1990, Wakefield et al., 1995). As described above, these cells are capable of producing nitrating intermediates and thus may function as cellular sources of tyrosine nitration. Proinflammatory cytokines such as IL-1β and TNFα are increased during VTE (Reitsma and Rosendaal., 2004) and are also known to induce nitric oxide expression either through eNOS or iNOS in endothelial or inflammatory cells (Busse and Mulsch., 1990). Thus, the over production of nitric oxide may facilitate its conversion to a nitrating intermediate. Studies with LPS injection into humans, an inflammatory process mediated by neutrophils and monocytes (Rivers et al., 1975, Boggs et al., 1968), produces increased levels of nitrated fibrinogen (Heffron et al., 2009). Furthermore, other

inflammatory thrombotic conditions, such as CAD and those at risk for thrombotic disease, like smokers have elevated nitrated fibrinogen levels within their respective patient population (Parastatidis et al., 2008, Vadseth et al., 2004). Thus, VTE patients may exhibit increased nitrated fibrinogen levels during acute thrombosis, which may represent a novel biomarker for VTE.

1.4 Specific Aims

Aim 1: Evaluate clot structure and functional properties in DVT and PE subjects, specifically focusing on those properties that might be involved in embolization.

Hypothesis: PE subjects exhibit unique clot structure and functional properties separate from DVT that that lead to instability and embolization.

DVT and PE are two pathological manifestations of the same disease, VTE. Thus far, the cause of embolization remains unknown. Research indicates that fibrin clot structure and functional properties are involved in both arterial and venous thrombotic disease and represent risk factors with pathological consequences. Furthermore, these properties may affect the ability of the clot to withstand shear forces within the vessel wall. Properties that are likely to be involved in embolization including viscoelastic properties, clot formation and lysis time, FXIII crosslinking and clot structure will be evaluated towards this end. Identification of differences in structure and functional clot properties between DVT and PE subjects may elucidate the mechanisms that regulate embolization.

Aim 2: Measure nitrated fibrinogen levels in VTE subjects, evaluating its role as a biomarker and risk factor for VTE.

Hypothesis: Nitrated fibrinogen levels are elevated in VTE, representing a unique marker of oxidative stress, inflammation, and coagulation in VTE.

VTE is a thrombotic disease characterized by activation of the coagulation cascade, production of inflammatory mediators, and the generation of oxidative and nitrative

intermediates. Thus far, specific markers of these processes in VTE are lacking. Previous studies have identified nitrated fibrinogen as not only a marker of these processes in CAD and smokers, but also involved in alterations in clot structure and function. Herein we evaluate the role of nitrated fibrinogen as a unique biomarker in VTE with specific pathological relevance to the disease.

.

CHAPTER 2

Increased fibrinolysis and acquisition of fibrin clot viscoelastic properties are associated with pulmonary embolism.

Marissa R. Martinez, Adam Cuker, Angela M. Mills, Amanda Crichlow, Richard T. Lightfoot, Irina N. Chernysh, Sekar Nagaswami, John W. Weisel, and Harry Ischiropoulos

Acknowledgements: The work was supported by grants from the National Institutes of Health HL54926, HL103918 and ES013508 NIEHS Center of Excellence in Environmental Toxicology to HI, and HL090774 to JWW. MRM is supported by the Hemostasis and Thrombosis training grant T32 HL07971. HI is the Gisela and Dennis Alter Research Professor of Pediatric Neonatology at the Children's Hospital of Philadelphia Research Institute. We would like to thank K. Hawkins for generously allowing us to use the AR-G2 rheometer and R. Litvinov for his help and advice on FXIII assays.

2.1 Abstract

Pulmonary embolism (PE) is a complication of deep vein thrombosis (DVT), although the cause of embolization remains relatively unknown. Fibrin clot structure and functional properties have been implicated in the pathology of venous thromboembolism. However, previous studies investigating the differences in properties between DVT and PE subjects remain incomplete. The goal of this study was to specifically evaluate fibrin properties that may contribute to embolization. Clot formation and lysis time, viscoelastic properties, activated factor XIII (FXIIIa) crosslinking, and clot structure were measured in the plasma from subjects with acute isolated DVT or PE. Clot formation and lysis time curves revealed faster lysis time in PE subjects, with no difference in lag time, rate of clot formation, or maximum absorbance. Compared with DVT, clots derived from PE subjects showed accelerated establishment of viscoelastic properties, documented by a decrease in lag time and an increase in the rate of viscoelastic property formation. Rate and extent of fibrin FXIIIa crosslinking were similar between DVT and PE subjects. Plasma fibrin clots from PE subjects exhibited lower fiber density compared to DVT. These data show that clot structure and functional properties differ between DVT and PE subjects, and may provide insights into possible mechanisms that regulate embolization.

2.2 Introduction

Deep vein thrombosis (DVT) is a thrombotic disease that mainly affects the large veins of the lower extremities and pelvis. DVT can result in partial or complete occlusion of venous circulation. One of the most serious complications of DVT is pulmonary embolism (PE), which occurs when whole or part of the thrombus embolizes and becomes lodged within the pulmonary vasculature, partially or completely blocking a pulmonary artery or branch. Together, DVT and PE comprise the thrombotic disease, venous thromboembolism (VTE). In contrast to DVT, death is a common outcome in PE and has been estimated to account for an in-hospital case fatality rate of 23 % (Anderson et al., 1991). Despite a common pathology relating PE to DVT, a cause for embolization remains unknown. Evidence does suggest however, that the specific location of the thrombus in DVT is related to risk of PE. Subjects with distal vein thrombosis, i.e. those with thrombi localized to only the calf veins, are at very low risk of PE (Moser and LeMoine., 1981, Kakkar et al., 1969, Moser et al., 1994). This is in contrast to those with proximal vein thrombosis or thrombi above the knee, who are at high risk for having or developing PE (Moser and LeMoine., 1981, Kakkar et al., 1969, Moser et al., 1994).

Recent research has focused on the relationship between fibrin clot structure and functional properties in relation to arterial thrombosis (Collet et al., 2006). Plasma clots derived *ex vivo* from subjects with premature coronary artery disease exhibited increased clot elastic properties, slower lysis, and increased fiber density compared to healthy controls (Collet et al., 2006). The authors further showed that clot structure and functional properties were associated with slower lysis and coronary artery disease. Although most similar studies have focused on arterial thrombosis (Silvain et al., 2011,

Vadseth et al., 2004, Machlus et al., 2011), some recent efforts have turned to venous thromboembolism (VTE) (Undas et al., 2009). Plasma clots from VTE subjects exhibited slower clot lysis time and decreased permeability as compared to relatives without disease and healthy controls. A sub-analysis of the data also showed that within the VTE-positive population, PE subjects had clots with increased porosity and exhibited faster lysis rates compared to DVT subjects (Undas et al., 2009). This data indicates that in VTE, clot structure and the resultant functional properties may be associated with thrombosis and embolization.

The goal of this study was to further investigate the relationship between fibrin clot structure and functional properties, expanding on the work by Undas *et al*. Specifically, this work evaluated for the first time the viscoelastic properties, clot formation and lysis time, and factor XIIIa crosslinking of fibrin employing plasma clots derived ex vivo from acute isolated DVT and PE subjects. Moreover, scanning electron microscopy was used to evaluate fiber clusters, bundles, density, and heterogeneities in fiber structure between plasma clots from acute isolated DVT and PE subjects. Differences in the establishment of viscoelastic properties and lysis time may contribute to embolization in PE subjects and provide additional insights into the pathways that contribute to pathology in VTE.

2.3 Materials and Methods

We performed a prospective cohort study from January 2010 to March 2012 of subjects 18 years of age or older presenting to the Hospital of the University of Pennsylvania emergency department with suspected acute DVT or PE. Exclusion criteria included history of VTE within the prior 4 weeks, unavailable for 90 day follow-up, current use of

anticoagulants, including any low molecular weight heparins or warfarin, and inability to provide informed consent. DVT was diagnosed by compression ultrasonography and PE by computed tomographic pulmonary angiography. Subjects 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 initial negative imaging were also considered positive for DVT and/or PE. Subjects with both distal and proximal vein thrombosis were classified as proximal since DVT is thought to progress from a distal to proximal location, whereby it is more likely to embolize (Kakkar et al., 1969). Subjects diagnosed with both DVT and PE were classified as PE, since a majority of PE cases are thought to arise from DVT (Hull et al., 1983). Three DVT subjects in our population reported a prior history of PE. These subjects were not excluded from the study as previous research has shown that 40 % of DVT subjects without chest symptoms of PE have evidence of PE upon lung scanning (Moser et al., 1994). Inclusion of these three subjects should bias our analysis towards the null hypothesis, and thus should not account for any observed differences in functional properties or structure. Secondary variables were also collected including demographic information, thrombotic risk factors, comorbidities, and medications. Prior to initiation of anticoagulant therapy, blood was collected from subjects in 3.2 % sodium citrate (BD Vacutainer, Franklin Lakes, NJ), and platelet poor plasma was prepared as described previously (Martinez et al., 2012) and stored at -80 °C. Due to sample volume limitations, not every patient sample was included in every assay. Because we hypothesized that functional properties would provide the most insight into the mechanisms that govern embolization, viscoelastic properties and clot formation and

lysis time assays were given priority for sample analysis. All samples available were used for these two assays. Samples for FXIIIa and SEM analysis were chosen at random, and do not differ in demographic variables or medication use compared with the full study population. The 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.

Fibrinogen concentration

Plasma fibrinogen levels were measured in DVT and PE subjects using an ELISA developed in our laboratory as previously described (Martinez et al., 2012, Heffron et al., 2009).

Clot formation and lysis time

Thrombin (0.25 U/ml), $CaCl_2$ (20 mM), and tissue plasminogen activator (tPA) (1.25 μg/ml) (American Diagnostica, Stamford, CT) combined in TBS were simultaneously added to plasma (0.1 ml, final) from DVT and PE subjects. Changes in turbidity due to clotting and lysis were monitored spectrophotometrically at 350 nm for 6 hrs. Lag time was defined as the time for absorbance to change to .01 (OD) (Pieters et al., 2008). The rate of clot formation was measured as the slope of the linear part of the clot formation and lysis time curve during the transition from the zero absorbance to the maximum absorbance. Lysis time was defined as the time difference between the midpoint of the zero to maximum absorbance transition and the midpoint of the maximum to zero absorbance transition (Lisman et al., 2005). Clots with higher maximum absorbance are likely to take longer to lyse. To account for this as well as individual sample variance,

lysis time was normalized form maximum absorbance (Collet et al., 2006). At a minimum each sample was measured in duplicate, and curves were averaged using OriginLab (Northampton, MA).

Viscoelastic properties

Viscoelastic properties were measured with a rheometer (AR-G2, TA Instruments, New Castle, DE) during plasma clot formation. Plasma clots were produced by the addition of thrombin (0.25 U/ml) (American Diagnostica, Stamford, CT) and $CaCl_2$ (20 mM) combined in TBS (0.05 M Tris, 0.14 M NaCl pH 7.4) to plasma (0.1 ml, final) between the stainless steel stage of the rheometer base and a 40 mm parallel plate. To avoid drying, the outside of the forming clot was surrounded by mineral oil (3.52 mPa·s) (Cannon Instrument Company, State College, PA), which is immiscible with plasma. A time-sweep test was performed for 1 h under an oscillation procedure of 2 % strain at an angular frequency of 5 radians per second. All measurements were temperature controlled by a circulating water bath at 25 °C. The storage modulus and the loss modulus, measures of elastic and viscous properties respectively, were measured at 3 s intervals. At a minimum each sample was measured in duplicate, and curves were averaged using OriginLab.

Factor XIIIa crosslinking

Plasma in 10 µl aliquots was clotted with thrombin (0.25 U/ml) and $CaCl_2$ (20 mM). The reaction was quenched with lithium dodecyl sulfate sample buffer (4X, 25 µl) and immediately boiled at 100° C for 5 min. Samples were diluted 100 fold and run on a NuPAGE® 4-12 % Bis-Tris gel (Invitrogen, Carlsbad, CA), followed by western blot

analysis with a primary polyclonal rabbit anti-human fibrinogen antibody (Dakocytomation, Glostrup, Denmark) and secondary goat anti-rabbit IRDye680® conjugated IgG antibody (Rockland, Gilbertsville, PA). The blot was viewed and quantified using the Odyssey infrared imaging system (LI-COR, Lincoln, NE). γ-dimer and α-polymer formation were normalized as a percent of total γ and α chain, respectively, at each time point.

Scanning electron microscopy

As described above, thrombin (0.25 U/ml) and $CaCl_2$ (20 mM) were added to plasma from DVT and PE subjects and allowed to clot for 2 hrs. Clots were then washed, fixed, dehydrated with serial ethanol dilutions followed by hexamethyldisilazane (Acros Organics, Fair Lawn, NJ), and sputter-coated with gold palladium as described previously (Weisel and Nagaswami., 1992). High definition micrographs were taken on a Philips/FEI XL20 scanning electron microscope (FEI, Hillsboro, Oregon). Nine to twelve micrographs were taken for each sample. Fiber density and fiber bundling were quantified using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). For fiber density, micrographs were overlaid with a 6x6 rectangular grid, with each rectangle being 3x4 μm^2 in size. Values were normalized for the number of images taken and are reported as the mean number of fibers per 3x4 μm^2 rectangle ± the standard error (SE). Fiber bundles and the number of fibers per bundle were counted for each image, resulting in a total area of 432 μm^2. These values were normalized for number of images and are reported as the mean number of bundles or number of fibers per bundle ± SE.

Statistics

All continuous variables were tested for Gaussian distribution by D'Agostino and Pearson omnibus normality test. For parametric distributions, an unpaired t-test was performed, and Welch's correction was applied for unequal variances where required. Values are expressed as the mean ± SE. For nonparametric distributions, Mann Whitney U test was performed, and the median and the interquartile range (IQR) are reported. For comparisons between more than two groups, Kruskal Wallis one-way Anova test was used, followed by testing between groups with Dunn's multiple comparison test. Values are expressed as mean ± SE. Correlations were assessed by Spearman rank correlation analysis. The presence of statistical outliers was determined by Grubb's outlier test. A p-value of 0.05 or less was considered statistically significant. All statistical calculations including linear regression analysis were generated and analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

2.4 Results

Study population characteristics

A total of 291 subjects with suspected VTE were recruited for this study. Only subjects positive for DVT or PE were included for experimental analysis. Of the 100 subjects with suspected DVT, 32 had a positive ultrasound and an additional 3 were determined to be positive on 90-day follow-up. Among the 191 subjects with suspected PE, 45 had a positive computed tomographic pulmonary angiography and 2 additional subjects were found to have new PE at 90 days. Patient demographics, disease history, risk factors, and medications are shown in Table 2-1. Significantly more DVT subjects were male

compared with PE. More DVT subjects had a previous history of peripheral artery disease, which is a reported risk factor for VTE (Sorensen et al., 2009, Eliasson et al., 2006). Immunosuppressant use, such as chemotherapeutics and glucocorticosteroids, was

Table 2-1. Study population demographics, history and medication use.

	DVT (n=35)	PE (n=47)	p-value
Age (yr)	57 (42-71)	56 (45-64)	
Sex (% male)	21 (60.0 %)	11 (23.4 %)	0.0012†
BMI (Kg/m^2)	28.0 (23.6-33.7)	27.8 (23.7-32.2)	
Race and ethnicity			
Caucasian	17 (48.6 %)	25 (53.2 %)	
African American	17 (48.6 %)	21 (44.7 %)	
Other	1 (2.9 %)	1 (2.1 %)	
Hispanic	2 (5.7 %)	0 (0.0 %)	
Disease history and risk factors			
H/O DVT	7 (20.0 %)	6 (12.8 %)	
H/O PE	3 (8.6 %)	7 (14.9 %)	
H/O CAD/MI/Angina	3 (8.6 %)	3 (6.4 %)	
H/O stroke	15 (7.5 %)	4 (8.0 %)	
Liver disease	3 (8.6 %)	1 (2.1 %)	
Rheumatoid arthritis	2 (5.7 %)	0 (0.0 %)	
Peripheral artery disease	6 (17.1 %)	1 (2.1 %)	0.0384†
Active cancer	13 (37.1 %)	19 (40.4 %)	
Recent surgery‡	8 (22.9 %)	11 (23.4 %)	
Immobilization > 6 weeks	7 (20.0 %)	9 (19.1 %)	
Air travel#	1 (2.9 %)	2 (4.3 %)	
Tobacco use	12 (34.3 %)	17 (36.2 %)	
Pregnancy	1 (2.9 %)	2 (4.3 %)	
Trauma	2 (5.7 %)	3 (6.4 %)	
Infection	2 (5.7 %)	1 (2.1 %)	
Medications			
Antiplatelet	8 (22.9 %)	6 (12.8 %)	
Statins	5 (14.3 %)	7 (14.9 %)	
Hormonal contraception	1 (7.1 %)	7 (19.4 %)	
Hormone therapy	3 (21.4 %)	1 (2.7 %)	
Antioxidants	2 (5.7 %)	5 (10.6 %)	
Immunosuppressants	7 (20.0 %)	2 (4.3 %)	0.0355†

Age and BMI are shown as median and the IQR. Comparisons were made by Mann Whitney U test. All other variables are shown as number and percentage within each group. †Comparisons were made by Fischer's exact test. ‡Within past 6 weeks. #Greater than 6 hours within past 6 weeks.

increased in DVT subjects. Other demographic variables, comorbidities, risk factors, and medication use were similar between DVT and PE subjects (Table 2-1).

Plasma fibrinogen concentration

Changes in fibrinogen concentration affect fibrin clot properties (Weisel and Nagaswami., 1992, Roberts et al., 1974). In addition, elevated fibrinogen levels are an accepted risk factor for arterial thrombosis, and may also play a role in VTE (Benderly et al., 1996, Folsom et al., 1997, van Hylckama Vlieg and Rosendaal., 2003). We measured fibrinogen concentration in the plasma of DVT and PE subjects by ELISA (Martinez et al., 2012, Heffron et al., 2009). Fibrinogen levels were similar between DVT and PE subjects (DVT: n=34, 5.20 ± 0.43 mg/ml; PE: n=38, 5.93 ± 0.54 mg/ml; p=NS), and did not differ between distal DVT, proximal DVT, or PE (Distal: n=9, 4.80 ± 0.60 mg/ml; Proximal: n=25, 5.35 ± 0.55 mg/ml; PE: n=38, 5.93 ± 0.54 mg/ml; p=NS).

Table 2-2. Clot formation and lysis time of fibrin clots in DVT and PE subjects.

	DVT n=24		PE n=28	p-value
	Distal DVT n=6	Proximal n=20		
Lag time (min)	16.16 ± 1.66		15.81 ± 2.81	NS
	13.58 ± 2.86	17.06 ± 2.00		NS
Abs_{Max} (OD)	0.65 ± 0.05		0.74 ± 0.05	NS
	0.63 ± 0.08	0.67 ± 0.06		NS
Rate of clot formation (OD/min)	0.16 ± 0.01		0.14 ± 0.01	NS
	0.15 ± 0.02	0.17 ± 0.02		NS
Lysis/Abs_{Max} (min/OD)	170.40 ± 17.80*		128.10 ± 10.73*	0.0487
	207.90 ± 29.61*	159.1 ± 21.09		0.0497

Lysis time was compared by unpaired t-test with Welch's correction for differing variances. All other variables were compared by unpaired t-test. *Comparisons for distal DVT, proximal DVT, and PE were made using Kruskal Wallis one-way anova, followed by Dunn's multiple comparison test.

Clot formation and lysis time

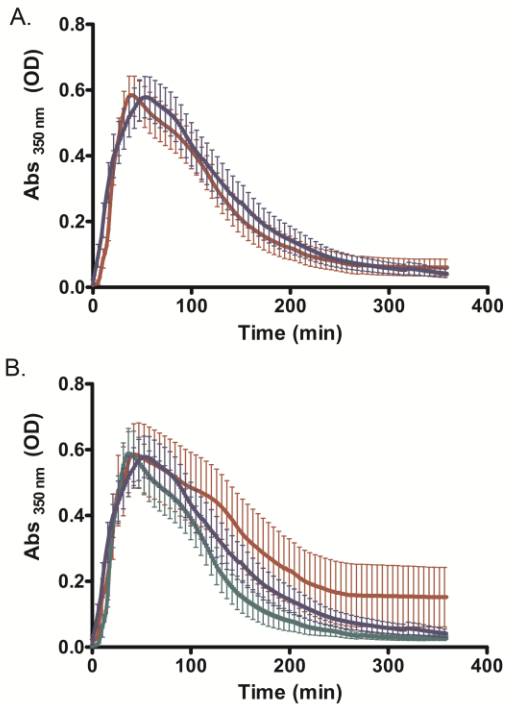

Figure 2-1. Averaged clot formation and lysis time curves from DVT and PE subjects. A) Clot formation and lysis curves for DVT (red, n=27) and PE subjects (blue, n=30). B) Clot formation and lysis time curves for distal DVT (red, n=6), proximal DVT (green, n=20), and PE (blue, n=30).

Previously published data have shown that lysis time is faster in PE compared to DVT subjects (Undas et al., 2009). Clot formation and lysis time were measured in the plasma of DVT and PE subjects by spectrophotometry. Lysis time was faster in PE subjects compared with DVT (Figure 2-1; Table 2-2). The remaining variables of the clot formation and lysis time curves, lag time, rate of clot formation, and maximum absorbance, were similar for both DVT and PE subjects (Table 2-2).

To further investigate if lysis time might contribute to embolization, DVT subjects were separated into distal and proximal DVT. One way Anova analysis showed that mean lysis times were different between the three groups (Figure 2-1; Table 2-2). Subsequent comparison between groups using Dunn's multiple comparison tests shows that lysis time was significantly slower in distal DVT compared with PE. No other clot formation and lysis time variables differed between distal DVT, proximal DVT, or PE.

Viscoelastic properties of fibrin clots

A clot's inherent viscoelastic properties may determine its ability to withstand shear forces within the venous lumen. Herein, the storage modulus and the loss modulus, which represents the elastic and viscous properties respectively, were measured throughout clot formation. Averaged viscoelastic property curves for DVT and PE subjects are shown in Figure 2-2. Initial establishment of viscoelastic properties is accelerated in plasma fibrin clots of PE subjects compared with DVT. Evaluation of individual elastic property curves obtained from PE subjects show a decrease in the lag time (Figure 2-2; Table 2-3). In addition, linear regression analysis of averaged curves demonstrates an increase in the rate of elastic property formation in PE subjects compared with DVT (Figure 2-2; Table 2-3). Similar results were obtained with viscous property curves. Despite differences in the initial phases of viscoelastic property formation, final elastic and viscous properties were similar between DVT and PE subjects.

Table 2-3. Kinetic analysis of elastic and viscous properties of fibrin clots in DVT and PE subjects.

		DVT (n=21)	PE (n=28)	p-value
G'	Lag time (min)	9.42 (6.59-14.91)	6.59 (2.43-10.25)	0.0186*
	Rate of formation (Pa/min)	7.35 ± 0.25	8.02 ± 0.05	0.0087§
	Final (Pa)	227.3 ± 38.44	273.0 ± 43.21	NS
G"	Lag time (min)	8.34 (6.59-14.03)	6.09 (2.26-11.25)	0.0299*
	Rate of formation (Pa/min)	0.51 ± 0.02	0.60 ± 0.01	<0.0019§
	Final (Pa)	12.22 ± 2.13	15.25 ± 2.08	NS

*Lag time was compared by Mann Whitney U test. §Rate of G' and G" formation were analyzed by linear regression analysis.

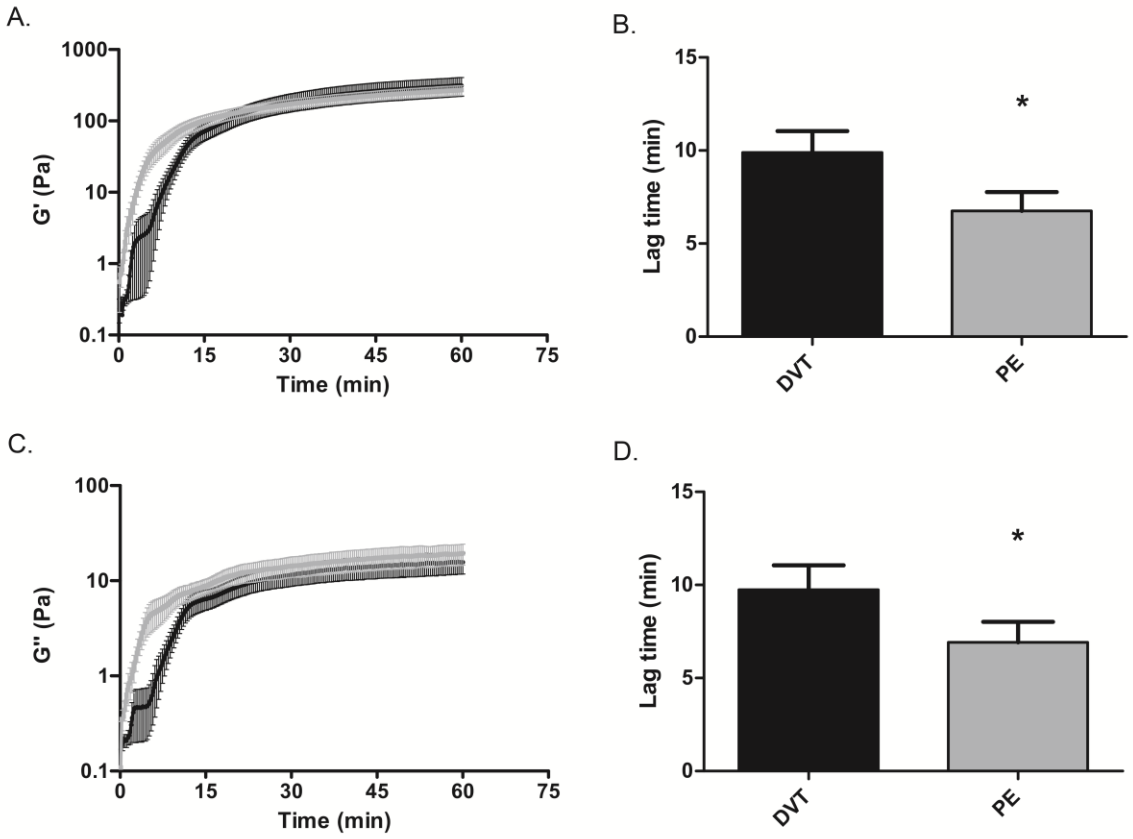

Figure 2-2. Viscoelastic properties in DVT and PE subjects. A) Formation of the clot elastic property (G') as a function of time in DVT (black, n=22) and PE (grey, n=27) subjects. B) Lag time is significantly longer in DVT subjects compared with PE (P=0.0186). C) Formation of the clot viscous property (G") as a function of time in DVT and PE subjects. B) Lag time is significantly longer in DVT than PE subjects (P=0.0299). Error bars represent the standard error of the mean.

Factor XIIIa crosslinking of fibrin

We observed increased establishment of viscoelastic properties and faster lysis rates in clots derived from PE subjects compared to DVT. One possible explanation may be related to FXIIIa crosslinking of fibrin, which has been shown to increase both the storage and loss moduli (Ryan et al., 1999), as well as delay lysis time (Lorand and Jacobsen., 1962, Gormsen et al., 1967). To determine the contribution of FXIIIa

crosslinking of fibrin to differences seen between DVT and PE subjects, the rate and extent of formation of γ-dimers and α-polymers were measured in the plasma of DVT (n=7) and PE (n=12) subjects over a 24 hr. period by western blot analysis. The formation of γ-dimers and α-polymers were normalized for total γ and α chain, respectively, at each time point. The rate and extent of γ-dimer formed was similar between DVT and PE subjects (Figure 2-3). Similar results were found for α-polymer (Figure 2-3). Correspondingly, loss of γ and α chains of fibrinogen as they became incorporated into γ-dimers and α-polymers, respectively, were similar between DVT and PE (data not shown).

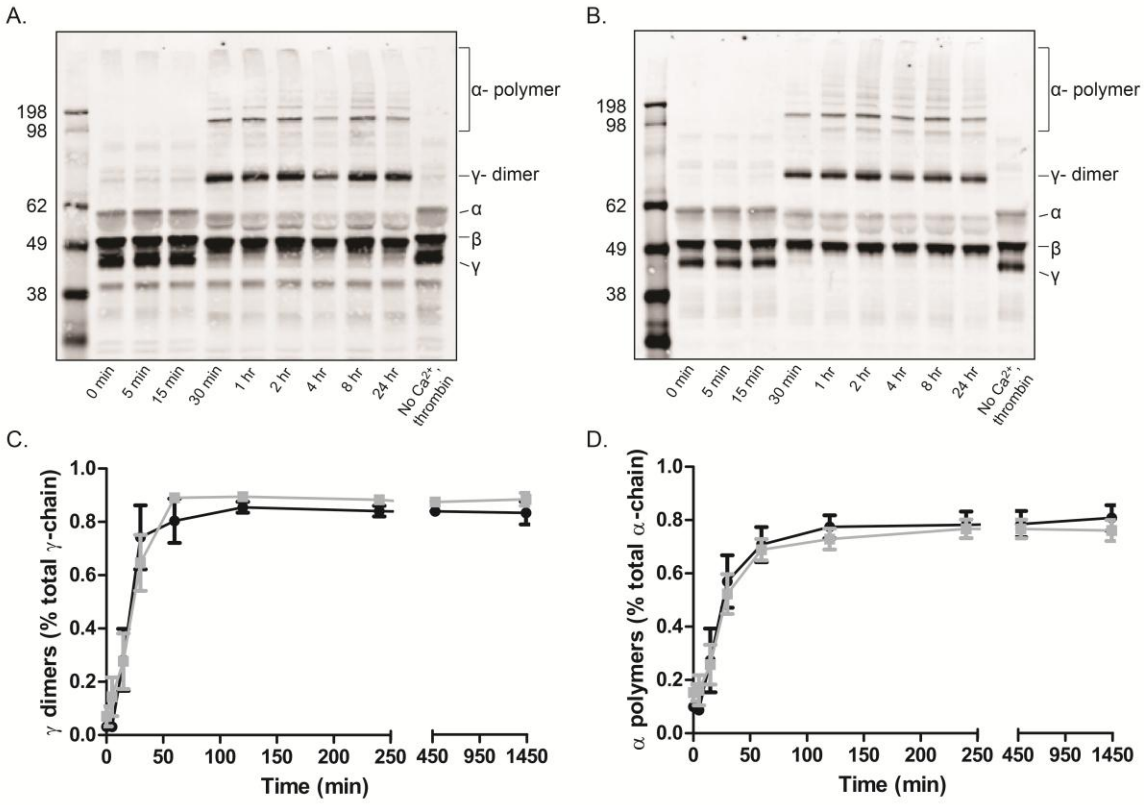

Figure 2-3. Factor XIIIa crosslinking of fibrin fibers within plasma clots as a function of time in DVT and PE subjects. Representative western blots from A) DVT and B) PE subjects; γ-dimers and α-polymers both appear in DVT and PE at 30 minutes. The γ chain is completely crosslinked by 1 hr, whereas some α chain remains at 24 hr. C) Formation of γ-dimers over time in DVT (black, n=7) and PE (grey, n=12) subjects. D) Formation of α-polymers over time in DVT and PE subjects.

Scanning electron microscopy to evaluate fibrin clot structure

Alterations in fibrin clot structure, including changes in fiber density, diameter, bundling, and clustering have been associated with risk of thrombosis (Parastatidis et al., 2008, Collet et al., 2006, Machlus et al., 2011) and are related to lysis rates. Previous studies have shown that clots derived from PE subjects showed increased porosity compared to DVT (Undas et al., 2009). Fibrin clot structure was evaluated in 7 DVT and 8 PE subjects by scanning electron microscopy. Due to their relationship with porosity (Blomback et al., 1989), fiber density and fiber bundling were quantified from scanning electron micrographs using Image J (Figure 2-4). Histograms of fiber density display a Gaussian distribution and show that clots from DVT subjects have a mean of 34.36 ± 0.14 fibers/area^2, while PE fibrin clots have a mean of 31.38 ± 0.33 fibers/area^2 ($p<0.0001$; Figure 2-4). Fiber density was not related to fibrinogen concentration, as Spearman rank correlation analysis showed no relationship between the two variables ($r=0.0071$; p=NS). Histograms of fiber bundling and the number of fibers per bundle were both asymmetric, and could therefore not be fit with Gaussian distributions (Figure 2-4). Comparison of means showed that neither fiber bundling (DVT: 3.73 ± 0.30 bundles/area^2, n=7; PE: 3.36 ± 0.62 bundles/area^2, n=8; p=NS) nor the number of fibers per bundle were significantly different between DVT and PE subjects (DVT: 4.25 ± 0.16 fibers/bundle, n=7; PE: 3.85 ± 0.13 fibers/bundle, n=8; p=NS). Fiber bundling was also not related to fibrinogen concentration, as Spearman rank correlation analysis showed no relationship between the two variables ($r=-0.1265$; p=NS).

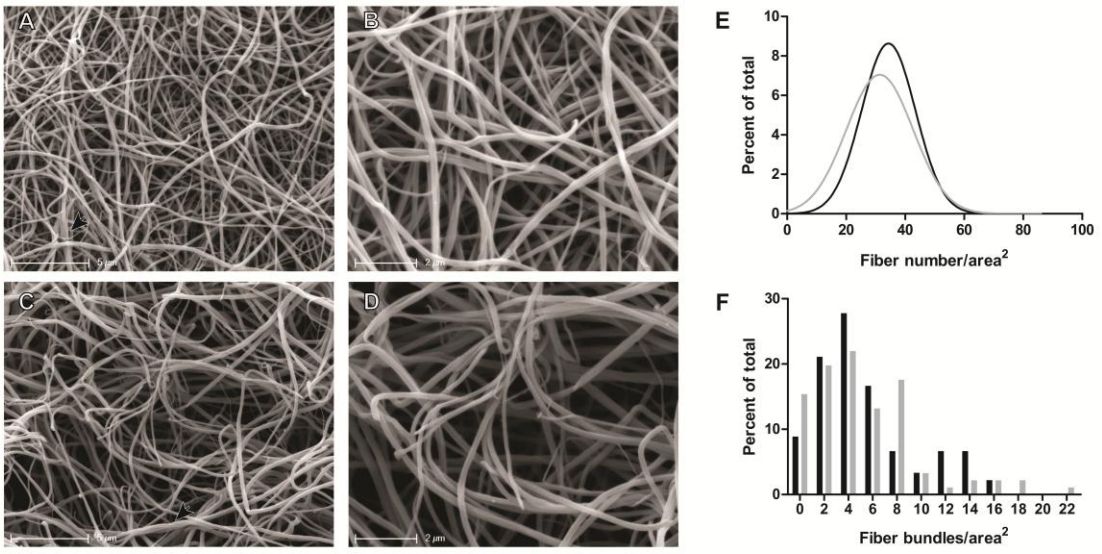

Figure 2-4. Scanning electron micrographs of clots formed from the plasma of DVT and PE subjects. A) Representative micrograph from an individual DVT subject. Magnification bar represents 5 μm. B) Same micrograph at higher magnification. Magnification bar represents 2 μm. C) Representative micrograph from an individual PE subject. Magnification bar represents 5 μm. D) Same micrograph at higher magnification. Magnification bar represents 2 μm. Bundles are indicated by arrowheads. E) Nonlinear regression fit of Gaussian distributions of fiber density for DVT (black, n=7) and PE (grey, n=8) subjects. F) Histogram of the distributions of fiber bundles in DVT (black, n=7) and PE subjects (grey, n=8). Histograms for fiber density and fiber bundling were normalized to the total number of fibers or bundles respectively, and are represented as the percent of total.

2.5 Discussion

DVT and PE encompass two pathological states of one disease, VTE, and likely have contrasting risk factors and pathology that define each condition (van Langevelde et al., 2012). Although much is known about the relationship between clot structure and arterial thrombosis, less is known about its role in VTE specifically concerning distinctions between DVT and PE. Furthermore, there is little information about embolization in general, despite the frequency of this condition and its clinical significance. This study addresses differences in clot structure and functional properties in this population which might elucidate properties that are liable to lead to embolization. In this study, both the storage and loss moduli were established at an earlier time point and at a faster rate in PE subjects compared to DVT. Plasma fibrin clots from PE subjects also exhibited faster

lysis times than DVT subjects. FXIIIa crosslinking was similar between DVT and PE subjects and is thus unlikely to account for the differences in viscoelastic properties and lysis. We also observed that clots derived from PE subjects exhibit decreased fiber density compared to DVT. Together these findings suggest that the formation of fibrin fibers differs between DVT and PE, which are apparent in the final clot structures determined by scanning electron microscopy. Furthermore, accelerated establishment of viscoelastic properties occurs in the absence of any observable changes in the rate of clot formation or lag time by spectrophotometry. This is likely due to the increased sensitivity of rheometry to detect changes in clot formation compared with spectrophotometry.

Clot formation and lysis are dynamic processes that occur simultaneously (Hoylaerts et al., 1982). During this process, tPA and plasminogen bind to fibrin, which acts as a cofactor in the conversion of plasminogen to plasmin (Hoylaerts et al., 1982). Larger fibrin structures are preferentially targeted compared to smaller ones, allowing for clot formation to proceed (Hoylaerts et al., 1982). During this process, tPA is limiting (Bannish et al., Acceptedb, Bannish et al., Accepteda), and clots with a decrease in the number of fibers being formed will have a higher tPA to fiber ratio. This may account, in part, for the accelerated rate of lysis in clots with decreased fiber density (Collet et al., 2000), and may also suggest that in some cases, lysis begins at an earlier time point. The earlier establishment of viscoelastic properties in PE subjects specifies faster formation of fiber structures compared to DVT. It is possible that the earlier those structures are established in PE, the faster lysis can occur, creating instability and embolization prior to full clot formation. Evidence documenting the time course of initiation of thrombosis to embolization is lacking in humans, and to date, no animal models of PE from an

established venous thrombus exist. However, recent studies utilizing intravital microscopy to monitor real-time thrombus formation in a laser-induced injury model have shown that embolization can occur as the thrombus is forming, prior to full clot formation (Neyman et al., 2008, Stolla et al., 2011). This is one possible explanation for changes seen in the initial, but not the final phases of elastic property formation. However, if embolization occurs at a time point after the clot has fully formed it is not clear how differences in viscoelastic properties relate to the tendency to embolize.

Among subjects presenting with acute DVT, identifying those at highest risk for PE could greatly inform treatment decisions. Although risk factors that characterize those subjects are lacking, evidence suggests that subjects with proximal vein thrombosis are at much higher risk of developing PE compared to subjects with distal vein thrombosis (Moser and LeMoine., 1981, Kakkar et al., 1969, Moser et al., 1994). In a sub-analysis of clot formation and lysis time properties, distal and proximal DVT subjects were compared for all clot formation and lysis time variables. Lysis time was significantly faster in PE compared to distal DVT, but not in proximal DVT. These data could indicate lysis time as a risk factor for PE. These conclusions must be viewed with caution however, due to small sample size. Additional studies with a larger population are necessary to confirm these findings.

In this study we used SEM to visualize clot structure which revealed that fiber density was decreased in clots from PE subjects relative to DVT, and no differences were seen in fiber bundling. The decrease in fiber density is consistent with the finding that clot permeability in PE subjects is increased compared to DVT (Undas et al., 2009). Fiber density is related to several factors including fibrinogen and thrombin concentration,

fibrinogen variants, post translational modifications of fibrinogen, and other plasma constituents (Weisel and Nagaswami., 1992, Parastatidis et al., 2008, Konings et al., 2011, Herbert et al., 1998, Dang et al., 1989, Langer et al., 1988). Fibrinogen concentration cannot be the source of changes in fiber density as it was comparable between DVT and PE subjects, similar to what has been shown in related studies (Undas et al., 2009). Additional studies are necessary to determine the causative factors that produce differences in fibrin clot structure, lysis, and viscoelastic properties between DVT and PE subjects.

In summary, compared with DVT, PE subjects exhibit faster lysis times, possibly due to lower fiber density, as well as an enhanced establishment of clot viscoelastic properties. These properties distinguish PE subjects from DVT and may define structural characteristics that delineate pathologies within VTE and predispose subjects to embolization.

CHAPTER 3

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

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

Acknowledgements: The work was supported by grants from the National Institutes of Health HL54926, HL103918 and ES013508 NIEHS Center of Excellence in Environmental Toxicology to HI, HL098055 and HL076491 to SLH, and HL103931 to WHT. MRM is supported by the Hemostasis and Thrombosis training grant T32 HL07971. HI is the Gisela and Dennis Alter Research Professor of Pediatric Neonatology at the Children's Hospital of Philadelphia Research Institute.

3.1 Abstract

The pathogenesis of venous thromboembolism (VTE) is linked to inflammation and oxidant production, although specific markers for these pathways with pathological relevance to VTE have not been explored. The coagulant protein fibrinogen is post-translationally modified by nitric oxide-derived oxidants to nitrated fibrinogen in both acute and chronic inflammatory states. Therefore, nitrated fibrinogen may serve as a marker of inflammation and oxidative stress in VTE. To test this hypothesis we enrolled subjects (n=251) presenting with suspected VTE at the Hospital of the University of Pennsylvania emergency department, 50 (19.9 %) of whom were positive by imaging or 90-day follow-up. Mean nitrated fibrinogen was elevated in VTE positive (62.7 nM, 95 % CI 56.6-68.8) compared to VTE negative subjects (54.2 nM, 95 % CI 51.4-57.1; $p < 0.01$). Subjects in the highest quartile of nitrated fibrinogen had an increased risk of VTE compared with subjects in the lowest quartile (OR 3.30; 95 % CI 1.25-8.68; $p<0.05$). This risk persisted after univariate adjustment for age, active cancer, and recent surgery, but not after multivariate adjustment. Mean fibrinogen levels measured either by the Clauss assay or by ELISA were not different between VTE negative and positive subjects. These data indicate that nitrated fibrinogen is an oxidative risk marker in VTE, providing a novel mechanistic link between oxidant production, inflammation, and VTE.

3.2 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 (Anderson et al., 1991, Salomon et al., 1999, Pabinger et al., 1992). VTE is a chronic disease with 30 % of patients experiencing a recurrent event over 10 years (Heit et al., 2000a, Schulman et al., 2006), and is estimated to account for between 100,000 and 180,000 deaths each year (Rathbun., 2009). 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 (Virchow., 1856). 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 pro-coagulant molecules. Electron micrographs of early thrombus formation show leukocyte adhesion to the vein wall (Schaub et al., 1984), presumably through the binding of P- and E-selectins that are expressed on activated endothelial cells (Myers et al., 2002, Geng et al., 1990). Leukocytes shed tissue factor-bearing microparticles, which induce coagulation and the production of a fibrin clot (Hrachovinova et al., 2003). 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 (Brennan et al., 2002, Hazen et al., 1999).

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 subjects with coronary artery disease (CAD) and in smokers (Vadseth et al., 2004, Parastatidis et al., 2007). Additionally, in humans injected with small amounts of lipopolysaccharide, plasma levels of nitrated fibrinogen increase and remain elevated for at least 72 hrs. post-injection (Heffron et al., 2009). 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 (Brennan et al., 2002). 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 structure and viscoelastic properties (Vadseth et al., 2004, Heffron et al., 2009, Parastatidis et al., 2007). 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 subjects 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.

3.3 Materials and Methods

Study 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. Subjects 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. Prior to initiation of anticoagulant therapy in the emergency department, blood was collected from subjects in 3.2 % sodium citrate (BD Vacutainer, Franklin Lakes, NJ). Blood was centrifuged at 150 g for 15 min, and the supernatant, platelet rich plasma, was transferred to a new tube. Platelet rich plasma was then centrifuged at 10,000 g 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 (Heffron et al., 2009). Briefly, 96-well plates were coated with 10 µg/ml anti-nitrotyrosine antibodies generated and described elsewhere (Heijnen et al., 2006) in 50 mM carbonate buffer pH 9, and incubated at 4 °C under constant rocking overnight. The next day, plates were washed with 50 mM Tris, 150 mM NaCl, 0.05 % Tween-20 (TBS-T). 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) that had been chemically nitrated and the levels of nitration were independently determined (Heffron et al., 2009). 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 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) followed by quenching of the reaction with 2 M sulfuric acid (Fisher Scientific, Fair Lawn, NJ). The absorbance was measured at 405 nm (Molecular devices, Sunnyvale, CA). Nitrated fibrinogen values were normalized to quality control plasma run with each plate. Each subject 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 inter-assay variations were 8.23 % and 9.32 %.

Plasma fibrinogen level

Fibrinogen levels were measured in plasma using an ELISA previously described (Heffron et al., 2009). Ninety-six well plates (Maxisorb, Nunc, Rochester, NY) were coated with 10 µg/ml mouse anti-fibrinogen monoclonal antibody (Heffron et al., 2009) in carbonate buffer pH 9, and incubated at 4° C, rocking overnight. The next day, 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, Stamford, CT) in TBS-T. Plasma was diluted (1:5000, 1:10000), added to the plate in triplicate, and incubated for 1 h 37° C. Plates were washed with TBS-T, then coated with 0.3 µg/ml HRP-conjugated rabbit-anti-human fibrinogen polyclonal antibody (DakoCytomation, Glostrup, Denmark) 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 inter-assay variations were 11.23% and 14.0%.

Plasma fibrinogen levels were measured by Clauss method (Clauss., 1957) using a Destiny Max coagulation analyzer according to manufacturer's protocol (Trinity Biotech, Co. Wicklow, Ireland).

D-dimer

Plasma D-dimer levels were measured at the Hospital of the University of Pennsylvania 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 (de Moerloose et al., 1996, Perrier et al., 1999). In accordance with the manufacturer's instructions, a D-dimer level <0.5 μg/ml was considered negative.

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

Statistics

Gaussian distribution was tested for all continuous variables using D'Agostino & Pearson omnibus normality test, which indicated non-parametric distributions for all variables within our data. Thus, comparisons between groups were made using 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 (OR) were compared by Fishers exact test. Trends in nitrated fibrinogen levels by quartile were compared by Chi-squared 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), or R-Statistical package (http://www.r-project.org/).

Table 3-1. Characteristics of VTE subject population

	Non-VTE (n=201)	VTE (n=50)	p-value
Age (yr)	47.6 ± 16.7	53.3 ± 16.4	0.0206*
Gender (% male)	63 (31.3 %)	23 (46.0 %)	
BMI (Kg/m^2)	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†
Recent surgery^	26 (12.9 %)	15 (30.0 %)	0.0089†
Immobilization > 6 weeks	30 (14.9 %)	11 (22.0 %)	
Air travel^	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 %)	
Medications			
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 mean ± SD or the number and percentage of sample group. VTE composition: DVT n=21; PE n=29. *Age and BMI were compared by Mann Whitney U test. †All other variables were compared by Fischer's exact test. ^Within past 6 weeks.

3.4 Results

Subject characteristics

A total of 251 subjects 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 subject was found to have new PE at 90 days. Subject demographics, disease history, risk factors, and medications are shown in Table 3-1. 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 subjects were significantly older compared with negative subjects and were more likely to have active cancer or recent surgery. Thrombophilia testing was not performed as part of the study. Nevertheless, three subjects reported a history of hereditary thrombophilia including one with protein C and two with protein S deficiency. Additionally, two subjects reported having antiphospholipid syndrome. No other measured demographic variables, comorbidities, risk factors, or medication use differed between VTE positive and negative subjects (Table 3-1).

Fibrinogen levels in VTE subjects

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 (Figure 3-1). Fibrinogen levels were also similar between DVT and PE subjects irrespective of the assay employed (Clauss: DVT:

3.8 mg/ml, 95 % CI 3.0-4.6, n=17; PE: 4.5 mg/ml, 95 % CI 3.6-5.4, n=27; ELISA: DVT: 5.5 mg/ml, 95 % CI 4.4-6.5, n=21; PE: 5.6 mg/ml, 95 % CI 4.7-6.5, n=28). ELISA-determined fibrinogen levels were higher than those reported by Clauss assay, which is consistent with findings in previous studies (Kallner et al., 2003).

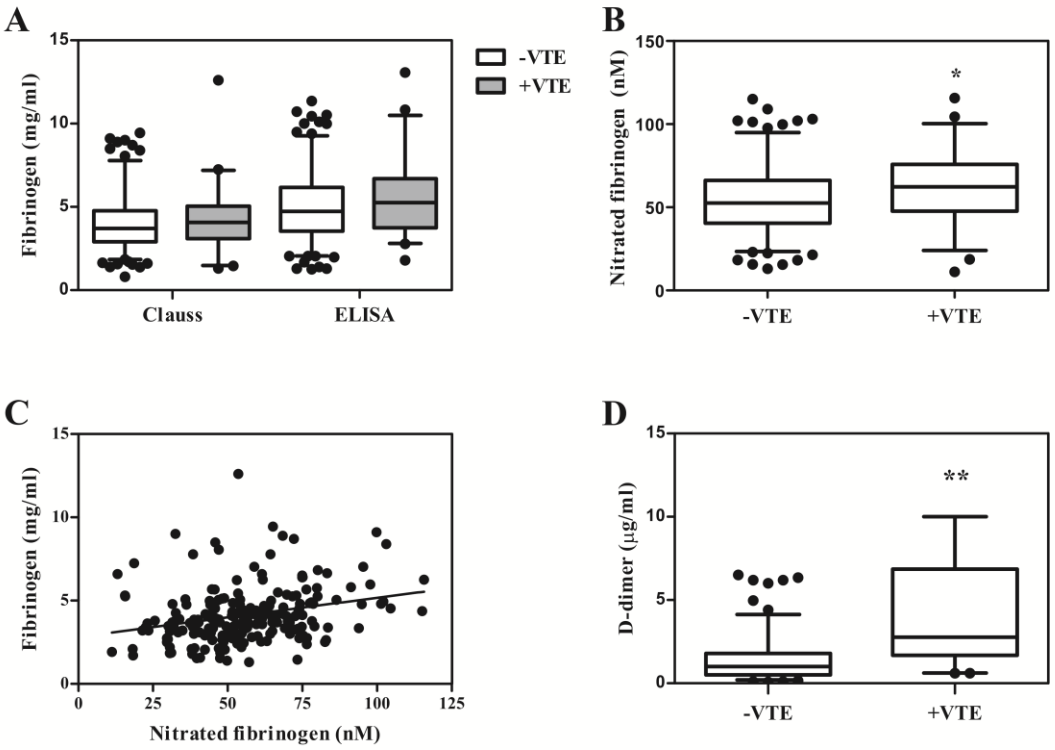

Figure 3-1. Fibrinogen, nitrated fibrinogen, and D-dimer levels in VTE negative and VTE positive subjects. A) Plasma fibrinogen levels were measured by two methods: ELISA, which measures fibrinogen antigen (VTE positive (+VTE): 5.5 mg/ml, 95 % CI 4.9-6.2, n=49; VTE negative (-VTE): 5.0 mg/ml, 95 % CI 4.7-5.3, n=194), and Clauss assay, which measures fibrin clotting time (+VTE: 4.3 mg/ml, 95 % CI 3.6-4.9, n=44; -VTE: 4.0 mg/ml, 95 % CI 3.8-4.2, n=185). Mean fibrinogen levels for each assay were compared by Mann-Whitney U test, p-values were nonsignificant for both comparisons. B) 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 by Vidas D-dimer exclusion assay were higher in +VTE (4.22 μg/ml, 95 % CI 3.23-5.20, n=42) versus -VTE subjects (1.42 μg/ml, 95 % CI 1.20-1.63, n=157). D-dimer levels were compard 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 the lower and upper bars represent the 5th and 95th percentiles, respectively.y.

Nitrated fibrinogen levels in VTE subjects

Nitrated fibrinogen was measured by an ELISA as described previously (Heffron et al., 2009). 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 (Heffron et al., 2009). To ensure the integrity of each assay, every 96-well plate included a quality control plasma standard, and intra- and inter-assay variation did not exceed 15 %. The mean levels of nitrated fibrinogen were significantly higher in VTE positive compared to VTE negative subjects (Figure 3-1). Nitrated fibrinogen levels were not different between DVT and PE subjects (DVT: 60.9 nM, 95 % CI 52.3-69.6, n=17; PE: 63.9 nM, 95 % CI 55.2-72.5, n=27).

Previous data indicated that one functional effect of fibrinogen tyrosine nitration was increased rate of fibrin formation. Since 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 (Figure 3-1). Nitrated fibrinogen correlated linearly with Clauss measured fibrinogen levels ($r=0.31$, 95 % CI 0.18-0.40, $n=207$; $p<0.0001$).

D-dimer levels in VTE subjects

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 (de Moerloose et al., 1996, Bounameaux et al., 1991, Di Nisio et al., 2007, Bozic et al., 2002, Declerck et al., 1987). D-dimer was measured with the Vidas ® D-dimer exclusion assay™ according to

manufacturer's instructions. As expected, mean D-dimer levels were significantly higher in VTE positive versus VTE negative subjects (Figure 3-1).

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 odds ratio of clinically documented VTE (Figure 3-2). 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 (χ^2 for trend 7.69; $p<0.01$) (Table 3-2). Subjects in the highest quartile were compared with those in the lowest quartile and were found to have an increased risk of VTE. This risk was maintained after univariate adjustment for the risk factors found to be elevated in our VTE positive subject population: advanced age, active cancer, and recent surgery (Table 3-2, models 1, 2, and 3). However, multivariate analysis with these risk factors did not reach statistical significance (Table 3-2, model 4). This may reflect the small size of our VTE positive subject population, where additional adjustments reduce the final sample size and eliminate statistical significance.

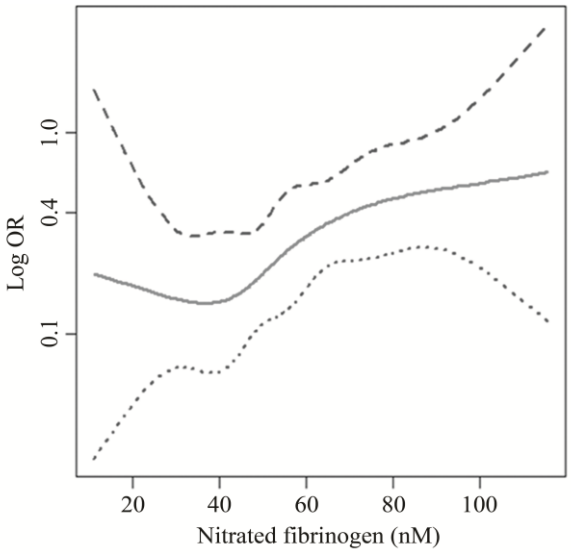

Figure 3-2. Relationship between the odds ratio of acute VTE and nitrated fibrinogen levels. Cubic spline regression model of the odds ratio of acute VTE as a function of nitrated fibrinogen after adjustment for comorbidity, demographics, and risk factors (n=226). Dotted lines represent the 95 % CI. $p<0.005$.

Table 3-2. Odds ratio of VTE by quartiles of nitrated fibrinogen.

Quartiles	**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).
Model 1: Adjusted for age.
Model 2: Adjusted for cancer.
Model 3: Adjusted for surgery.
Model 4: Adjusted for age, cancer, surgery.
Odds ratios were compared by Fischer's exact test.
† $p<0.05$

3.5 Discussion

Previous studies have implied that inflammation and oxidative processes may be mechanistically linked to the pathogenesis of VTE (Wakefield et al., 1995, Nordenholz et al., 2008, Parastatidis et al., 2008, Brennan et al., 2002, Vadseth et al., 2004, Heffron et al., 2009, Hazen et al., 1999, Henke et al., 2004, Lo et al., 1995, Eiserich et al., 1998, Pignatelli et al., 2001). Nitration of proteins and specifically, nitrated fibrinogen has been quantified in the plasma of subjects that experience inflammatory changes and oxidative burden (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009). 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 subjects with acute VTE compared to those found to be 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 previous studies, the levels of nitrated fibrinogen in VTE negative subjects are higher than would be expected in healthy non-smoking controls (Parastatidis et al., 2008, Vadseth et al., 2004). This is likely due to the presence of inflammatory and oxidative conditions in the VTE negative population, including CAD and smoking that are associated with increased plasma concentration of nitrated fibrinogen (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009). 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 (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009). 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. When compared with subjects 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 subject 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 subjects 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 subjects (Pignatelli et al., 2001). 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 (Anderson et al., 1991, Heit et al., 2000b) and were more prevalent among VTE positive than 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 subjects 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 those 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* (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009, Parastatidis et al., 2007, Pignatelli et al., 2001). Tyrosine nitration even in the presence of other oxidized amino acids was shown to significantly accelerate clot formation and factor XIII cross-

linking (Vadseth et al., 2004). Incorporation of nitrated fibrinogen molecules into fibrin clots was documented by immune-electron microscopy and was found to alter clot architecture in a dose-dependent manner (Parastatidis et al., 2008, Vadseth et al., 2004). Changes in structure were accompanied by changes in viscoelastic properties and a decrease in dissolution by the fibrinolytic systems (Parastatidis et al., 2008, Vadseth et al., 2004). Removal of the nitrated fibrinogen molecules restored the kinetics of fibrin clot formation and architecture (Parastatidis et al., 2008). These profound effects on fibrin clot structure were produced by a relative 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 (Parastatidis et al., 2008). 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 (Doolittle and Pandi., 2006, Geer et al., 2007, Litvinov et al., 2007). This acceleration is likely 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 β chain (Parastatidis et al., 2008). 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 from other proteins that require nucleation events to form protein fibers (Hodara et al., 2004). These previous studies suggest that the elevated levels of nitrated fibrinogen in VTE positive subjects 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 subjects consistent with previous studies (de Moerloose et al., 1996, Bounameaux et al., 1991, Di Nisio et al., 2007, Bozic et al., 2002, Declerck et al., 1987). Sensitivity and specificity for D-dimer at 0.5 µg/ml cutoff in this subject 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™ (de Moerloose et al., 1996, Di Nisio et al., 2007, Freyburger et al., 1998).

Fibrinogen is an independent risk factor and predictor of CAD and associated morbidity and mortality (Benderly et al., 1996, Folsom et al., 1997). However, its role in VTE remains unclear. The Leiden Thrombophilia Study (LETS) showed elevated fibrinogen levels measured by Clauss assay in DVT subjects compared with healthy controls (Koster et al., 1994). However, in later investigations within LETS, increased risk of DVT with increasing fibrinogen levels was only found in subjects >45 yrs. In subjects <45 yrs., fibrinogen levels in the 95th percentile did not confer increased risk of DVT (van Hylckama Vlieg and Rosendaal., 2003). Furthermore, in two separate studies in subjects with suspected PE, the levels of fibrinogen were significantly lower in subjects diagnosed as positive versus negative for the disease (Kucher et al., 2003b, Kucher et al., 2003a). In this study, fibrinogen levels were similar between VTE positive and negative groups, and there were no differences in DVT versus PE subjects. Subject population or sample size may account for the differences between this study and LETS, where DVT subjects were compared to a large number of healthy age and sex-matched controls. Likewise, subject population may also explain the dissimilarity between this study and the two previous studies investigating fibrinogen in PE (Kucher et al., 2003b, Kucher et al., 2003a).

Overall, fibrinogen nitration in the VTE subjects certainly reflects active inflammatory and oxidative states that could favor a pro-coagulant environment resulting in fibrin structures with altered biochemical and biophysical properties and structure. These alterations in fibrin clots may profoundly impact the pathology of VTE.

CHAPTER 4

Summary and Discussion

4.1 Fibrin clot properties and structure are differentially altered in VTE subjects

Deep vein thrombosis (DVT) and pulmonary embolism (PE) share a common etiology, but have divergent outcomes. Thus, it is likely that there are coinciding, as well as distinct characteristics that define each condition (van Langevelde et al., 2012). Previous studies have shown that alterations in clot structure produce changes in the functional properties of clots including clot formation, lysis, and viscoelastic properties. Furthermore, these clot characteristics were also related to risk for arterial thrombosis and venous thromboembolism (VTE) (Parastatidis et al., 2008, Collet et al., 2006, Undas et al., 2009). While the cause of embolization remains unknown, it is likely related to the ability of the clot to withstand the shear forces within the vessel. Thus, alterations in clot structure may differentiate patients that embolize from those that do not. This is the first study to evaluate clot structure and functional properties in DVT and PE subjects. Compared to subjects with isolated DVT, clots formed from PE subjects exhibited enhanced establishment of viscoelastic properties as evidenced by a decrease in the lag time and an increase in the rate of both elastic and viscous property formation. In spectrophotometry assays, clots formed from PE subjects showed a faster lysis time, but did not differ in lag time, rate of clot formation, or maximum absorbance. Activated factor XIII (FXIIIa) crosslinking was also similar between the two groups. Fibrin fiber density was decreased in PE subjects, although fiber bundling remained unaffected.

4.1.1 Fibrinolysis may regulate embolization

Impaired fibrinolysis has been associated with both arterial and venous thrombotic diseases (Parastatidis et al., 2008, Collet et al., 2006, Undas et al., 2009, Machlus et al.,

2011). Inability of the body's own internal mechanisms to clear thrombi can result in occlusion of vessels and lead to outcomes such as DVT, PE, and myocardial infarction. This is the proposed mechanism by which elevated levels of circulating fibrinogen become a risk factor for arterial thrombotic diseases (Machlus et al., 2011). However, the paradigm appears to take a different course concerning embolic subjects. We observed faster lysis times in PE subjects compared to DVT. Concomitantly, lysis time was faster in PE compared to distal DVT, but not proximal DVT, where patients are disposed to embolization (Moser and LeMoine., 1981, Kakkar et al., 1969, Moser et al., 1994). Although clot formation and lysis time were not evaluated in a non-VTE population in this study, previous studies have observed slower lysis rates in both DVT and PE subjects compared with healthy controls (Undas et al., 2009). Together, these previous studies and ours suggest that 1) slower lysis alone is a risk factor for thrombosis and 2) changes in propensity for lysis may predispose a clot towards embolization. These changes may be due to differences in shear stress, alterations in venous endothelium, or alterations in clot structure due to local inflammatory or coagulant mediators.

Fibrin clot structure is the most important determinant of lysis rates. Clots composed of thinner fibers that are more densely packed or heterogeneous clot structures with bundles or clusters tend to lyse at a slower rate (Collet et al., 2000, Parastatidis et al., 2008). The converse is also true, where thick fibers that are less densely packed lyse more quickly (Collet et al., 2000). This is due to the lateral transection of plasmin across fibers as opposed to lysis from the outside in of individual fibers (Collet et al., 2000). PE subjects exhibited decreased fiber density compared to DVT, which may account for the faster lysis observed.

4.1.2 Clots formed from PE subjects establish viscoelastic properties faster than DVT

Previous studies evaluating the role of viscoelastic properties in arterial thrombotic diseases have observed changes in final viscoelastic properties (Collet et al., 2006). Specifically, clots from premature coronary artery disease subjects have increased elastic properties i.e. stiffer final clot structure. In this study, clots from PE subjects exhibited increased rate of the establishment of both the elastic and viscous properties, with no differences in final viscoelastic properties. To date, no studies have evaluated clot viscoelastic properties in VTE compared to controls, or between DVT and PE subjects. Thus, these findings may represent unique clot functional properties involved in embolization.

Viscoelastic properties are influenced by clot structure. In this study, clots from PE subjects had decreased fiber density with no differences in bundling and no apparent cluster formation. Previous studies have shown that clots composed of thinner fibers with increased fiber density have increased elastic properties (Collet et al., 2006, Roberts et al., 1974). Heterogeneous clot structures composed of fiber clusters or bundles also tend to have a higher elastic component (Parastatidis et al., 2008, Bale et al., 1985). Earlier establishment of viscoelastic properties in PE subjects suggests that there are distinctions in the fibers formed during clot formation, which is evident by differences in fiber density by SEM. However, the relationship between viscoelastic properties and clot structure is complicated by other mediators such as coagulation factors, fibrinogen variants and post-translational modifications, and will be discussed further below.

Additional studies will be necessary to address if and how these mediators affect viscoelastic properties and clot structure in DVT and PE subjects.

Clot formation and lysis are processes of coagulation that occur simultaneously. Together, tissue plasminogen activator (tPA) and plasminogen bind to fibrin forming a ternary complex (Hoylaerts et al., 1982). Here fibrin acts a cofactor for the conversion of plasminogen to plasmin. In addition, tPA and plasminogen preferentially target larger fibrin structures, which allow coagulation to proceed. The faster rate of establishment of viscoelastic properties in PE subjects may allow tPA and plasmin to bind and begin lysis at an earlier time point. This process would likely lead to incomplete or unstable structures and possibly embolization. Evidence for a mechanism of embolization is lacking, but studies using intravital microscopy evaluating models of both arterial and venous thrombosis with nitrogen laser injury have observed embolization as a byproduct of thrombosis, occurring during thrombus formation rather than after the thrombus has been established (Neyman et al., 2008, Stolla et al., 2011). This might be one way in which changes in the initial phases of viscoelastic property formation contribute to embolization. However, if embolization occurs at a time point after the clot has fully formed it is not clear how these differences relate to the tendency to embolize.

4.1.3 FXIIIa crosslinking of fibrin does not contribute to differences in fibrin clot properties

FXIII is a transglutaminase activated by thrombin cleavage of its N-terminal activation peptide, resulting in FXIIIa crosslinking of fibrin γ and α chains to create γ-dimers and α-polymers, respectively (Nakamura et al., 1975). The main function of FXIIIa crosslinking

is to provide stability and prevent degradation of the fibrin clot. Clots that are not crosslinked by FXIIIa are lysed at a faster rate (Lorand and Jacobsen., 1962, Gormsen et al., 1967). The rate and extent of FXIIIa crosslinking of α and γ chains of fibrin in clots within our DVT and PE population were evaluated, and no differences were found between the two groups. Thus, the faster lysis rate observed in clots formed from PE subjects cannot be attributed to FXIIIa crosslinking of fibrin. However, FXIIIa also crosslinks α_2-antiplasmin (α2-PI) to fibrin, which also delays lysis time (Lorand and Jacobsen., 1962, Gormsen et al., 1967). Further studies are required to determine if α2-PI is crosslinked to fibrin in DVT and PE subjects and how this effects lysis time.

FXIII also provides the clot with a large extensibility, and in its absence, protofibrils slide past one another (Gerth et al., 1974, Brown et al., 2009). Overall, FXIIIa crosslinking enhances the elastic properties of clots (Gerth et al., 1974, Nelb et al., 1976), which may have functional consequences on the ability of the clot to withstand shear forces of blood flow within the vessel. Because FXIIIa crosslinking was similar between DVT and PE subjects, it is unlikely that the differences in viscoelastic properties are attributed to FXIIIa crosslinking of fibrin.

4.1.4 Fibrinogen variants, fibrinogen post-translational modifications, and plasma constituents may contribute to differences between DVT and PE

Although differences in viscoelastic properties, lysis time, and fiber density were observed between DVT and PE subjects, the cause of these alterations remains unknown. Fibrinogen concentration, variants, and post-translational modifications, and other plasma constituents have been shown to affect fibrin clot formation and structure. One of the

main effectors of fibrin clot structure is fibrinogen concentration. Increased fibrinogen levels result in faster clot formation, slower lysis, an increased elastic property, and a dense fibrin meshwork composed of thinner fibers (Weisel and Nagaswami., 1992, Collet et al., 2006, Machlus et al., 2011). Elevated fibrinogen levels are considered as a risk factor for arterial thrombosis (Machlus et al., 2011, Benderly et al., 1996, Folsom et al., 1997), however fibrinogen levels and risk of VTE remain unclear. We and others did not observe differences in plasma fibrinogen levels between VTE negative and positive subjects (Martinez et al., 2012, van Hylckama Vlieg and Rosendaal., 2003), or between DVT and PE. Thus, plasma fibrinogen levels are unlikely to account for the differences observed between DVT and PE subjects.

A naturally occurring splice variant of the γ chain is γ', which contains an additional 20 amino acids in place of the last 4 (Wolfenstein-Todel and Mosesson., 1981). About 10 % of fibrinogen molecules contain γ/γ' heterodimers, while < 1 % contain γ'/γ' homodimers (Mosesson et al., 1972). Clots formed from γ'/γ' have thinner fibers, smaller pores, more bundling and free fiber ends (Gersh et al., 2009).The additional 20 amino acids are negatively charged and contain two tyrosine sulfation sites that increase thrombin binding affinity, and may account for the antithrombin activity of fibrin (Meh et al., 2001). Studies have shown that subjects with lower γ' levels are at increased risk of DVT (Uitte de Willige et al., 2005). To date, no studies have evaluated γ' levels in DVT and PE, but the ratio of plasma γ'/γ fibrinogen may affect clot structure and thus, the propensity for embolization.

Fibrinogen is modified by various post-translational modifications including sulfation (above), homocysteinylation, tyrosine nitration, and oxidation (Vadseth et al., 2004, Collet et al., 2004, Sauls et al., 2006, Shimizu et al., 1986). Homocysteine (Hcy) is a sulfur containing amino acid that is involved in the metabolic cycle of methionine. Homocysteinylation occurs through addition of the Hcy cyclic thioester, Hcy thiolactone to fibrinogen (Sauls et al., 2006). Twelve homocysteinylated lysines were identified on all three chains of fibrinogen after incubation of isolated fibrinogen with Hcy thiolactone (Sauls et al., 2006). These fibrin clots showed fibers with decreased mass to length ratio and impaired fibrinolysis (Sauls et al., 2006). Further studies will be required to identify the mechanisms by which Hcy thiolactone affects fibrinogen, and how it relates to VTE.

The effects of tyrosine nitration and oxidation on fibrinogen are discussed at length below. Briefly, several studies have demonstrated that tyrosine nitration increases fibrin clot formation (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009, Gole et al., 2000). While tyrosine nitration is increased in VTE positive subjects compared to VTE negative subjects, no differences in tyrosine nitration were found between DVT and PE and it is thus unlikely to account for the differences in clot properties and structure. Oxidation of fibrinogen delays fibrin clot formation (Vadseth et al., 2004, Zieve and Solomon., 1966, Inada et al., 1978), but has yet to be evaluated in a VTE population. DVT and PE subjects both experienced elevated levels of nitrated fibrinogen. Consequently, oxidation is unlikely to account for differences between DVT and PE subjects since nitration negates the effects of fibrinogen oxidation (Vadseth et al., 2004).

Factor XII (FXII) is one of the initial mediators of the intrinsic pathway of the coagulation cascade, becoming activated (FXIIa) upon exposure to a damaged surface (Figure 1-1). FXIIa also directly interacts with fibrinogen during clot formation, resulting in dose-dependent delays in lag time and an increase in fiber density (Konings et al., 2011). Polyphosphate is a linear polymer of inorganic phosphate that is stored in platelet dense granules and released upon platelet activation (Ruiz et al., 2004). Polyphosphate interacts with fibrin to produce thicker fibers, a stiffer clot structure, and delays in lysis, which were independent of FXIIIa crosslinking (Smith and Morrissey., 2008). Fibrin interactions with FXIIa, polyphosphate, or other plasma constituents have yet to be evaluated between DVT and PE, but may have important consequences for clot structure and tendency to embolize.

4.1.5 Shear rates may affect embolization in proximal veins

Given the increased propensity for embolization in proximal but not distal vein thrombosis, it is possible that different factors affect local thrombus formation. One obvious effector might be shear stress, which is related by the formula $\tau = 8 \cdot \mu \cdot \frac{u}{\delta}$ where τ is shear stress, μ is viscosity, u is velocity, and δ is the diameter of the vessel. As the blood is pumped back towards the heart from the lower veins through the upper veins, vessel segments enjoin and diameter increases. Thus far, no studies have compared shear stress in the proximal versus distal veins. However, using the formula above, if velocity and viscosity remain constant while diameter increases due to vessel enjoining, vascular shear stress should be reduced. Stasis or reduced shear rate is known to cause expression of adhesion molecules, recruitment of proinflammatory mediators, and secretion of

coagulation factors (Parmar et al., 2006). These local effectors may thus influence clot formation or propagation, increasing the tendency to embolize.

4.1.6 Future directions

VTE is a thrombotic disorder that has been thought to progress via endothelial cell activation, the recruitment of leukocytes, activation of the coagulation cascade followed by the production of fibrin, and also interactions with platelets (von Bruhl et al., 2012). Chapter 2 describes differences in fibrin clot structure and functional properties in DVT and PE subjects, with the goals of further understanding embolization. However, the coagulation cascade and the production of fibrin are only one component in a complex processes involving many cell types that play important roles in mediating thrombosis. Furthermore, fibrin(ogen)-cellular interactions may have consequences for not only fibrin clot structure, but possibly embolization. Endothelial cells produce tissue factor when activated, ultimately resulting in the production of the prothrombinase complex for the cleavage of fibrinogen to fibrin (Grabowski et al., 1993, Campbell et al., 2009). Fibrinogen co-cultured with TNFα-activated endothelial cell monolayers display fibrin clot structures that have increased fiber density close to the cells that diminishes with increasing distance (Campbell et al., 2009). Heterogeneity in fibrin clot structure may alter lysis rates, where tPA and plasmin lyse clots faster at the periphery and progressively decrease in speed towards the core. Increased fiber density towards the endothelium may provide stability during clot formation, whereas clots created by diffuse tissue factor production may result in clots with diminished fiber density, making them liable for embolization. Although the direct interaction of neutrophils and monocytes

with fibrinogen and the resultant effects on clot structure have yet to be determined, addition of neutrophils prior to clot formation decreased lag time while also diminishing clot strength (Perrin et al., 2008). In addition, proteins derived from activated neutrophils such as lysosomal cationic protein, enhanced lateral aggregation of fibrin protofibrils, increasing fiber diameter (Carr et al., 1986). Thus, variations in local leukocyte concentration or the proteins that they secrete during activation may influence fibrin clot structure and stability. Although the interactions between fibrinogen and platelets have been well studied, their effects on clot structure or embolization are unknown. Classically, fibrinogen binds to αIIbβ3 on platelets, creating bridges that bind platelet together (Marguerie et al., 1979). In venous thrombosis, platelets directly interact with both the endothelium and leukocytes (von Bruhl et al., 2012). Platelet-leukocyte interactions promote leukocyte recruitment and the releases of neutrophil extracellular traps (NETs), which are extracellular chromatin fibers and histones that function in this setting to concentrate procoagulant mediators like tissue factor and FXIIa (von Bruhl et al., 2012). Fibrin is also concentrated at the NETs, and it remains to be determined what effects this may have on fibrin clot structure. Again, the concentration of initiating coagulation factors near the endothelial cell surface could alter fibrin clot density, ultimately affecting thrombus stability.

Ideally our studies on clot structure in DVT and PE would be extended to an *in vivo* model of thrombosis, where all cellular and plasma constituents are present. One possible model might involve the use fibrinogen deficient ($Fg^{-/-}$) mice (Iwaki et al., 2002). Fibrinogen isolated from either DVT or PE subjects would be fluorescently tagged and injected into $Fg^{-/-}$ mice. Thrombosis would be initiated by a method that does not denude

the endothelium such as electrical stimulation, and time to thrombus formation, lysis, and the number of emboli formed could be evaluated by intravital microscopy. In addition, fibrin clot structure and thrombus cellular composition could be evaluated by SEM and procoagulant factors and inflammatory mediators could be measured by histology.

Another approach to evaluate the *in vivo* structure of DVT and PE thrombi would be to obtain thrombi via either thrombectomy or embolectomy during the course of treatment or during necropsy. Ideally, thrombi would be collected from distal DVT, proximal DVT, and PE for a comparison of clot structure and cellular composition during changes in the propensity for embolization. Although no study has evaluated *in vivo* thrombi between DVT and PE patients, some studies have evaluated each separately. Early studies evaluating the structures of 50 valve-pocket thrombi from the femoral veins of 41 post-mortem subjects observed heterogeneity amongst the thrombi collected (Sevitt., 1974). Some thrombi had little or no cellular invasion. Most others had two main regions, red areas composed of fibrin and erythrocytes representing the initiating point, and white areas composed mainly of platelets and fibrin(ogen) representing sites of thrombus propagation. Thrombus structure has also been evaluated in one PE patient undergoing embolectomy (Undas et al., 2010). Although there was no comparison group, the thrombus obtained during embolectomy was in itself heterogeneous, with the inner thrombus composed of tightly packed thick fibrin fibers and cellular debris, likely from erythrocytes, and the periphery was composed of many erythrocytes in a thin fibrin network with few platelets. The authors did not observe the characteristic layering of ‘white’ and ‘red’ thrombi that has been previously described for venous and arterial thrombi (Sevitt., 1974, Silvain et al., 2011). These data may indicate that not only fibrin

clot structure, but also cellular constituents may have an effect on propensity for embolization.

4.2 Nitrated fibrinogen is a biomarker for VTE

VTE is a thrombotic disease where thrombus initiation is characterized by the recruitment of inflammatory mediators including neutrophils, monocytes and cytokines (von Bruhl et al., 2012). Neutrophils contain myeloperoxidase (MPO), which is capable of generating nitrative intermediates (van der Vliet et al., 1997). Previous studies in models of inflammatory challenge, coronary artery disease, and smokers have found fibrinogen to be post-translationally modified by reactive nitrogen intermediates, creating nitrated fibrinogen. Despite the relationship between oxidant production, inflammation, and thrombosis in VTE, no specific markers encompassing these processes has been identified. The goal of this study was to evaluate nitrated fibrinogen as a biomarker of VTE. The study population consisted of subjects presenting with symptoms of VTE to the emergency department at the Hospital of the University of Pennsylvania. Nitrated fibrinogen levels were significantly elevated in the plasma of subjects that were found to be positive for acute DVT or PE. Cubic spline analysis demonstrated a positive relationship between nitrated fibrinogen levels and odds of VTE. When divided into quartiles, subjects in the highest quartile of nitrated fibrinogen had increased odds of VTE compared to subjects in the lowest quartile. This relationship persisted after univariate regression analysis for those variables that were elevated in VTE positive subjects: advanced age, active cancer, and recent surgery. However, significance was lost when the three variables were combined in multivariate regression analysis. These data

support a role for inflammation and oxidant production in VTE, and establish nitrated fibrinogen as a biomarker of these processes.

4.2.1 Nitrated fibrinogen levels are elevated in VTE positive subjects

Elevated nitrated fibrinogen levels in VTE positive subjects were not dependent on changes in plasma fibrinogen concentration as no differences were observed between VTE positive and negative subjects with either fibrinogen assay. Nitrated fibrinogen levels were higher in our VTE negative population than in previous studies where VTE subjects were compared to healthy controls (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009). Our VTE negative group consisted of subjects that presented with symptoms of VTE, but were not found to have DVT by compression ultrasound (CUS) or PE by computed tomographic pulmonary angiography (CTPA). Many of the VTE negative subjects had previous history of coronary artery disease (CAD), smoking, infection, and other inflammatory conditions that likely affected nitrated fibrinogen levels. Because arterial thrombosis, smoking, infection etc. are risk factors for VTE and have been shown to produce nitrated fibrinogen (Parastatidis et al., 2008, Vadseth et al., 2004, Heffron et al., 2009), this suggests that nitrative intermediates are produced both in VTE as well as in those at risk for disease and may ultimately contribute to disease pathogenesis.

4.2.2 Nitrated fibrinogen is a risk factor for VTE

Cubic spline curves demonstrated a strong, positive relationship between nitrated fibrinogen levels and odds of VTE. In addition, VTE positive subjects in the highest quartile of nitrated fibrinogen had an increased risk of VTE compared with those in the

lowest quartile. Regression analysis showed that this risk persisted after univariate, but not multivariate adjustment for the variables found to be elevated in the VTE positive group: advanced age, active cancer, and recent surgery. This suggests that those with advanced age, active cancer, and recent surgery also experience elevated nitrated fibrinogen levels independent of VTE. Indeed previous studies have documented elevated nitrated fibrinogen in lung cancer subjects (Pignatelli et al., 2001). Further studies evaluating nitrated fibrinogen in advanced age, recent surgery, and other cancer types will be necessary to address these findings. Alternatively, the loss of significance may be due to limited numbers within our subject population. Because the VTE positive subject group is relatively small, adjustment further reduces the sample size and eliminates statistical significance. Additional studies with a larger population size would be necessary to address this.

4.2.3 Nitrated fibrinogen may affect clot formation in VTE

Nitrated fibrinogen has been associated with both arterial thrombosis (Vadseth et al., 2004), as well as those at risk for disease such as smokers (Parastatidis et al., 2008). Neutrophils and monocytes, which are capable of producing nitrating intermediates, are key mediators in the initial phases of venous thrombus formation (von Bruhl et al., 2012, Kakkar et al., 1969, Brennan et al., 2002, Hazen et al., 1999). Furthermore, nitrated fibrinogen levels increase in response to inflammation (Heffron et al., 2009). The effects of nitrated fibrinogen remain to be elucidated for VTE, however previous studies have investigated these effects *in vitro* (Vadseth et al., 2004) and *ex vivo* in the plasma of smokers (Parastatidis et al., 2008). When fibrinogen was nitrated with either 3-morpholinosydnonimine (SIN-1), which produces nitric oxide and superoxide that react

to form peroxynitrite, or with MPO, hydrogen peroxide, and nitrite, a decrease in lag time and an increase in both the rate of fibrin polymerization and final absorbance was observed during turbidity assays when compared with unmodified fibrinogen (Vadseth et al., 2004). Fibrinogen treated with MPO and hydrogen peroxide alone i.e. oxidizing conditions, produced impairment of fibrin polymerization. Furthermore, oxidized fibrinogen that was subsequently nitrated resulted in a reversal of the impaired fibrin polymerization, producing polymerization rates similar to nitration alone. The clot structure formed from nitrated fibrinogen is composed of thick, twisted fiber bundles and large pores, which is in contrast to the homogenous structure composed of thin fibers created from fibrinogen treated with MPO and hydrogen peroxide. Although nitration and oxidation of fibrinogen produce disparate effects on clot structure, G' was similar between the two groups and significantly lower than control, indicating a decrease in clot stiffness. Although no differences in lysis rate or lysis product formed were seen between control and nitrated fibrinogen *in vitro*, injection of microemboli into mice composed of fibrin treated with SIN-1 followed by bolus injection of tPA showed enhanced lytic susceptibility compared to emboli of nascent fibrin. No differences were observed between nitrated and control fibrinogen for fibrinopeptide A (FpA) or B (FpB) release or FXIIIa crosslinking.

In smokers, elevated nitrated fibrinogen levels showed a dose-dependent increase in the rate of fibrin polymerization that was reversed when nitrated molecules were removed via immunoprecipitation with antinitrotyrosine antibodies (Parastatidis et al., 2008). Scanning electron micrographs showed fiber clustering that was also reversed by antibody immunoprecipitation. Nitrated fibrinogen levels were positively correlated with

G', G", tan δ, and inversely correlated with fibrinolysis rate. Mass spectrometric analysis revealed Tyr^{292} and Tyr^{422} as nitrated in a majority of the samples analyzed. These residues are located in the carboxy terminus of the β chain of fibrinogen near the 'b' hole. Use of the 'B' knob mimetic peptide accelerated lateral aggregation in all samples, but was positively correlated with nitrated fibrinogen levels. The effects of *in vivo* nitration in smokers show some similarities to the *in vitro* modified fibrinogen, such as increased rate of fibrin polymerization. However, there are some disparities between the two studies (G', G", tan δ, clot structure) that might be related to the particular tyrosines that are susceptible to nitration or the extent of nitration in each system. In this study, nitrated fibrinogen levels were positively correlated with Clauss-measured fibrinogen levels. Because Clauss measures fibrinogen based on clotting, this correlation may suggest that fibrinogen from VTE positive subjects also exhibits gain of function effects for fibrin polymerization due to nitration. However, VTE subjects might also exhibit unique functional properties and clot structures that are related to the selectivity and extent of the tyrosines that are nitrated.

Many of the same pathways that produce nitrating intermediates also produce reactive oxygen intermediates. Reactive oxygen intermediates have been implicated in vascular disease pathology and hence, much attention has been paid to the role and function of oxidative modifications of coagulation factors, especially fibrinogen. In contrast to nitration, oxidation of fibrinogen by hematoporphyrins, photosensitive dyes, and chloramines, which release singlet oxygen ($^1O_2$), impair fibrin polymerization in a dose-dependent manner (Zieve and Solomon., 1966, Inada et al., 1978). Photooxidized fibrin monomer does not bind fibrinogen as well as nascent fibrin monomer, despite similar

release of FpA and FpB (Inada et al., 1978). His16, which is located within the 'B' knob site of the Bβ chain of fibrinogen was the only amino acid found to be modified upon oxidation (Shimizu et al., 1986). Oxidation of fibrinogen also has functional effects on lysis. Pro-urokinase is activated faster in the presence of oxidized fibrin, and plasmin lyses oxidized fibrin at a faster rate. This implies a pro-fibrinolytic capacity for oxidized fibrinogen (Stief., 1993). Overall, the effect of oxidation on fibrinogen appears to be a loss of function, rather than gain of function as seen with nitration. Nitration levels were similar between DVT and PE in this study, but oxidation of fibrinogen in VTE has yet to be determined. Nonetheless, oxidation is unlikely to account for fibrin clot structure and functional property differences observed between DVT and PE subjects. Because both DVT and PE patients have elevated nitrated fibrinogen levels, the effect of nitration on fibrin clot structure and functional properties are likely supersede the effects of oxidation (Vadseth et al., 2004).

4.2.4 Plasma fibrinogen levels are not associated with VTE

Elevated plasma fibrinogen levels are a risk factor for CAD and its associated morbidity and mortality (Benderly et al., 1996, Folsom et al., 1997). Some studies have suggested a similar role for fibrinogen in VTE (Koster et al., 1994). Compared to healthy control subjects, fibrinogen levels measured by Clauss assay were significantly higher in DVT subjects, and were thus identified as a risk factor for DVT (Koster et al., 1994). However, later studies in the same subject population showed that fibrinogen was only a risk factor for DVT in subjects >45 years of age (van Hylckama Vlieg and Rosendaal., 2003). Conversely, in PE subjects, plasma fibrinogen levels were found to be lower compared to

healthy controls (Kucher et al., 2003b, Kucher et al., 2003a). Plasma fibrinogen levels in this study population were similar between VTE positive and negative subjects, and between DVT and PE. Discrepancies between this study and others may be due to overall differences in subject population (Kucher et al., 2003b, Kucher et al., 2003a), or to differences in the comparison group, where DVT was compared to healthy age and sex-matched controls (van Hylckama Vlieg and Rosendaal., 2003).

4.2.5 Future directions

D-dimer levels were significantly elevated in VTE positive compared with VTE negative subjects. At an exclusionary cutoff of 0.5 µg/ml, sensitivity and specificity for D-dimer in this population were 100 % (95 % CI 91.6-100) and 22.3 % (95 % CI 16.1-29.6), respectively. D-dimer remains the standard non-invasive biomarker for diagnosis of acute DVT and PE due to its high sensitivity, especially in ELFA and ELISA-based tests (77-100 %) (Di Nisio et al., 2007). However, specificity of D-dimer, regardless of test employed, remains lower and more variable (7-94 %) (Di Nisio et al., 2007). Lack of specificity for D-dimer requires that many patients undergo unnecessary imaging, which is both costly and also exposes patients to ionizing radiation in the case of CTPA and possibly allergy to the contrast agent (Lensing et al., 1990). Identification of a novel biomarker with specificity to VTE could greatly reduce these concerns. Attempts have been made to identify such markers. Prothrombin is activated to thrombin by the cleavage of prothrombin fragment 1+2 (PF 1+2) by the prothrombinase complex containing factors Xa and Va, Ca^{2+}, and phospholipids. In a study that enrolled 135 consecutive outpatients with suspected lower extremity DVT, sensitivity of PF 1+2 was 100 %, but specificity

was only 11 % (Bozic et al., 2002). Similar results with high sensitivity and low specificity have been found for thrombin-antithrombin III complex, P-selectin, and VCAM-1 (Bozic et al., 2002). Thus the search remains ongoing for non-invasive diagnostic markers. Recently, 50 potential biomarkers were evaluated in 304 consecutive subjects presenting with VTE, 22 of which were positive (Nordenholz et al., 2008). Of the 50 proteins identified, only D-dimer, c-reactive protein (CRP), and MPO were potential markers. Further analysis found that MPO in combination with D-dimer did not increase sensitivity (100 %), but did increase specificity from 59 % to 73 %, which would have reduced the need for vascular imaging by 13 % (Mitchell et al., 2008). Due to our small sample size, we did not perform receiver operating characteristic curves or calculate sensitivity and specificity. However, the identification of MPO as a possible marker of VTE supports a role for inflammatory and oxidant markers in the diagnosis of VTE. Our observation that nitrated fibrinogen is a risk factor for VTE may lend itself to larger studies, pursuing it as a diagnostic marker, possibly in combination with D-dimer or MPO.

Further evidence for the production of reactive nitrogen intermediates during inflammation comes from the identification of antibodies that recognize nitrated epitopes in atherosclerotic lesions and the circulation of CAD patients, as well as in plasma from acute lung injury patients (Thomson et al., 2007). Furthermore, antigen presenting cells display nitrated peptides by MHC class II molecule I-A, eliciting a CD4 T-cell response (Herzog et al., 2005). Although these processes have yet to be evaluated in VTE, the recognition of nitrated fibrinogen by antinitrotyrosine antibodies and the subsequent presentation of nitrated epitopes in VTE may represent a novel interplay between oxidant

production, inflammation, and thrombosis that has not yet been explored. In patients undergoing vein stripping for primary varicosity, dendritic cells, which are antigen presenting cells, were observed in patients with varicose veins and/or superficial thrombophlebitis, but not in vein segments from healthy subjects (Cherian et al., 1999). Dendritic cells were localized with T-cells and macrophages around areas of neovascularization. Although this study was performed in superficial thrombophlebitis and varicose veins, both of these conditions are risk factors for VTE (Heit et al., 2000b, Decousus et al., 2010). In addition, neovascularization subsequent to thrombosis has also been observed in a rat model of DVT (Wakefield et al., 1999) and it is probable that similar innate and adaptive immune responses occur for thrombus resolution in DVT. Thus, it is possible that nitrated fibrinogen is recognized by antibodies and targeted for antigen presentation in VTE, resulting in recruitment of adaptive immune cells for thrombus resolution. Additional studies would be required to address this.

4.3 Risk factors for VTE

Many variables have been associated with risk of VTE including patient demographics, disease history, and medication use. Previous studies have shown that the risk of VTE increases exponentially as a function of age, with a relative risk of 1.9 per 10 year increase (Anderson et al., 1991). In accordance with these and other findings, VTE positive subjects in this study were older (Table 3-1) and no differences in age were observed between DVT and PE. In our subject population males were significantly more prevalent in DVT subjects, whereas females were more prevalent in PE subjects. Tormene et al. similarly showed that men were at higher risk of isolated DVT and women

at higher risk of PE (Tormene et al., 2009). However, other studies indicate that DVT is more prevalent in women and PE in men (Kniffin et al., 1994), while others show no differences in prevalence between sexes (Anderson et al., 1991, Silverstein et al., 1998). Thus far it appears that the relationship between sex and risk for either DVT or PE remains unresolved.

DVT subjects within our population had an increased incidence of peripheral artery disease compared with PE, despite being a risk factor for both DVT and PE (Sorensen et al., 2009, Sorensen et al., 2011). In a population based study, subjects with a history of arterial thrombotic events including myocardial infarction and stroke were at risk for VTE three months following an event (Sorensen et al., 2009, Sorensen et al., 2011). A recent study also suggests that heart disease is an independent risk factor for PE in the absence of venous thrombosis and suggests that PE might also arise from the heart itself rather than the venous system. If this is the case, clots from these subjects might share structural characteristics that are similar to arterial clots. Arterial and venous thrombotic diseases appear to share some, but not all clot structural characteristics (Collet et al., 2006, Undas et al., 2009) and elucidating differences may help identify factors that uniquely contribute to each disease, and possibly embolization.

In a cohort of 625 cases of VTE with age and sex-matched controls, malignant neoplasm increased risk of VTE by 4-fold, with a more than 6-fold increased risk for subjects on chemotherapy (Heit et al., 2000b). In that same study, surgery increased the risk of VTE by more than 22-fold (Heit et al., 2000b). Increased incidence of both cancer and surgery were found for VTE positive subjects in this study (Table 3-1), and incidences were similar between DVT and PE. The use of immunomodulatory agents including

chemotherapeutics are implicated as risk factors for VTE. In a landmark study (Levine et al., 1988), stage II breast cancer subjects were randomized to either 12 weeks of multi drug chemotherapy regimen that included cyclophosphamide, methotrexate, fluorouracil, vincristine, prednisone, doxorubicin plus tamoxifen or a similar 36 week regimen minus tamoxifen. During the course of treatment, 5 and 9 subjects developed VTE during the course of either the 12 or 36 week treatment, respectively (Levine et al., 1988). No thrombotic events occurred before or after the treatment course, implicating a direct relationship between chemotherapeutics and VTE. Research has also indicated that treatment of multiple myeloma with either thalidomide or lenalidomide coupled with dexamethasone increases rates of VTE (Dimopoulos et al., 2007, Rajkumar et al., 2006). Several subjects in this study reported taking immunomodulators such as prednisone, vincristine, fluorouracil, lenalidomide and dexamethasone. Although use of immunomodulators increases risk of both DVT and PE, in our study DVT subjects reported a higher use of these agents. Previous studies have indicated that hormonal contraception or hormone replacement therapies containing estrogen-derivatives are risk factors for VTE (Gerstman et al., 1990, Daly et al., 1996). In our population, incidences of hormonal contraception or replacement therapy were similar between VTE negative and positive subjects and between DVT and PE.

4.4 VTE, antioxidants, and pharmaceutics

Several medications including anti-platelet therapies, statins, and antioxidants that are used in the treatment of arterial diseases, such as CAD, have also been effective at reducing the risk of VTE. The effects of these medications may be in part due to lowering oxidant burden in diseased patients. Given as a prophylactic to hip fracture surgery,

aspirin lowers the risk of a first DVT by 29 % and PE by 43 % compared with placebo (Anonymous 2000). Aspirin also attenuates lipid peroxidation and cytotoxicity of bovine aortic endothelial cells caused by SIN-1-oxidized low-density lipoprotein (Hermann et al., 1999). Statin use in females reduces the risk of VTE events compared to non-statin users (relative hazard=.45; P=0.02), and offsets the use of hormone replacement therapy (relative hazard=1.34; P=0.45) (Herrington et al., 2002). In an interventional study statins were given to subjects with borderline high (>130 mg/dL) cholesterol for 12 weeks and nitrotyrosine levels were measured prior to and after statin therapy. Levels of nitrotyrosine decreased by 25 % following statin use, and were similar in magnitude to reduction in total cholesterol and low-density lipoprotein particle number (Shishehbor et al., 2003). The Women's Healthy Study evaluated the effects of the antioxidant vitamin E on VTE by randomizing 39,876 women to 600 IU of vitamin E or placebo and followed them over 10 years (Glynn et al., 2007). Compared to placebo, vitamin E reduced relative hazard of VTE by 21 %. Antioxidant supplementation of 100 IU/kg vitamin E and 1 g vitamin C daily diminished neovascularization of the myocardium, reduced VEGF expression and reduced the amount of nitrotyrosine containing proteins in hypercholesterolemic pigs (Zhu et al., 2004). Although antiplatelets, statins, and antioxidant usage were similar between VTE negative and positive subjects, and between DVT and PE subjects in our cohort, future studies may investigate the relationships between these medications and their effects on oxidant burden and disease outcome.

In conclusion, these studies show that fibrin clot structure and functional properties differ between DVT and PE subjects and provide further insight on the mechanisms that govern

embolization. These studies also identify nitrated fibrinogen as a unique biomarker of thrombosis, oxidant production, and inflammation in VTE.

References

Anonymous 2000. Prevention of pulmonary embolism and deep vein thrombosis with low dose aspirin: Pulmonary Embolism Prevention (PEP) trial. *Lancet* 355:1295-1302.

Anonymous 1997. Low-molecular-weight heparin in the treatment of patients with venous thromboembolism. The Columbus Investigators. *N. Engl. J. Med.* 337:657-662.

Fenton, H.J.H. 1894. LXXIII. Oxidation of tartaric acid in presence of iron. *J. Chem. Soc., Trans.* 899-910.

Ali, T., J. Humphries, K. Burnand, B. Sawyer, C. Bursill, K. Channon, D. Greaves, B. Rollins, I.F. Charo, and A. Smith. 2006. Monocyte recruitment in venous thrombus resolution. *J. Vasc. Surg.* 43:601-608.

Anderson, F.A.,Jr, H.B. Wheeler, R.J. Goldberg, D.W. Hosmer, N.A. Patwardhan, B. Jovanovic, A. Forcier, and J.E. Dalen. 1991. 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.

Aslan, M., T.M. Ryan, T.M. Townes, L. Coward, M.C. Kirk, S. Barnes, C.B. Alexander, S.S. Rosenfeld, and B.A. Freeman. 2003. Nitric oxide-dependent generation of reactive species in sickle cell disease. Actin tyrosine induces defective cytoskeletal polymerization. *J. Biol. Chem.* 278:4194-4204.

Babior, B.M., J.T. Curnutte, and B.J. McMurrich. 1976. The particulate superoxide-forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. *J. Clin. Invest.* 58:989-996.

Baldus, S., J.P. Eiserich, A. Mani, L. Castro, M. Figueroa, P. Chumley, W. Ma, A. Tousson, C.R. White, D.C. Bullard, M.L. Brennan, A.J. Lusis, K.P. Moore, and B.A. Freeman. 2001. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J. Clin. Invest.* 108:1759-1770.

Bale, M.D., M.F. Muller, and J.D. Ferry. 1985. Rheological studies of creep and creep recovery of unligated fibrin clots: comparison of clots prepared with thrombin and ancrod. *Biopolymers* 24:461-482.

Bannish, B.E., J.P. Keener, and A.L. Fogelson. Accepted a. Modelling fibrinolysis: a 3-D stochastic multiscale model. *Math. Med. Biol.*

Bannish, B.E., J.P. Keener, M. Woodbury, J.W. Weisel, and A.L. Fogelson. Accepted b. Modelling fibrinolysis: 1-D continuum models. *Math. Med. Biol.*

Bates, S.M., R. Jaeschke, S.M. Stevens, S. Goodacre, P.S. Wells, M.D. Stevenson, C. Kearon, H.J. Schunemann, M. Crowther, S.G. Pauker, R. Makdissi, G.H. Guyatt, and American College of Chest Physicians. 2012. Diagnosis of DVT: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 141:e351S-418S.

Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall, and B.A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A.* 87:1620-1624.

Benderly, M., E. Graff, H. Reicher-Reiss, S. Behar, D. Brunner, and U. Goldbourt. 1996. 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.

Bettelheim, F.R. 1956. The clotting of fibrinogen. II. Fractionation of peptide material liberated. *Biochim. Biophys. Acta* 19:121-130.

Biro, E., K.N. Sturk-Maquelin, G.M. Vogel, D.G. Meuleman, M.J. Smit, C.E. Hack, A. Sturk, and R. Nieuwland. 2003. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. *J. Thromb. Haemost.* 1:2561-2568.

Blomback, B., K. Carlsson, B. Hessel, A. Liljeborg, R. Procyk, and N. Aslund. 1989. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. *Biochim. Biophys. Acta* 997:96-110.

Blomback, B., B. Hessel, and D. Hogg. 1976. Disulfide bridges in nh2 -terminal part of human fibrinogen. *Thromb. Res.* 8:639-658.

Blomback, B., B. Hessel, D. Hogg, and L. Therkildsen. 1978. A two-step fibrinogen--fibrin transition in blood coagulation. *Nature* 275:501-505.

Blomback, B., B. Hessel, S. Iwanaga, J. Reuterby, and M. Blomback. 1972. Primary structure of human fibrinogen and fibrin. I. Clevage of fibrinogen with cyanogen bromide. Isolation and characterization of NH 2 -terminal fragments of the ("A") chain. *J. Biol. Chem.* 247:1496-1512.

Boggs, D.R., J.C. Marsh, P.A. Chervenick, G.E. Cartwright, and M.M. Wintrobe. 1968. Neutrophil releasing activity in plasma of normal human subjects injected with endotoxin. *Proc. Soc. Exp. Biol. Med.* 127:689-693.

Bok, R.A., and W.F. Mangel. 1985. Quantitative characterization of the binding of plasminogen to intact fibrin clots, lysine-sepharose, and fibrin cleaved by plasmin. *Biochemistry* 24:3279-3286.

Bos, A., R. Wever, and D. Roos. 1978. Characterization and quantification of the peroxidase in human monocytes. *Biochim. Biophys. Acta* 525:37-44.

Bounameaux, H., P. Cirafici, P. de Moerloose, P.A. Schneider, D. Slosman, G. Reber, and P.F. Unger. 1991. Measurement of D-dimer in plasma as diagnostic aid in suspected pulmonary embolism. *Lancet* 337:196-200.

Bozic, M., A. Blinc, and M. Stegnar. 2002. 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.

Brennan, M.L., W. Wu, X. Fu, Z. Shen, W. Song, H. Frost, C. Vadseth, L. Narine, E. Lenkiewicz, M.T. Borchers, A.J. Lusis, J.J. Lee, N.A. Lee, H.M. Abu-Soud, H. Ischiropoulos, and S.L. Hazen. 2002. 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.

Brown, A.E., R.I. Litvinov, D.E. Discher, P.K. Purohit, and J.W. Weisel. 2009. Multiscale mechanics of fibrin polymer: gel stretching with protein unfolding and loss of water. *Science* 325:741-744.

Brown, J.H., N. Volkmann, G. Jun, A.H. Henschen-Edman, and C. Cohen. 2000. The crystal structure of modified bovine fibrinogen. *Proc. Natl. Acad. Sci. U. S. A.* 97:85-90.

Budzynski, A.Z., S.A. Olexa, and B.V. Pandya. 1983. Fibrin polymerization sites in fibrinogen and fibrin fragments. *Ann. N. Y. Acad. Sci.* 408:301-314.

Burton, R.A., G. Tsurupa, R.R. Hantgan, N. Tjandra, and L. Medved. 2007. NMR solution structure, stability, and interaction of the recombinant bovine fibrinogen alphaC-domain fragment. *Biochemistry* 46:8550-8560.

Busse, R., and A. Mulsch. 1990. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* 275:87-90.

Campbell, R.A., K.A. Overmyer, C.H. Selzman, B.C. Sheridan, and A.S. Wolberg. 2009. Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability. *Blood* 114:4886-4896.

Carr, M.E.,Jr, D.A. Gabriel, J.C. Herion, and H.R. Roberts. 1986. Granulocyte lysosomal cationic protein alters fibrin assembly: a possible mechanism for granulocyte control of clot structure. *J. Lab. Clin. Med.* 107:199-203.

Cherian, S.M., Y.V. Bobryshev, S.J. Inder, R.S. Lord, and K.W. Ashwell. 1999. Dendritic cells in venous pathologies. *Angiology* 50:393-402.

Chuang, Y.J., R. Swanson, S.M. Raja, and S.T. Olson. 2001. Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin. *J. Biol. Chem.* 276:14961-14971.

Chung, S.I., M.S. Lewis, and J.E. Folk. 1974. Relationships of the catalytic properties of human plasma and platelet transglutaminases (activated blood coagulation factor XIII) to their subunit structures. *J. Biol. Chem.* 249:940-950.

Ciuti, G., E. Grifoni, A. Pavellini, D. Righi, R. Livi, F. Perfetto, R. Abbate, D. Prisco, and A.M. Pignone. 2012. Incidence and characteristics of asymptomatic distal deep vein thrombosis unexpectedly found at admission in an Internal Medicine setting. *Thromb. Res.* 130:591-595.

Clauss, A. 1957. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematologica* 237-246.

Cogo, A., A.W. Lensing, P. Prandoni, and J. Hirsh. 1993. Distribution of thrombosis in patients with symptomatic deep vein thrombosis. Implications for simplifying the diagnostic process with compression ultrasound. *Arch. Intern. Med.* 153:2777-2780.

Collet, J.P., Y. Allali, C. Lesty, M.L. Tanguy, J. Silvain, A. Ankri, B. Blanchet, R. Dumaine, J. Gianetti, L. Payot, J.W. Weisel, and G. Montalescot. 2006. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler. Thromb. Vasc. Biol.* 26:2567-2573.

Collet, J.P., C. Nagaswami, D.H. Farrell, G. Montalescot, and J.W. Weisel. 2004. Influence of gamma' fibrinogen splice variant on fibrin physical properties and fibrinolysis rate. *Arterioscler. Thromb. Vasc. Biol.* 24:382-386.

Collet, J.P., D. Park, C. Lesty, J. Soria, C. Soria, G. Montalescot, and J.W. Weisel. 2000. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. *Arterioscler. Thromb. Vasc. Biol.* 20:1354-1361.

Corrin, B., and A.G. Nicholson. 2011. Pathology of the lungs. Churchill Livingston Elsevier, 778pp.

Cottrell, B.A., D.D. Strong, K.W. Watt, and R.F. Doolittle. 1979. Amino acid sequence studies on the alpha chain of human fibrinogen. Exact location of cross-linking acceptor sites. *Biochemistry* 18:5405-5410.

Crabtree, G.R., and J.A. Kant. 1982. Coordinate accumulation of the mRNAs for the alpha, beta, and gamma chains of rat fibrinogen following defibrination. *J. Biol. Chem.* 257:7277-7279.

Cushman, M., E.S. O'Meara, A.R. Folsom, and S.R. Heckbert. 2009. Coagulation factors IX through XIII and the risk of future venous thrombosis: the Longitudinal Investigation of Thromboembolism Etiology. *Blood* 114:2878-2883.

Cushman, M., A.W. Tsai, R.H. White, S.R. Heckbert, W.D. Rosamond, P. Enright, and A.R. Folsom. 2004. Deep vein thrombosis and pulmonary embolism in two cohorts: the longitudinal investigation of thromboembolism etiology. *Am. J. Med.* 117:19-25.

Dalen, J.E., C.I. Haffajee, J.S. Alpert 3rd, J.P. Howe, I.S. Ockene, and J.A. Paraskos. 1977. Pulmonary embolism, pulmonary hemorrhage and pulmonary infarction. *N. Engl. J. Med.* 296:1431-1435.

Daly, E., M.P. Vessey, M.M. Hawkins, J.L. Carson, P. Gough, and S. Marsh. 1996. Risk of venous thromboembolism in users of hormone replacement therapy. *Lancet* 348:977-980.

Dang, C.V., C.K. Shin, W.R. Bell, C. Nagaswami, and J.W. Weisel. 1989. Fibrinogen sialic acid residues are low affinity calcium-binding sites that influence fibrin assembly. *J. Biol. Chem.* 264:15104-15108.

de Moerloose, P., S. Desmarais, H. Bounameaux, G. Reber, A. Perrier, G. Dupuy, and J.L. Pittet. 1996. Contribution of a new, rapid, individual and quantitative automated D-dimer ELISA to exclude pulmonary embolism. *Thromb. Haemost.* 75:11-13.

Declerck, P.J., P. Mombaerts, P. Holvoet, M. De Mol, and D. Collen. 1987. Fibrinolytic response and fibrin fragment D-dimer levels in patients with deep vein thrombosis. *Thromb. Haemost.* 58:1024-1029.

Decousus, H., I. Quere, E. Presles, F. Becker, M.T. Barrellier, M. Chanut, J.L. Gillet, H. Guenneguez, C. Leandri, P. Mismetti, O. Pichot, A. Leizorovicz, and POST (Prospective Observational Superficial Thrombophlebitis) Study Group. 2010. Superficial venous thrombosis and venous thromboembolism: a large, prospective epidemiologic study. *Ann. Intern. Med.* 152:218-224.

Denicola, A., B.A. Freeman, M. Trujillo, and R. Radi. 1996. Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* 333:49-58.

Di Nisio, M., A. Squizzato, A.W. Rutjes, H.R. Buller, A.H. Zwinderman, and P.M. Bossuyt. 2007. Diagnostic accuracy of D-dimer test for exclusion of venous thromboembolism: a systematic review. *J. Thromb. Haemost.* 5:296-304.

Dimopoulos, M., A. Spencer, M. Attal, H.M. Prince, J.L. Harousseau, A. Dmoszynska, J. San Miguel, A. Hellmann, T. Facon, R. Foa, A. Corso, Z. Masliak, M. Olesnyckyj, Z. Yu, J. Patin, J.B. Zeldis, R.D. Knight, and Multiple Myeloma (010) Study Investigators. 2007. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. *N. Engl. J. Med.* 357:2123-2132.

Doolittle, R.F., and L. Pandi. 2006. 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.

Eiserich, J.P., M. Hristova, C.E. Cross, A.D. Jones, B.A. Freeman, B. Halliwell, and A. van der Vliet. 1998. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391:393-397.

Eliasson, A., D. Bergqvist, M. Bjorck, S. Acosta, N.H. Sternby, and M. Ogren. 2006. Incidence and risk of venous thromboembolism in patients with verified arterial thrombosis: a population study based on 23,796 consecutive autopsies. *J. Thromb. Haemost.* 4:1897-1902.

Elms, M.J., I.H. Bunce, P.G. Bundesen, D.B. Rylatt, A.J. Webber, P.P. Masci, and A.N. Whitaker. 1986. Rapid detection of cross-linked fibrin degradation products in plasma using monoclonal antibody-coated latex particles. *Am. J. Clin. Pathol.* 85:360-364.

Folsom, A.R., K.K. Wu, W.D. Rosamond, A.R. Sharrett, and L.E. Chambless. 1997. Prospective study of hemostatic factors and incidence of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* 96:1102-1108.

Fowler, W.E., R.R. Hantgan, J. Hermans, and H.P. Erickson. 1981. Structure of the fibrin protofibril. *Proc. Natl. Acad. Sci. U. S. A.* 78:4872-4876.

Francalanci, I., P. Comeglio, A.A. Liotta, A.P. Cellai, S. Fedi, E. Parretti, G. Mello, D. Prisco, and R. Abbate. 1995. D-dimer concentrations during normal pregnancy, as measured by ELISA. *Thromb. Res.* 78:399-405.

Freyburger, G., H. Trillaud, S. Labrouche, P. Gauthier, S. Javorschi, P. Bernard, and N. Grenier. 1998. 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. Haemost.* 79:32-37.

Furchgott, R.F., and S. Bhadrakom. 1953. Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J. Pharmacol. Exp. Ther.* 108:129-143.

Furchgott, R.F., and J.V. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376.

Furie, B.C., M. Blumenstein, and B. Furie. 1979. Metal binding sites of a gamma-carboxyglutamic acid-rich fragment of bovine prothrombin. *J. Biol. Chem.* 254:12521-12530.

Gaffney, P.J., F. Joe, and M. Mahmoud. 1980. Giant fibrin fragments derived from crosslinked fibrin: structure and clinical implication. *Thromb. Res.* 20:647-662.

Garman, A.J., and R.A. Smith. 1982. The binding of plasminogen to fibrin: evidence for plasminogen-bridging. *Thromb. Res.* 27:311-320.

Geer, C.B., A. Tripathy, M.H. Schoenfisch, S.T. Lord, and O.V. Gorkun. 2007. Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. *J. Thromb. Haemost.* 5:2344-2351.

Geng, J.G., M.P. Bevilacqua, K.L. Moore, T.M. McIntyre, S.M. Prescott, J.M. Kim, G.A. Bliss, G.A. Zimmerman, and R.P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* 343:757-760.

Gersh, K.C., C. Nagaswami, J.W. Weisel, and S.T. Lord. 2009. The presence of gamma' chain impairs fibrin polymerization. *Thromb. Res.* 124:356-363.

Gerstman, B.B., J.M. Piper, J.P. Freiman, D.K. Tomita, D.L. Kennedy, W.J. Ferguson, and R.C. Bennett. 1990. Oral contraceptive oestrogen and progestin potencies and the incidence of deep venous thromboembolism. *Int. J. Epidemiol.* 19:931-936.

Gerth, C., W.W. Roberts, and J.D. Ferry. 1974. Rheology of fibrin clots. II. Linear viscoelastic behavior in shear creep. *Biophys. Chem.* 2:208-217.

Glynn, R.J., P.M. Ridker, S.Z. Goldhaber, R.Y. Zee, and J.E. Buring. 2007. Effects of random allocation to vitamin E supplementation on the occurrence of venous thromboembolism: report from the Women's Health Study. *Circulation* 116:1497-1503.

Goldstein, Sara, and Czapski Gidon. 1999. Viscosity effects on the reaction of peroxynitrite with CO_2: Evidence for radical formation in a solvent cage. *J. Am. Chem. Soc.* 121:2444-2447.

Gole, M.D., J.M. Souza, I. Choi, C. Hertkorn, S. Malcolm, R.F. Foust 3rd, B. Finkel, P.N. Lanken, and H. Ischiropoulos. 2000. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278:L961-7.

Gormsen, J., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1967. Enzymic lysis of plasma clots: The influence of fibrin stabilization on lysis rates. *Arch. Biochem. Biophys.* 120:654-665.

Gow, A., D. Duran, S.R. Thom, and H. Ischiropoulos. 1996. Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch. Biochem. Biophys.* 333:42-48.

Grabowski, E.F., D.B. Zuckerman, and Y. Nemerson. 1993. The functional expression of tissue factor by fibroblasts and endothelial cells under flow conditions. *Blood* 81:3265-3270.

Gryglewski, R.J., R.M. Palmer, and S. Moncada. 1986. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 320:454-456.

Hafter, R., R. Schrock, R. von Hugo, and H. Graeff. 1985. Measurement of crosslinked fibrin derivatives in plasma and ascitic fluid with monoclonal antibodies against D dimer using EIA and latex test. *Scand. J. Clin. Lab. Invest. Suppl.* 178:137-144.

Hall, C.E., and H.S. Slayter. 1959. The fibrinogen molecule: its size, shape, and mode of polymerization. *J. Biophys. Biochem. Cytol.* 5:11-16.

Hantgan, R.R., and J. Hermans. 1979. Assembly of fibrin. A light scattering study. *J. Biol. Chem.* 254:11272-11281.

Hazen, S.L., R. Zhang, Z. Shen, W. Wu, E.A. Podrez, J.C. MacPherson, D. Schmitt, S.N. Mitra, C. Mukhopadhyay, Y. Chen, P.A. Cohen, H.F. Hoff, and H.M. Abu-Soud. 1999. 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.

Heffron, S.P., I. Parastatidis, M. Cuchel, M.L. Wolfe, M.G. Tadesse, E.R. Mohler 3rd, H. Ischiropoulos, D.J. Rader, and M.P. Reilly. 2009. Inflammation induces fibrinogen nitration in experimental human endotoxemia. *Free Radic. Biol. Med.* 47:1140-1146.

Heijboer, H., A. Cogo, H.R. Buller, P. Prandoni, and J.W. ten Cate. 1992. Detection of deep vein thrombosis with impedance plethysmography and real-time compression ultrasonography in hospitalized patients. *Arch. Intern. Med.* 152:1901-1903.

Heijnen, H.F., E. van Donselaar, J.W. Slot, D.M. Fries, B. Blachard-Fillion, R. Hodara, R. Lightfoot, M. Polydoro, D. Spielberg, L. Thomson, E.A. Regan, J. Crapo, and H. Ischiropoulos. 2006. 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.

Heit, J.A., A. Cohen, and F.J. Anderson. 2005. Estimated annual number of incident and recurrent, non-fatal and fatal venous thromboembolism (VTE) events in the US. *Blood* 106:267a. (Abstr.)

Heit, J.A., D.N. Mohr, M.D. Silverstein, T.M. Petterson, W.M. O'Fallon, and L.J. Melton 3rd. 2000a. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. *Arch. Intern. Med.* 160:761-768.

Heit, J.A., M.D. Silverstein, D.N. Mohr, T.M. Petterson, W.M. O'Fallon, and L.J. Melton 3rd. 2000b. Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study. *Arch. Intern. Med.* 160:809-815.

Heit, J.A., M.D. Silverstein, D.N. Mohr, T.M. Petterson, W.M. O'Fallon, and L.J. Melton 3rd. 1999. Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. *Arch. Intern. Med.* 159:445-453.

Henke, P.K., A. Varga, S. De, C.B. Deatrick, J. Eliason, D.A. Arenberg, P. Sukheepod, P. Thanaporn, S.L. Kunkel, G.R. Upchurch Jr, and T.W. Wakefield. 2004. Deep vein thrombosis resolution is modulated by monocyte CXCR2-mediated activity in a mouse model. *Arterioscler. Thromb. Vasc. Biol.* 24:1130-1137.

Herbert, C.B., C. Nagaswami, G.D. Bittner, J.A. Hubbell, and J.W. Weisel. 1998. Effects of fibrin micromorphology on neurite growth from dorsal root ganglia cultured in three-dimensional fibrin gels. *J. Biomed. Mater. Res.* 40:551-559.

Hermann, M., S. Kapiotis, R. Hofbauer, M. Exner, C. Seelos, I. Held, and B. Gmeiner. 1999. Salicylate inhibits LDL oxidation initiated by superoxide/nitric oxide radicals. *FEBS Lett.* 445:212-214.

Herrington, D.M., E. Vittinghoff, F. Lin, J. Fong, F. Harris, D. Hunninghake, V. Bittner, H.G. Schrott, R.S. Blumenthal, R. Levy, and HERS Study Group. 2002. Statin therapy, cardiovascular events, and total mortality in the Heart and Estrogen/Progestin Replacement Study (HERS). *Circulation* 105:2962-2967.

Herzog, J., Y. Maekawa, T.P. Cirrito, B.S. Illian, and E.R. Unanue. 2005. Activated antigen-presenting cells select and present chemically modified peptides recognized by unique CD4 T cells. *Proc. Natl. Acad. Sci. U. S. A.* 102:7928-7933.

Hewat, E.A., L. Tranqui, and R.H. Wade. 1983. Electron microscope structural study of modified fibrin and a related modified fibrinogen aggregate. *J. Mol. Biol.* 170:203-222.

Hodara, R., E.H. Norris, B.I. Giasson, A.J. Mishizen-Eberz, D.R. Lynch, V.M. Lee, and H. Ischiropoulos. 2004. Functional consequences of alpha-synuclein tyrosine nitration: diminished binding to lipid vesicles and increased fibril formation. *J. Biol. Chem.* 279:47746-47753.

Hoylaerts, M., D.C. Rijken, H.R. Lijnen, and D. Collen. 1982. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J. Biol. Chem.* 257:2912-2919.

Hrachovinova, I., B. Cambien, A. Hafezi-Moghadam, J. Kappelmayer, R.T. Camphausen, A. Widom, L. Xia, H.H. Kazazian Jr, R.G. Schaub, R.P. McEver, and D.D. Wagner. 2003. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat. Med.* 9:1020-1025.

Huang, S., Z. Cao, and E.W. Davie. 1993. The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen. *Biochem. Biophys. Res. Commun.* 190:488-495.

Hull, R.D., J. Hirsh, C.J. Carter, R.M. Jay, P.E. Dodd, P.A. Ockelford, G. Coates, G.J. Gill, A.G. Turpie, D.J. Doyle, H.R. Buller, and G.E. Raskob. 1983. Pulmonary angiography, ventilation lung scanning, and venography for clinically suspected pulmonary embolism with abnormal perfusion lung scan. *Ann. Intern. Med.* 98:891-899.

Humphries, S.E., A.M. Imam, T.P. Robbins, M. Cook, B. Carritt, C. Ingle, and R. Williamson. 1984. The identification of a DNA polymorphism of the alpha fibrinogen gene, and the regional assignment of the human fibrinogen genes to 4q26-qter. *Hum. Genet.* 68:148-153.

Ignarro, L.J., G.M. Buga, K.S. Wood, R.E. Byrns, and G. Chaudhuri. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U. S. A.* 84:9265-9269.

Inada, Y., B. Hessel, and B. Blomback. 1978. Photooxidation of fibrinogen in the presence of methylene blue and its effect on polymerization. *Biochim. Biophys. Acta* 532:161-170.

Ischiropoulos, H., L. Zhu, and J.S. Beckman. 1992. Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* 298:446-451.

Iwaki, T., M.J. Sandoval-Cooper, M. Paiva, T. Kobayashi, V.A. Ploplis, and F.J. Castellino. 2002. Fibrinogen stabilizes placental-maternal attachment during embryonic development in the mouse. *Am. J. Pathol.* 160:1021-1034.

John, M.A., M.J. Elms, E.J. O'Reilly, D.B. Rylatt, P.G. Bundesen, and C.J. Hillyard. 1990. The simpliRED D dimer test: a novel assay for the detection of crosslinked fibrin degradation products in whole blood. *Thromb. Res.* 58:273-281.

Jy, W., L.L. Horstman, F. Wang, R.C. Duncan, and Y.S. Ahn. 1995. Platelet factor 3 in plasma fractions: its relation to microparticle size and thromboses. *Thromb. Res.* 80:471-482.

Kakkar, V.V., C.T. Howe, C. Flanc, and M.B. Clarke. 1969. Natural history of postoperative deep-vein thrombosis. *Lancet* 2:230-232.

Kallner, A., N. Egberg, A. Yu, R. Merzoug-Djilali, I. Blomberg, G. Jorneskog, and M. Blomback. 2003. Are results of fibrinogen measurements transferable? *Clin. Chem. Lab. Med.* 41:804-808.

Kant, J.A., S.T. Lord, and G.R. Crabtree. 1983. Partial mRNA sequences for human A alpha, B beta, and gamma fibrinogen chains: evolutionary and functional implications. *Proc. Natl. Acad. Sci. U. S. A.* 80:3953-3957.

Kearon, C., E.A. Akl, A.J. Comerota, P. Prandoni, H. Bounameaux, S.Z. Goldhaber, M.E. Nelson, P.S. Wells, M.K. Gould, F. Dentali, M. Crowther, S.R. Kahn, and American College of Chest Physicians. 2012. Antithrombotic therapy for VTE disease: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 141:e419S-94S.

Kearon, C., J.S. Ginsberg, J.A. Julian, J. Douketis, S. Solymoss, P. Ockelford, S. Jackson, A.G. Turpie, B. MacKinnon, J. Hirsh, M. Gent, and Fixed-Dose Heparin (FIDO) Investigators. 2006. Comparison of fixed-dose weight-adjusted unfractionated heparin and low-molecular-weight heparin for acute treatment of venous thromboembolism. *JAMA* 296:935-942.

Killewich, L.A., G.R. Bedford, K.W. Beach, and D.E. Strandness Jr. 1989. Diagnosis of deep venous thrombosis. A prospective study comparing duplex scanning to contrast venography. *Circulation* 79:810-814.

Klebanoff, S.J., and C.C. Shepard. 1984. Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on Mycobacterium leprae. *Infect. Immun.* 44:534-536.

Klug, D., J. Rabani, and I. Fridovich. 1972. A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. *J. Biol. Chem.* 247:4839-4842.

Kniffin, W.D.,Jr, J.A. Baron, J. Barrett, J.D. Birkmeyer, and F.A. Anderson Jr. 1994. The epidemiology of diagnosed pulmonary embolism and deep venous thrombosis in the elderly. *Arch. Intern. Med.* 154:861-866.

Kollman, J.M., L. Pandi, M.R. Sawaya, M. Riley, and R.F. Doolittle. 2009. Crystal structure of human fibrinogen. *Biochemistry* 48:3877-3886.

Konings, J., J.W. Govers-Riemslag, H. Philippou, N.J. Mutch, J.I. Borissoff, P. Allan, S. Mohan, G. Tans, H. Ten Cate, and R.A. Ariens. 2011. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood* 118:3942-3951.

KOPEC, M., E. TEISSEYRE, and G. DUDEK-WOJCIECHOUSKA. 1973. Studies on the "double D" fragment from stabilised bovine fibrin. *Thromb. Res.* 2:283.

Koster, T., F.R. Rosendaal, P.H. Reitsma, P.A. van der Velden, E. Briet, and J.P. Vandenbroucke. 1994. 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. Haemost.* 71:719-722.

Krakow, W., G.F. Endres, B.M. Siegel, and H.A. Scheraga. 1972. An electron microscopic investigation of the polymerization of bovine fibrin monomer. *J. Mol. Biol.* 71:95-103.

Kucher, N., H.P. Kohler, T. Dornhofer, D. Wallmann, and B. Lammle. 2003a. Accuracy of D-dimer/fibrinogen ratio to predict pulmonary embolism: a prospective diagnostic study. *J. Thromb. Haemost.* 1:708-713.

Kucher, N., V. Schroeder, and H.P. Kohler. 2003b. 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. Haemost.* 90:434-438.

Kuller, L.H., R. Tracy, W. Belloso, S. De Wit, F. Drummond, H.C. Lane, B. Ledergerber, J. Lundgren, J. Neuhaus, D. Nixon, N.I. Paton, J.D. Neaton, and INSIGHT SMART Study Group. 2008. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med.* 5:e203.

Lane, D.A., I.R. MacGregor, M. VanRoss, G. Cella, and V.V. Kakkar. 1979. Molecular weight dependence of the anticoagulant properties of heparin: intravenous and subcutaneous administration of fractionated heparins to man. *Thromb. Res.* 16:651-662.

Langer, B.G., J.W. Weisel, P.A. Dinauer, C. Nagaswami, and W.R. Bell. 1988. Deglycosylation of fibrinogen accelerates polymerization and increases lateral aggregation of fibrin fibers. *J. Biol. Chem.* 263:15056-15063.

Leffell, M.S., and J.K. Spitznagel. 1974. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. *Infect. Immun.* 10:1241-1249.

Lensing, A.W., P. Prandoni, H.R. Buller, D. Casara, A. Cogo, and J.W. ten Cate. 1990. Lower extremity venography with iohexol: results and complications. *Radiology* 177:503-505.

Levine, M.N., M. Gent, J. Hirsh, A. Arnold, M.D. Goodyear, W. Hryniuk, and S. De Pauw. 1988. The thrombogenic effect of anticancer drug therapy in women with stage II breast cancer. *N. Engl. J. Med.* 318:404-407.

Lisman, T., P.G. de Groot, J.C. Meijers, and F.R. Rosendaal. 2005. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood* 105:1102-1105.

Litvinov, R.I., O.V. Gorkun, D.K. Galanakis, S. Yakovlev, L. Medved, H. Shuman, and J.W. Weisel. 2007. Polymerization of fibrin: Direct observation and quantification of individual B:b knob-hole interactions. *Blood* 109:130-138.

Lo, S.K., A. Cheung, Q. Zheng, and R.L. Silverstein. 1995. Induction of tissue factor on monocytes by adhesion to endothelial cells. *J. Immunol.* 154:4768-4777.

Lorand, L. 1951. 'Fibrino-peptide'; new aspects of the fibrinogen-fibrin transformation. *Nature* 167:992-993.

Lorand, L., and A. Jacobsen. 1962. Accelerated lysis of blood clots. *Nature* 195:911-912.

Lymar, S.,V., and J. Hurst K. 1995. Rapid reaction between peroxonitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* 117:8867-8868.

Machlus, K.R., J.C. Cardenas, F.C. Church, and A.S. Wolberg. 2011. Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis in mice. *Blood* 117:4953-4963.

Magnusson, S., L. Sottrup-Jensen, T.E. Petersen, H.R. Morris, and A. Dell. 1974. Primary structure of the vitamin K-dependent part of prothrombin. *FEBS Lett.* 44:189-193.

Marguerie, G.A., E.F. Plow, and T.S. Edgington. 1979. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 254:5357-5363.

Martel, N., J. Lee, and P.S. Wells. 2005. Risk for heparin-induced thrombocytopenia with unfractionated and low-molecular-weight heparin thromboprophylaxis: a meta-analysis. *Blood* 106:2710-2715.

Martinez, M., A. Cuker, A. Mills, R. Lightfoot, Y. Fan, W.H. Tang, S.L. Hazen, and H. Ischiropoulos. 2012. Nitrated fibrinogen is a biomarker of oxidative stress in venous thromboembolism. *Free Radic. Biol. Med.* 53:230-236.

McCord, J.M., and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244:6049-6055.

McCord, J.M., and I. Fridovich. 1968. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* 243:5753-5760.

McGuinness, C.L., J. Humphries, M. Waltham, K.G. Burnand, M. Collins, and A. Smith. 2001. Recruitment of labelled monocytes by experimental venous thrombi. *Thromb. Haemost.* 85:1018-1024.

Medved, L.V., O.V. Gorkun, and P.L. Privalov. 1983. Structural organization of C-terminal parts of fibrinogen A alpha-chains. *FEBS Lett.* 160:291-295.

Meh, D.A., K.R. Siebenlist, S.O. Brennan, T. Holyst, and M.W. Mosesson. 2001. The amino acid sequence in fibrin responsible for high affinity thrombin binding. *Thromb. Haemost.* 85:470-474.

Mitchell, A.M., K.E. Nordenholz, and J.A. Kline. 2008. Tandem measurement of D-dimer and myeloperoxidase or C-reactive protein to effectively screen for pulmonary embolism in the emergency department. *Acad. Emerg. Med.* 15:800-805.

Monreal, M., E. Lafoz, A. Olive, L. del Rio, and C. Vedia. 1994. Comparison of subcutaneous unfractionated heparin with a low molecular weight heparin (Fragmin) in patients with venous thromboembolism and contraindications to coumarin. *Thromb. Haemost.* 71:7-11.

Mos, I.C., F.A. Klok, L.J. Kroft, A. DE Roos, O.M. Dekkers, and M.V. Huisman. 2009. Safety of ruling out acute pulmonary embolism by normal computed tomography pulmonary angiography in patients with an indication for computed tomography: systematic review and meta-analysis. *J. Thromb. Haemost.* 7:1491-1498.

Moser, K.M., P.F. Fedullo, J.K. LitteJohn, and R. Crawford. 1994. Frequent asymptomatic pulmonary embolism in patients with deep venous thrombosis. *JAMA* 271:223-225.

Moser, K.M., and J.R. LeMoine. 1981. Is embolic risk conditioned by location of deep venous thrombosis? *Ann. Intern. Med.* 94:439-444.

Mosesson, M.W., J.P. DiOrio, M.F. Muller, J.R. Shainoff, K.R. Siebenlist, D.L. Amrani, G.A. Homandberg, J. Soria, C. Soria, and M. Samama. 1987. Studies on the ultrastructure of fibrin lacking fibrinopeptide B (beta-fibrin). *Blood* 69:1073-1081.

Mosesson, M.W., J.S. Finlayson, and R.A. Umfleet. 1972. Human fibrinogen heterogeneities. 3. Identification of chain variants. *J. Biol. Chem.* 247:5223-5227.

Myers, D.,Jr, D. Farris, A. Hawley, S. Wrobleski, A. Chapman, L. Stoolman, R. Knibbs, R. Strieter, and T. Wakefield. 2002. Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis. *J. Surg. Res.* 108:212-221.

Nakamura, S., S. Iwanaga, and T. Suzuki. 1975. On the activation of bovine plasma factor XIII. Amino acid sequence of the peptide released by thrombin and the terminal residues of the subunit polypeptides. *J. Biochem.* 78:1247-1266.

Nelb, G.W., C. Gerth, and J.D. Ferry. 1976. Rheology of fibrin clots. III. Shear creep and creep recovery of fine ligated and coarse unligated closts. *Biophys. Chem.* 5:377-387.

Neyman, M., J. Gewirtz, and M. Poncz. 2008. Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots. *Blood* 112:1101-1108.

Nichols, B.A., D.F. Bainton, and M.G. Farquhar. 1971. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J. Cell Biol.* 50:498-515.

Nordenholz, K.E., A.M. Mitchell, and J.A. Kline. 2008. 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.

Nossel, H.L., I. Yudelman, R.E. Canfield, V.P. Butler Jr, K. Spanondis, G.D. Wilner, and G.D. Qureshi. 1974. Measurement of fibrinopeptide A in human blood. *J. Clin. Invest.* 54:43-53.

Olaisen, B., P. Teisberg, and T. Gedde-Dahl Jr. 1982. Fibrinogen gamma chain locus is on chromosome 4 in man. *Hum. Genet.* 61:24-26.

Pabinger, I., S. Brucker, P.A. Kyrle, B. Schneider, H.C. Korninger, H. Niessner, and K. Lechner. 1992. 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.

Palabrica, T., R. Lobb, B.C. Furie, M. Aronovitz, C. Benjamin, Y.M. Hsu, S.A. Sajer, and B. Furie. 1992. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 359:848-851.

Palmer, R.M., A.G. Ferrige, and S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526.

Parastatidis, I., L. Thomson, A. Burke, I. Chernysh, C. Nagaswami, J. Visser, S. Stamer, D.C. Liebler, G. Koliakos, H.F. Heijnen, G.A. Fitzgerald, J.W. Weisel, and H. Ischiropoulos. 2008. Fibrinogen beta-chain tyrosine nitration is a prothrombotic risk factor. *J. Biol. Chem.* 283:33846-33853.

Parastatidis, I., L. Thomson, D.M. Fries, R.E. Moore, J. Tohyama, X. Fu, S.L. Hazen, H.F. Heijnen, M.K. Dennehy, D.C. Liebler, D.J. Rader, and H. Ischiropoulos. 2007. Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. *Circ. Res.* 101:368-376.

Parmar, K.M., H.B. Larman, G. Dai, Y. Zhang, E.T. Wang, S.N. Moorthy, J.R. Kratz, Z. Lin, M.K. Jain, M.A. Gimbrone Jr, and G. Garcia-Cardena. 2006. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J. Clin. Invest.* 116:49-58.

Perrier, A., S. Desmarais, M.J. Miron, P. de Moerloose, R. Lepage, D. Slosman, D. Didier, P.F. Unger, J.V. Patenaude, and H. Bounameaux. 1999. Non-invasive diagnosis of venous thromboembolism in outpatients. *Lancet* 353:190-195.

Perrin, J., L. Morlon, C. Vigneron, and M. Marchand-Arvier. 2008. Influence of polymorphonuclear leukocytes on the plasma clot formation as evaluated by thromboelastometry (ROTEM). *Thromb. Res.* 121:647-652.

Pieters, M., N. Covic, F.H. van der Westhuizen, C. Nagaswami, Y. Baras, D. Toit Loots, J.C. Jerling, D. Elgar, K.S. Edmondson, D.G. van Zyl, P. Rheeder, and J.W. Weisel. 2008. Glycaemic control improves fibrin network characteristics in type 2 diabetes - a purified fibrinogen model. *Thromb. Haemost.* 99:691-700.

Pignatelli, B., C.Q. Li, P. Boffetta, Q. Chen, W. Ahrens, F. Nyberg, A. Mukeria, I. Bruske-Hohlfeld, C. Fortes, V. Constantinescu, H. Ischiropoulos, and H. Ohshima. 2001. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res.* 61:778-784.

Pratt, K.P., H.C. Cote, D.W. Chung, R.E. Stenkamp, and E.W. Davie. 1997. The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal gamma chain fragment complexed with the peptide Gly-Pro-Arg-Pro. *Proc. Natl. Acad. Sci. U. S. A.* 94:7176-7181.

Prutz, W.A., H. Monig, J. Butler, and E.J. Land. 1985. Reactions of nitrogen dioxide in aqueous model systems: oxidation of tyrosine units in peptides and proteins. *Arch. Biochem. Biophys.* 243:125-134.

Pryce, D.M., and B.E. Heard. 1956. The distribution of experimental pulmonary emboli in the rabbit. *J. Pathol. Bacteriol.* 71:15-25.

Rajkumar, S.V., E. Blood, D. Vesole, R. Fonseca, P.R. Greipp, and Eastern Cooperative Oncology Group. 2006. Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. *J. Clin. Oncol.* 24:431-436.

Rakoczi, I., B. Wiman, and D. Collen. 1978. On the biological significance of the specific interaction between fibrin, plasminogen and antiplasmin. *Biochim. Biophys. Acta* 540:295-300.

Rathbun, S. 2009. Cardiology patient pages. The Surgeon General's call to action to prevent deep vein thrombosis and pulmonary embolism. *Circulation* 119:e480-2.

Reitsma, P.H., and F.R. Rosendaal. 2004. Activation of innate immunity in patients with venous thrombosis: the Leiden Thrombophilia Study. *J. Thromb. Haemost.* 2:619-622.

Rivers, R.P., W.E. Hathaway, and W.L. Weston. 1975. The endotoxin-induced coagulant activity of human monocytes. *Br. J. Haematol.* 30:311-316.

Robbins, K.C., L. Summaria, B. Hsieh, and R.J. Shah. 1967. The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. *J. Biol. Chem.* 242:2333-2342.

Roberts, W.W., O. Kramer, R.W. Rosser, F.H. Nestler, and J.D. Ferry. 1974. Rheology of fibrin clots. I. Dynamic viscoelastic properties and fluid permeation. *Biophys. Chem.* 1:152-160.

Ruckley, C.V., J.D. Cash, and P.C. Das. 1970. Fibrinogen-fibrin degradation products in venous thromboembolic disease. *Br. J. Surg.* 57:860-861.

Ruiz, F.A., C.R. Lea, E. Oldfield, and R. Docampo. 2004. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J. Biol. Chem.* 279:44250-44257.

Ryan, E.A., L.F. Mockros, J.W. Weisel, and L. Lorand. 1999. Structural origins of fibrin clot rheology. *Biophys. J.* 77:2813-2826.

Salomon, O., D.M. Steinberg, A. Zivelin, S. Gitel, R. Dardik, N. Rosenberg, S. Berliner, A. Inbal, A. Many, A. Lubetsky, D. Varon, U. Martinowitz, and U. Seligsohn. 1999. Single and combined prothrombotic factors in patients with idiopathic venous thromboembolism: prevalence and risk assessment. *Arterioscler. Thromb. Vasc. Biol.* 19:511-518.

Santos, C.X., M.G. Bonini, and O. Augusto. 2000. Role of the carbonate radical anion in tyrosine nitration and hydroxylation by peroxynitrite. *Arch. Biochem. Biophys.* 377:146-152.

Sauls, D.L., E. Lockhart, M.E. Warren, A. Lenkowski, S.E. Wilhelm, and M. Hoffman. 2006. Modification of fibrinogen by homocysteine thiolactone increases resistance to fibrinolysis: a potential mechanism of the thrombotic tendency in hyperhomocysteinemia. *Biochemistry* 45:2480-2487.

Schaub, R.G., C.A. Simmons, M.H. Koets, P.J. Romano 2nd, and G.J. Stewart. 1984. Early events in the formation of a venous thrombus following local trauma and stasis. *Lab. Invest.* 51:218-224.

Schielen, W.J., H.P. Adams, K. van Leuven, M. Voskuilen, G.I. Tesser, and W. Nieuwenhuizen. 1991a. The sequence gamma-(312-324) is a fibrin-specific epitope. *Blood* 77:2169-2173.

Schielen, W.J., H.P. Adams, M. Voskuilen, G.I. Tesser, and W. Nieuwenhuizen. 1991b. The sequence A alpha-(154-159) of fibrinogen is capable of accelerating the t-PA catalysed activation of plasminogen. *Blood Coagul. Fibrinolysis* 2:465-470.

Schulman, S., P. Lindmarker, M. Holmstrom, G. Larfars, A. Carlsson, P. Nicol, E. Svensson, B. Ljungberg, S. Viering, S. Nordlander, B. Leijd, K. Jahed, M. Hjorth, O. Linder, and M. Beckman. 2006. 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. Haemost.* 4:734-742.

Schwartz, M.L., S.V. Pizzo, R.L. Hill, and P.A. McKee. 1971. The subunit structures of human plasma and platelet factor XIII (fibrin-stabilizing factor). *J. Biol. Chem.* 246:5851-5854.

Sevitt, S. 1974. The structure and growth of valve-pocket thrombi in femoral veins. *J. Clin. Pathol.* 27:517-528.

Shainoff, J.R., and B.N. Dardik. 1983. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. *Ann. N. Y. Acad. Sci.* 408:254-268.

Shimizu, A., Y. Saito, and Y. Inada. 1986. Distinctive role of histidine-16 of the B beta chain of fibrinogen in the end-to-end association of fibrin. *Proc. Natl. Acad. Sci. U. S. A.* 83:591-593.

Shishehbor, M.H., R.J. Aviles, M.L. Brennan, X. Fu, M. Goormastic, G.L. Pearce, N. Gokce, J.F. Keaney Jr, M.S. Penn, D.L. Sprecher, J.A. Vita, and S.L. Hazen. 2003. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* 289:1675-1680.

Silvain, J., J. Collet, C. Nagaswami, F. Beygui, K.E. Edmondson, A. Bellemain-Appaix, G. Cayla, A. Pena, D. Brugier, O. Barthelemy, G. Montalescot, and J.W. Weisel. 2011. Composition of coronary thrombus in acute myocardial infarction. *Journal of the American College of Cardiology* 57:1359-1367.

Silverstein, M.D., J.A. Heit, D.N. Mohr, T.M. Petterson, W.M. O'Fallon, and L.J. Melton 3rd. 1998. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. *Arch. Intern. Med.* 158:585-593.

Smith, S.A., and J.H. Morrissey. 2008. Polyphosphate enhances fibrin clot structure. *Blood* 112:2810-2816.

Sobel, J.H., and M.A. Gawinowicz. 1996. Identification of the alpha chain lysine donor sites involved in factor XIIIa fibrin cross-linking. *J. Biol. Chem.* 271:19288-19297.

Sohn, H.Y., F. Krotz, S. Zahler, T. Gloe, M. Keller, K. Theisen, T.M. Schiele, V. Klauss, and U. Pohl. 2003. Crucial role of local peroxynitrite formation in neutrophil-induced endothelial cell activation. *Cardiovasc. Res.* 57:804-815.

Sorensen, H.T., E. Horvath-Puho, T.L. Lash, C.F. Christiansen, R. Pesavento, L. Pedersen, J.A. Baron, and P. Prandoni. 2011. Heart disease may be a risk factor for pulmonary embolism without peripheral deep venous thrombosis. *Circulation* 124:1435-1441.

Sorensen, H.T., E. Horvath-Puho, K.K. Sogaard, S. Christensen, S.P. Johnsen, R.W. Thomsen, P. Prandoni, and J.A. Baron. 2009. Arterial cardiovascular events, statins, low-dose aspirin and subsequent risk of venous thromboembolism: a population-based case-control study. *J. Thromb. Haemost.* 7:521-528.

Souza, J.M., E. Daikhin, M. Yudkoff, C.S. Raman, and H. Ischiropoulos. 1999. Factors determining the selectivity of protein tyrosine nitration. *Arch. Biochem. Biophys.* 371:169-178.

Spraggon, G., S.J. Everse, and R.F. Doolittle. 1997. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature* 389:455-462.

Stein, T.P., M.J. Leskiw, and H.W. Wallace. 1978. Measurement of half-life human plasma fibrinogen. *Am. J. Physiol.* 234:D504-10.

Stewart, G.J., W.G. Ritchie, and P.R. Lynch. 1974. Venous endothelial damage produced by massive sticking and emigration of leukocytes. *Am. J. Pathol.* 74:507-532.

Stief, T.W. 1993. Oxidized fibrin stimulates the activation of pro-urokinase and is the preferential substrate of human plasmin. *Blood Coagul. Fibrinolysis* 4:117-121.

Stolla, M., L. Stefanini, R.C. Roden, M. Chavez, J. Hirsch, T. Greene, T.D. Ouellette, S.F. Maloney, S.L. Diamond, M. Poncz, D.S. Woulfe, and W. Bergmeier. 2011. The kinetics of alphaIIbbeta3 activation determines the size and stability of thrombi in mice: implications for antiplatelet therapy. *Blood* 117:1005-1013.

Szabo, C., and A.L. Salzman. 1995. Endogenous peroxynitrite is involved in the inhibition of mitochondrial respiration in immuno-stimulated J774.2 macrophages. *Biochem. Biophys. Res. Commun.* 209:739-743.

Thomson, L., J. Christie, C. Vadseth, P.N. Lanken, X. Fu, S.L. Hazen, and H. Ischiropoulos. 2007. Identification of immunoglobulins that recognize 3-nitrotyrosine in patients with acute lung injury after major trauma. *Am. J. Respir. Cell Mol. Biol.* 36:152-157.

Thorsen, S., P. Glas-Greenwalt, and T. Astrup. 1972. Differences in the binding to fibrin of urokinase and tissue plasminogen activator. *Thromb. Diath. Haemorrh.* 28:65-74.

Tormene, D., S. Barbar, M. Perlati, B. Brandolin, and P. Simioni. 2009. Sex-related type of venous thromboembolism in patients with and without thrombophilia. *Thromb. Haemost.* 102:177-178.

Tsurupa, G., and L. Medved. 2001. Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) alpha C-domains. *Biochemistry* 40:801-808.

Tsurupa, G., L. Tsonev, and L. Medved. 2002. Structural organization of the fibrin(ogen) alpha C-domain. *Biochemistry* 41:6449-6459.

Uitte de Willige, S., M.C. de Visser, J.J. Houwing-Duistermaat, F.R. Rosendaal, H.L. Vos, and R.M. Bertina. 2005. Genetic variation in the fibrinogen gamma gene increases the risk for deep venous thrombosis by reducing plasma fibrinogen gamma' levels. *Blood* 106:4176-4183.

Undas, A., E. Stepien, P. Rudzinski, and J. Sadowski. 2010. Architecture of a pulmonary thrombus removed during embolectomy in a patient with acute pulmonary embolism. *J. Thorac. Cardiovasc. Surg.* 140:e40-1.

Undas, A., K. Zawilska, M. Ciesla-Dul, A. Lehmann-Kopydlowska, A. Skubiszak, K. Ciepluch, and W. Tracz. 2009. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood* 114:4272-4278.

Vadseth, C., J.M. Souza, L. Thomson, A. Seagraves, C. Nagaswami, T. Scheiner, J. Torbet, G. Vilaire, J.S. Bennett, J.C. Murciano, V. Muzykantov, M.S. Penn, S.L. Hazen, J.W. Weisel, and H. Ischiropoulos. 2004. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J. Biol. Chem.* 279:8820-8826.

van Dalen, C.J., M.W. Whitehouse, C.C. Winterbourn, and A.J. Kettle. 1997. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem. J.* 327 (Pt 2):487-492.

van der Vliet, A., J.P. Eiserich, B. Halliwell, and C.E. Cross. 1997. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* 272:7617-7625.

van Hylckama Vlieg, A., and F.R. Rosendaal. 2003. High levels of fibrinogen are associated with the risk of deep venous thrombosis mainly in the elderly. *J. Thromb. Haemost.* 1:2677-2678.

van Langevelde, K., L.E. Flinterman, A. van Hylckama Vlieg, F.R. Rosendaal, and S.C. Cannegieter. 2012. Broadening the factor V Leiden paradox: pulmonary embolism and deep-vein thrombosis as 2 sides of the spectrum. *Blood* 120:933-946.

Varma, M.R., A.J. Varga, B.S. Knipp, P. Sukheepod, G.R. Upchurch, S.L. Kunkel, T.W. Wakefield, and P.K. Henke. 2003. Neutropenia impairs venous thrombosis resolution in the rat. *J. Vasc. Surg.* 38:1090-1098.

Virchow, R. 1856. Thrombose und Embolie. Gefässentzündung und septische Infektion. *In* Frankfurt am Main: Von Meidinger & Sohn, 219-732.

von Bruhl, M.L., K. Stark, A. Steinhart, S. Chandraratne, I. Konrad, M. Lorenz, A. Khandoga, A. Tirniceriu, R. Coletti, M. Kollnberger, R.A. Byrne, I. Laitinen, A. Walch, A. Brill, S. Pfeiler, D. Manukyan, S. Braun, P. Lange, J. Riegger, J. Ware, A. Eckart, S. Haidari, M. Rudelius, C. Schulz, K. Echtler, V. Brinkmann, M. Schwaiger, K.T. Preissner, D.D. Wagner, N. Mackman, B. Engelmann, and S. Massberg. 2012. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J. Exp. Med.* 209:819-835.

Wakefield, T.W., M.J. Linn, P.K. Henke, A.M. Kadell, C.A. Wilke, S.K. Wrobleski, M. Sarkar, M.D. Burdick, D.D. Myers, and R.M. Strieter. 1999. Neovascularization during venous thrombosis organization: a preliminary study. *J. Vasc. Surg.* 30:885-892.

Wakefield, T.W., R.M. Strieter, C.A. Wilke, A.M. Kadell, S.K. Wrobleski, M.D. Burdick, R. Schmidt, S.L. Kunkel, and L.J. Greenfield. 1995. Venous thrombosis-associated inflammation and attenuation with neutralizing antibodies to cytokines and adhesion molecules. *Arterioscler. Thromb. Vasc. Biol.* 15:258-268.

Wallerath, T., I. Gath, W.E. Aulitzky, J.S. Pollock, H. Kleinert, and U. Forstermann. 1997. Identification of the NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets. *Thromb. Haemost.* 77:163-167.

Wang, W. 2011. Identification of respective lysine donor and glutamine acceptor sites involved in factor XIIIa-catalyzed fibrin alpha chain cross-linking. *J. Biol. Chem.* 286:44952-44964.

Weisel, J.W. 1986. Fibrin assembly. Lateral aggregation and the role of the two pairs of fibrinopeptides. *Biophys. J.* 50:1079-1093.

Weisel, J.W., and C. Nagaswami. 1992. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys. J.* 63:111-128.

Wells, P.S., D.R. Anderson, J. Bormanis, F. Guy, M. Mitchell, L. Gray, C. Clement, K.S. Robinson, and B. Lewandowski. 1997. Value of assessment of pretest probability of deep-vein thrombosis in clinical management. *Lancet* 350:1795-1798.

Whipple, G.H., and S.H. Hurwitz. 1911. Fibrinogen of the Blood as Influenced by the Liver Necrosis of Chloroform Poisoning. *J. Exp. Med.* 13:136-161.

Whitaker, A.N., J.R. McFarlane, E.A. Rowe, K. Lee, and P.P. Masci. 1985. Measurement of autoantibodies against fibrinogen and fibrin degradation products by enzyme-linked immunoassay. *Thromb. Haemost.* 53:80-85.

Wolfenstein-Todel, C., and M.W. Mosesson. 1981. Carboxy-terminal amino acid sequence of a human fibrinogen gamma-chain variant (gamma'). *Biochemistry* 20:6146-6149.

Yang, Z., J.M. Kollman, L. Pandi, and R.F. Doolittle. 2001. Crystal structure of native chicken fibrinogen at 2.7 A resolution. *Biochemistry* 40:12515-12523.

Yee, V.C., K.P. Pratt, H.C. Cote, I.L. Trong, D.W. Chung, E.W. Davie, R.E. Stenkamp, and D.C. Teller. 1997. Crystal structure of a 30 kDa C-terminal fragment from the gamma chain of human fibrinogen. *Structure* 5:125-138.

Zhang, J.Z., and C.M. Redman. 1992. Identification of B beta chain domains involved in human fibrinogen assembly. *J. Biol. Chem.* 267:21727-21732.

Zhu, X.Y., M. Rodriguez-Porcel, M.D. Bentley, A.R. Chade, V. Sica, C. Napoli, N. Caplice, E.L. Ritman, A. Lerman, and L.O. Lerman. 2004. Antioxidant intervention attenuates myocardial neovascularization in hypercholesterolemia. *Circulation* 109:2109-2115.

Zieve, P.D., and H.M. Solomon. 1966. Effect of hematoporphyrin and light on human fibrinogen. *Am. J. Physiol.* 210:1391-1395.

Zouki, C., S.L. Zhang, J.S. Chan, and J.G. Filep. 2001. Peroxynitrite induces integrin-dependent adhesion of human neutrophils to endothelial cells via activation of the Raf-1/MEK/Erk pathway. *FASEB J.* 15:25-27.