CHARACTERIZATION OF γ' FIBRINOGEN AS A CARDIOVASCULAR RISK MARKER

By

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BMI – body mass index

CAD – coronary artery disease

CRP – C-reactive protein

C-terminus – carboxyl terminus

CV – coefficient of variation

CVD – cardiovascular disease

DEAE – diethylaminoethyl

ELISA – enzyme-linked immunosorbent assay

FXIII/FXIIIa – factor XIII / activated factor XIII

HDL – high-density lipoprotein

HRP – horseradish peroxidase

hsCRP – high-sensitivity C-reactive protein

ICAM-1 – intercellular adhesion molecule 1

ICC – intra-class correlation coefficient

IoI – index of individuality

IL-1β – interleukin-1β

IL-6 – interleukin-6

INF-γ – interferon-γ

LDL – low-density lipoprotein

MI – myocardial infarction

NMR – nuclear magnetic resonance

N-terminus – amino terminus

PAR1 – protease-activated receptor 1

PAVE – Periodontitis And Vascular Events

PI3K – phosphatidylinositol 3-kinase

RU – resonance units

SD – standard deviation

SE – standard error

SNPs – single nucleotide polymorphisms

SPR – surface plasmon resonance

SR – serine/arginine-rich

TNF-α – tumor necrosis factor-α

tPA – tissue plasminogen activator

VC – validity coefficient

VCAM-1 – vascular cell adhesion molecule 1

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Abstract

The γ' isoform of fibrinogen is associated with cardiovascular disease (CVD) and may be useful for cardiovascular risk prediction. This isoform is produced through the incorporation of a splice variant of the fibrinogen γ chain into the molecule, and its levels vary between individuals. It has been proposed that γ' fibrinogen may increase the risk of thrombosis through its interactions with thrombin, the major blood coagulation enzyme. In this dissertation, the role of electrostatic interactions in this binding was investigated using surface plasmon resonance technology. Our findings indicate that the binding of exosite II of thrombin to the γ' chain is mediated by the ensemble of negatively-charged residues in the γ' chain carboxyl terminus, and that no single charged residue is absolutely required. As CVD appears to be an inflammatory process, the relationship between γ' fibrinogen and inflammation was explored in a cohort of subjects from the Periodontitis and Vascular Events (PAVE) study. These subjects had prevalent CVD and periodontal disease, a common source of chronic inflammation. In this group, significant correlations were found between γ' fibrinogen and C-reactive protein (CRP), a systemic marker of inflammation, both as a continuous variable and as a dichotomized variable using the "high-risk" classification of CRP > 3 mg/L. Additionally, γ' fibrinogen was associated with the localized inflammation of the gingiva, but was not associated with total fibrinogen levels. To gain further insight into the potential use of γ' fibrinogen as a cardiovascular risk factor, we measured its levels, along with other biochemical risk markers, in a group of 15 healthy individuals over the course of a year. Both the

with-subject and between-subject variabilities of all of the markers were assessed. The variability profile of γ' fibrinogen in this study was very similar to that of low-density lipoprotein cholesterol. High-density lipoprotein cholesterol had the lowest proportion of within-subject variability, making it the most reliable marker measured, while C-reactive protein had the highest proportion of within-subject variance. It was estimated that eight replicate measurements of CRP would be required to achieve the same validity as a single HDL cholesterol measurement. The work presented in this dissertation helps to clarify the role of γ' fibrinogen as a potential risk factor on both a molecular level, by examining its interaction with thrombin, and an epidemiological level, by assessing its relationship with chronic inflammation and the sources of variance in its measurement. The results of this research indicate that this protein has the potential to be a valuable risk marker for CVD and inflammation, but future work will be needed to confirm and extend these findings.

Chapter 1. Introduction

1.1 Fibrinogen Structure and Production

1.1.1 Fibrinogen Structure

Fibrinogen is a fibrous glycoprotein with a mass of about 340,000 Da. It is composed of two each of the three polypeptide chains, termed Aα, Bβ, and γ, with both halves of the dimeric fibrinogen molecule containing one of each type of chain. The nomenclature for the Aα and Bβ chains reflects the fact that small peptides, called fibrinopeptides A and B are cleaved from fibrinogen by thrombin, while there is no cleavage of the γ chain. These six chains are connected by 29 disulfide bonds (1), and oriented with their amino-termini near the center of the protein. Both the Bβ and the γ chain contain an N-linked glycosylation site (Bβ364 and γ52), giving the full protein four oligosaccharide chains of the biantennary type (2).

The predominant structure of the protein is α-helical coiled-coil, with nodular regions at the center of the molecule, where the fibrinopeptides A and B are located, and at the ends, where the globular C-terminal domains of the Bβ and γ reside (Figure 1.1). The gives fibrinogen the appearance, by electron microscopy, of three beads on a string, with the "beads" being the nodular regions at the ends and center, and the "string" representing the rod-like coiled-coil regions in between. The C-terminus of the Aα chains, known as the αC domain, has only been crystallized as a

fragment, so its structure in the intact molecule is not well understood (3). It consists of both an extended and a globular region, with the globular regions of each chain interacting with one another and with the central region of the molecule (4, 5).

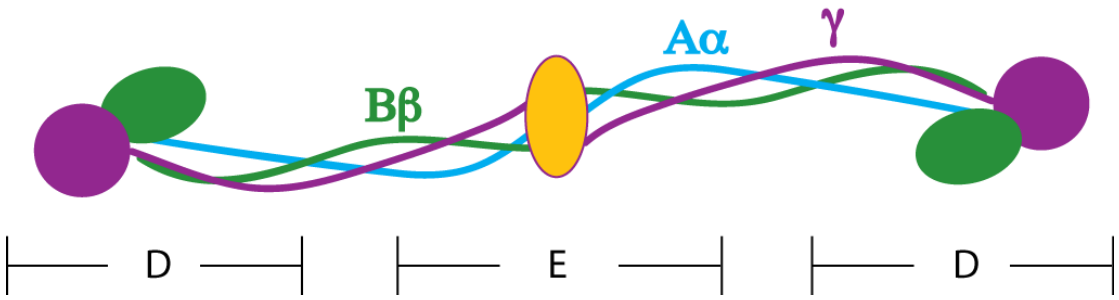

Figure 1.1. Structure of fibrinogen. The Aα chains are shown here in blue, the Bβ chains in green and the γ chains in purple.

Proteolytic fragments produced by the degradation of fibrin clots have contributed to fibrinogen structure terminology. Plasmin, a fibrinolytic enzyme, cleaves fibrinogen in the middle of both coiled-coil regions. This eventually produces three fragments: two D fragments, each containing a C-terminal nodule and about half of one coiled-coil region, and one E fragment with the central region and the remaining coiled-coils on both sides.

1.1.2 Fibrinogen Synthesis, Assembly, and Secretion

The genes for the three polypeptide chains that make up fibrinogen are clustered together on chromosome 4, bands q23-q32 (Figure 1.2) (6). The γ chain gene, *FGG*, is the furthest upstream and is composed of ten exons. The transcript of *FGG* is

processed to produce two polypeptide variants through alternative splicing, known as γA and γ'. This is discussed in greater detail in section 1.4. In the middle of the cluster is the fibrinogen Aα gene, *FGA*, consisting of five exons. Alternate splicing of the sixth intron of this gene produces an Aα chain with an extended C-terminus found in 1-2% of adult proteins (7). *FGB*, the Bβ chain gene, has eight exons and is transcribed in the opposite direction to that of *FGG* and *FGA* upstream. The tight clustering of the fibrinogen genes is unusual in eukaryotic systems, and this arrangement, along with the homologous nature of the genes, indicates that they arose through duplication and divergence of an ancestral gene (6).

Figure 1.2. Arrangement of fibrinogen genes on chromosome 4.

Fibrinogen is synthesized predominantly in the liver at a rate of 1.7 – 5.0 grams per day (8). The mRNA expression for the three chains is coordinately regulated, with hepatocytes producing relatively equal proportions of each transcript (9). While the complete mechanism of this regulation has not been elucidated, it is likely to involve the highly homologous sequences in the 5' untranslated regions of the three genes

containing binding sites for several general and liver-specific transcription factors (10-12).

After transcription, the newly synthesized chains are assembled into the complete fibrinogen molecule in the rough endoplasmic reticulum of hepatocytes. This occurs in a stepwise fashion, with the formation of two-chain complexes, followed by the three-chain half-proteins (Aα, Bβ, γ), which then dimerize to form functional fibrinogen (13, 14). In human hepatocellular carcinoma cells, the availability of the Bβ chain appears to be the rate-limiting factor in fibrinogen assembly (15), and increasing the Bβ chain production in these cells through the transfection of Bβ chain cDNA not only increases fibrinogen assembly and secretion, it stimulates the synthesis of the Aα and γ chains, such that their relative surplus is maintained (16). Elevating the levels of the Aα or γ chains using transfected cDNA also increases the synthesis of all three chains, but to a lesser extent than that seen with the Bβ chain cDNA (17).

Fully assembled fibrinogen is secreted by the liver into the plasma, where it circulates at a concentration of about 2.5 g/L and has a half-life of 3-5 days. While most fibrinogen circulates in plasma, it is also found in platelets, lymph nodes and interstitial fluid. There was some debate about the origin of the fibrinogen stored in platelet α-granules, specifically whether it is synthesized by megakaryocytes, the large, nucleated precursors to platelets, or taken up into platelets from the plasma through endocytosis. However, at this point, evidence points toward integrin αIIbβ3-mediated endocytosis as the main source of platelet fibrinogen (18).

1.2 Fibrinogen and Hemostasis

Fibrinogen plays a vital role in the formation of a blood clot, where it is involved in both primary and secondary hemostasis. This function is so crucial that individuals with a rare congenital absence of fibrinogen, known as afibrinogenemia, cannot form normal blood clots and are at high risk of death from uncontrolled bleeding. Even relatively minor mutations that cause decreased levels of fibrinogen or interfere with fibrinogen function can cause significant bleeding complications.

1.2.1 Primary Hemostasis

Primary hemostasis describes the formation of a platelet plug following vascular injury. Platelets initially adhere to the exposed subendothelium through interactions with extracellular matrix proteins, including the molecule von Willebrand factor, which attaches to collagen and, under conditions of high shear (rapid blood flow through narrow vessels), tethers platelets by binding to their GPIb receptors. When platelets are exposed to collagen, signaling cascades are initiated that cause the platelets to become activated. This activation results in a number of dramatic changes to the platelets, both inside and out. Activated platelets change shape, extending finger-like projections called filopodia, then flattening and spreading into lamellipodia to form a monolayer over the injured area. Activation also causes platelets to release factors that promote the activation and aggregation of nearby platelets. In addition, these platelets activate receptors on their membranes, including the integrin αIIbβ3 that binds to the C-terminal residues of the γ chains of circulating fibrinogen. The result of this process is the production of

a soft plug composed mainly of platelets and fibrinogen, which occurs very quickly after injury, usually within the first minute. The platelet plug formed in primary hemostasis is fragile and requires the actions of secondary hemostasis for stabilization.

1.2.2 Secondary Hemostasis

Secondary hemostasis, also known as blood coagulation, is the process by which a series of enzymes and cofactors are sequentially activated, ultimately resulting in the production of an insoluble fibrin clot. The numerous sequential activation steps involved in this process, much like those in a cellular signaling cascade, allow for considerable amplification of the initial signal. This provides a mechanism by which a relatively large blood clot can be formed very quickly in response to injury.

Historically, the coagulation cascade has been divided into two pathways, the intrinsic pathway and the extrinsic pathway, which converge into a final common pathway (Figure 1.3). It was originally thought that these two pathways were equally important in the initiation of blood coagulation, but it is now recognized that the extrinsic pathway, triggered by tissue factor, is the main physiological pathway for initiation of coagulation, while the intrinsic pathway plays a role mainly in amplification of the cascade.

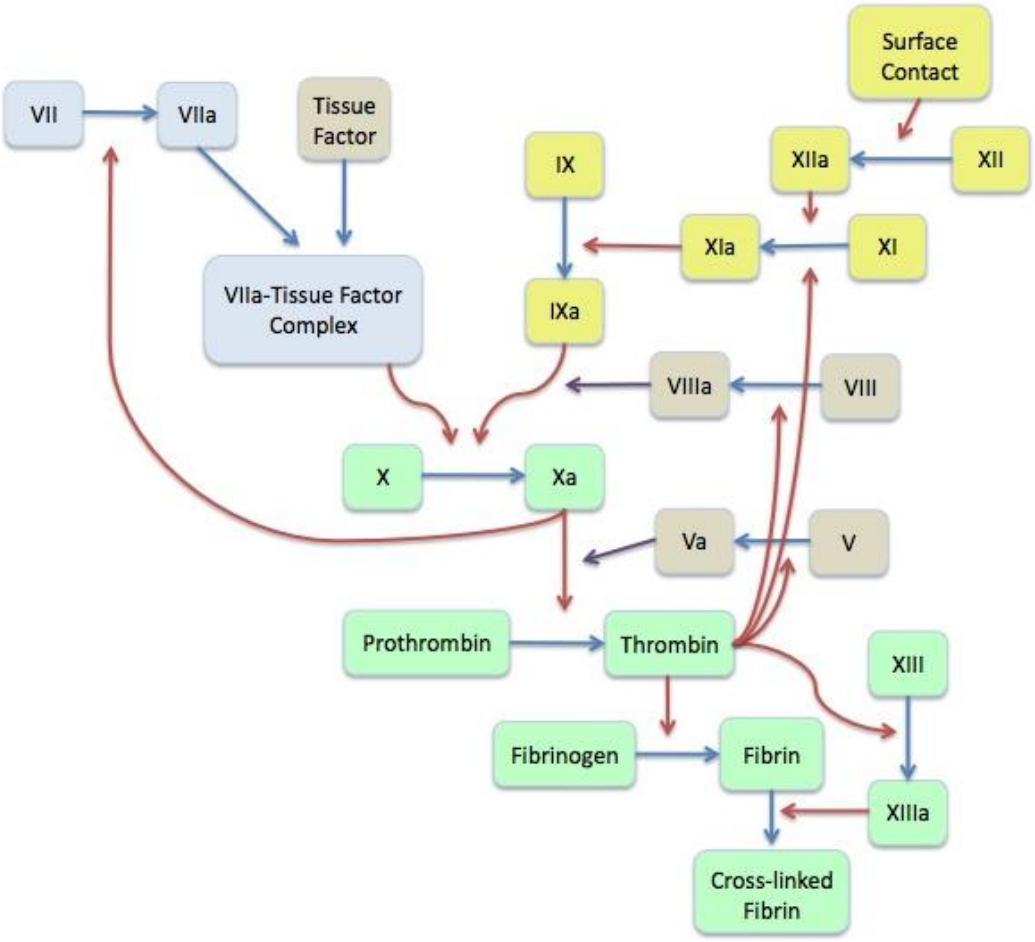

Figure 1.3. The coagulation cascade. The extrinsic/tissue factor pathway is shown in blue boxes, the intrinsic/contact pathway is shown in yellow, and the final common pathway is shown in green. Cofactors are in beige boxes. Blue arrows indicate conversion of a factor to an active form, and red arrows indicate the enzyme(s) involved in this conversion. Purple arrows denote the participation of required cofactors.

The extrinsic pathway, also known as the tissue factor pathway, is activated when blood is exposed to tissue factor. Tissue factor is a transmembrane glycoprotein found in many tissues and is particularly abundant in the walls of blood vessels, but is not expressed on the endothelial lining of the vessels and so does not generally come into contact with normally circulating blood. When blood vessels are injured, tissue factor quickly binds with factor VII from the blood. A small amount of factor VII circulates in its active form, factor VIIa, which can form the factor VIIa-tissue factor complex, also known as the extrinsic Xase complex. This complex activates factor X, which along with its cofactor, factor Va, forms the prothrombinase complex.

The formation of the prothrombinase complex leads to the common pathway of coagulation. As the name suggests, the prothrombinase complex cleaves prothrombin, converting it to thrombin, which plays a central role in hemostasis. Thrombin is responsible for converting soluble fibrinogen to insoluble fibrin, the final product of the coagulation cascade, in a process that will be described in more detail in the next section. Additionally, thrombin is a potent activator of platelets and thus functions in promoting primary hemostasis as well. Factor XIII, the final enzyme of the coagulation cascade, is also activated by thrombin and it acts to stabilize the clot by cross-linking fibrin molecules.

The thrombin formed initially by the tissue factor and common pathways can then act to propagate further thrombin production by way of the intrinsic pathway. The first step in the intrinsic pathway is the activation of factor XII through the actions of

several proteins in conjunction with exposure to a negatively-charged surface. Factor XIIa can then activate factor XI to factor XIa, which, in turn, activates factor IX. Factor IXa, with its cofactor, factor VIIIa, can then form the intrinsic complex Xase, which activates factor X, leading to the common pathway.

The activation of factor XII, the first step in the intrinsic pathway, does not appear to be necessary for normal blood coagulation and may be more involved in inflammatory processes. However, when thrombin is generated through activation of the extrinsic pathway, it can promote further thrombin production through the intrinsic pathway by activating factors XI and VIII. This provides a mechanism to rapidly produce a large thrombin burst in response to injury, promoting swift amplification of both primary and secondary hemostasis, which results in the formation of a stable clot composed mainly of platelets and blood cells held in a fibrin meshwork.

While the robust production of active thrombin is important for hemostasis, uncontrolled thrombin activation would lead to undesirable outcomes such as thrombosis or disseminated intravascular coagulation. To prevent this from happening, thrombin also functions in an anticoagulant capacity by activating protein C. Activated protein C proteolytically inactivates factors Va and VIIIa, cofactors needed for thrombin generation, thus inhibiting both the progression and amplification of the coagulation cascade (19). Thrombin activation of protein C is mediated by thrombomodulin, found on the surface of endothelial cells. Binding to thrombomodulin both prevents thrombin from cleaving its procoagulant substrates

and increases its affinity for protein C by over 1000-fold (20). Thrombin bound to thrombomodulin is also more vulnerable to inactivation by antithrombin.

1.2.3 Conversion of Fibrinogen to Fibrin Clot

Fibrinogen is converted to fibrin through the cleavage of fibrinopeptides A and B from the N-termini of the Aα and Bβ chains by thrombin, a serine protease. Thrombin binding to fibrinogen involves both its catalytic site and a nearby region on the thrombin B chain called exosite I that binds to fibrinogen's E region (21, 22). Fibrinopeptide A appears to generally be cleaved first by thrombin, with the majority of fibrinopeptide B cleavage occurring afterward (23, 24). The release of fibrinopeptide B is substantially increased after fibrin polymerization (25, 26).

When fibrinopeptide A is cleaved from fibrinogen, a binding site 'A' is exposed, which interacts with a site 'a' located in the C-terminal γ chain nodule. Similarly, release of fibrinopeptide B allows binding between the newly exposed 'B' binding site and the corresponding site 'b' in C-terminal region of the β chain. The A:a binding between the central domain of one fibrin molecule with the end of another leads to the aggregation of the monomers into half-staggered protofibrils (Figure1.4). These two-stranded protofibrils can then associate laterally to form fibrin fibers, a process that is enhanced by the removal of the B fibrinopeptides (27) and interactions between the αC domains of adjacent protofibrils (28). It appears that lateral aggregation is limited by the twisting of the fibers, which requires newly

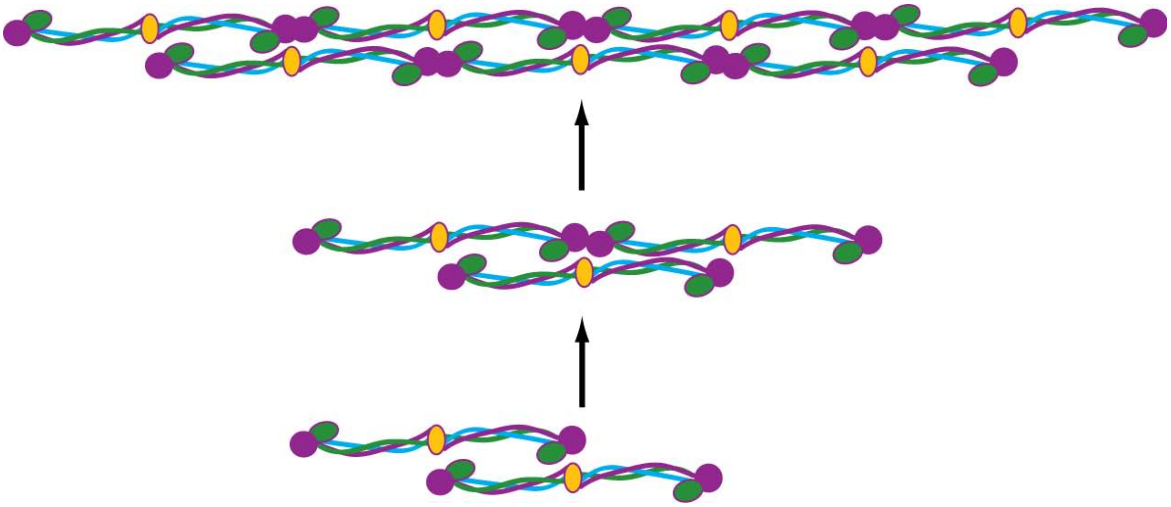

Figure 1.4. Fibrin polymerization. Interactions between the central region of one fibrin monomer and the C-terminal domains of another drive aggregation into protofibrils.

added protofibrils to stretch further as the fiber diameter increases (29). These fibers branch and spread, forming a three-dimensional network that interacts with platelets and red blood cells to produce a functional clot (Figure 1.5).

The fibrin clot is stabilized by the actions of factor XIIIa, a transglutaminase activated by thrombin. Factor XIIIa forms covalent bonds between glutamine and lysine residues on adjacent fibrin monomers. During factor XIIIa crosslinking, bonds are first formed between the γ chains, specifically γGln398/399 and γLys 406, followed more slowly by bonds between αC domains at a number of residues (30). These bonds serve to strengthen the fibrin clot mechanically and to provide resistance to proteolysis.

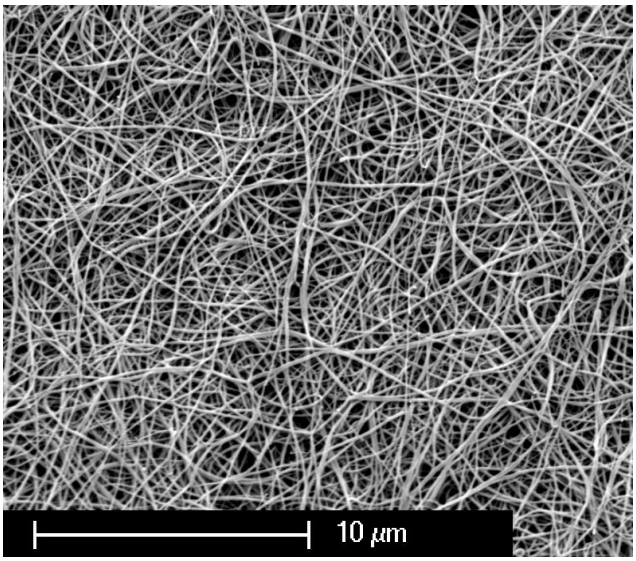

Figure 1.5. Fibrin clot structure. A scanning electron micrograph of a fibrin clot produced with purified fibrinogen and thrombin.

1.2.4 Fibrinolysis

Even as blood clot is being formed, events leading to its eventual destruction are put into motion. While the swift formation of a blood clot is vital to preventing blood loss during injury, it is intended as a temporary fix and coagulation must be carefully regulated to preserve circulation to the tissues and to break down the clot when it is no longer needed. This breakdown is called fibrinolysis.

The main enzyme responsible for fibrinolysis is plasmin, a serine protease. Plasmin circulates in the blood in its inactive zymogen form, plasminogen. Plasminogen binds to lysine and arginine residues on fibrin(ogen) and thus becomes incorporated into a blood clot during coagulation. Activation of plasminogen to plasmin in blood requires the formation of a ternary complex consisting of fibrin, plasminogen and tissue-type plasminogen activator, another serine protease present in endothelial cells and secreted in response to injury. Once activated, plasmin cleaves fibrin at specific proteolytic sites, yielding fibrin degradation

products that have given rise to fibrin(ogen) structural designations, including the D and E regions described earlier.

Much like in the coagulation cascade, positive feedback is an important mechanism during fibrinolysis. As activated plasmin begins to cleave fibrin, C-terminal lysine residues are created that provide more binding sites for plasmin. Plasmin also cleaves tissue-type plasminogen activator, converting it to a more active form, which in turn speeds plasminogen activation, amplifying the process (31).

There are various inhibitors that are also responsible for regulating fibrinolysis. The protease inhibitors α2-antiplasmin and α2-macroglobulin inhibit fibrin degradation through direct inactivation of plasmin. Plasminogen activator inhibitors 1 and 2 function by inactivating tissue-type plasminogen activator. Another fibrinolysis inhibitor, the descriptively named 'thrombin-activatable fibrinolysis inhibitor', acts by binding to plasminogen and cleaving C-terminal lysine and arginine residues from fibrin, impeding this positive feedback mechanism. As one might expect, this inhibitor is activated by thrombin and helps to prevent premature fibrinolysis. Though it does not interact directly with any of the fibrinolytic enzymes, factor XIIIa is an important inhibitor of fibrinolysis as well. It not only crosslinks fibrin molecules in the blood clot, it covalently links α2-antiplasmin to fibrin. Both of these functions significantly reduce fibrinolysis (32).

1.3 Fibrinogen, Inflammation and Cardiovascular Disease

1.3.1 Inflammation in the Development and Progression of Atherosclerosis

In past decades, it was thought that atherosclerosis was simply a disease of passive lipid accumulation, with high cholesterol levels driving the formation of fatty lesions in the arterial wall. Effective lipid-lowering therapies were expected to greatly reduce coronary artery disease (CAD) by the end of the 20th century (33). It is now recognized that inflammation is intimately involved in both the initiation and progression of atherosclerotic cardiovascular disease (CVD) and that, in spite of the important role played by lipids, this disease is better characterized as an inflammatory process than disorder of lipid accumulation (34). Indeed, inflammatory and immune responses are involved in the development of atherosclerotic lesions from their earliest beginnings.

Endothelial cell activation in a blood vessel's innermost layer, called the intima, appears to be the initiating process in the formation of an atherosclerotic lesion. This activation is associated with many traditional cardiovascular risk factors. Accumulation and modification of low-density lipoprotein (LDL) particles in the intima is one of the best known and studied causes of endothelial dysfunction, and hypercholesterolemia, particularly a high LDL level, has been recognized as an important cardiovascular risk factor for decades. Endothelial dysfunction can be caused by other mechanisms as well, such as hyperglycemia, smoking, hypertension, injury, and by bacterial and viral infections (34). It is also worth noting that vessel sites that experience turbulent blood flow, coronary artery branch points, for

example, have been shown to have increased endothelial activation, and that these areas are often the sites of lesion development (35), indicating a mechanical component to this process.

The healthy vascular endothelium generally functions to prevent thrombus formation in flowing blood by providing a 'non-stick' surface to platelets and coagulation factors. However, activation of these endothelial cells through various mechanisms leads to an increase in their expression of inflammatory genes and adhesion molecules. Platelets bind to the endothelium through interaction with these adhesion molecules, including P-selectin and CD41, and this binding occurs very quickly after endothelial activation (36). The interaction between platelets and adhesion proteins may promote further activation of the endothelial cells.

Activated endothelium also expresses adhesion molecules that target leukocytes, particularly vascular-cell adhesion molecule I (VCAM-1). Monocytes and lymphocytes bind to these adhesion proteins and migrate into the arterial intima. Platelets play a role in monocyte recruitment as well, by forming bridges between monocytes and endothelial cells and by secreting chemokines that trigger monocyte arrest on the inflamed endothelium (37). The activated endothelium secretes macrophage colony-stimulating factor, triggering monocytes in the area to differentiate in macrophages. This accumulation of immune cells at a site of endothelial dysfunction is the beginning of an atherosclerotic lesion, or plaque.

Macrophages are one of the most common cell types in the growing plaque. They express a variety of receptors that play a role in lesion development, which include

toll-like and scavenger receptors. Toll-like receptors, which are also present on mast cells, dendritic cells and endothelial cells, generally recognize pathogen-associated molecular patterns, and may be activated by bacterial toxins, heat shock proteins or oxidized LDL (38). Activation of toll-like receptors generates inflammatory responses directly, by triggering a signaling cascade that leads to the expression of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β, and interleukin-6 (IL-6), as well as proteases and reactive oxygen and nitrogen species. Scavenger receptors are responsible for internalizing a variety of particles, including modified LDL. Macrophages take up lipoprotein particles through these scavenger receptors and, when activated, through liquid-phase endocytosis (39). As the macrophage's uptake of cholesterol outpaces the cell's ability to process and remove it, lipid accumulates as cytosolic droplets. This process transforms the cell into a foam cell, the hallmark cell of atherosclerosis, so named for the foamy appearance produced by these droplets. Foam cells add to the inflammatory response by secreting cytokines, reactive oxygen species, proteases and exposing surface tissue factor.

Lymphocytes are another common cell type in an atherosclerotic lesion, with T cells representing about 10% of the cells in a plaque (40). T cells are recruited to the plaque through many of the same chemokines and adhesion molecules that attract monocytes. Once in the intima, T-cells can become activated through interaction with antigen, mainly LDL-derived microparticles (33), presented by macrophages and other cells expressing major histocompatibility (MHC) class II molecules. Activated T cells release pro-inflammatory cytokines, including interferon-γ and

various interleukins, further amplifying the immune response through a variety of mechanisms, such as the interferon-γ induced expression of MHC class II proteins on vascular endothelial and smooth muscle cells (41), and activation of macrophages.

Atherosclerosis is a progressive, chronic disease that often begins early in life. The first grossly visible lesions in the development of atherosclerosis are called fatty streaks. These small yellowish-white patches or streaks are found on the intimal surface of arteries, particularly the aorta and the coronary arteries. Fatty streaks are composed mainly of immune cells, predominantly lipid-laden macrophages and T cells. Additionally, small quantities of lipid may be found in the lesion's smooth muscle cells and in extracellular droplets. These lesions are not only found in adults, but are common in children and infants as well. Fatty streaks are estimated to be present in the aortas of nearly early every child in North America over the age of three (42, 43). Many of these early lesions do not progress to become advanced lesions, but those that do are generally found in areas of the vessel wall subject to mechanical forces that drive progression of the lesions by increasing lipid influx and adaptive intimal thickening (44).

As the lesion progresses, it becomes an atheroma, and there is further accumulation of lipid and recruitment of immune cells by the mechanisms discussed previously. Cytokines secreted by activated macrophages and T cells promote proliferation and migration of smooth muscle cells to the intima from the middle layer of the vessel wall. These smooth muscle cells produce extracellular matrix molecules, which form a fibrous cap surrounding the plaque. There is evidence that some smooth

muscle cells can also transform into foam cells through ingestion of lipids (45). The interior of the plaque contains mainly foam cells and extracellular lipid droplets. In advanced lesions, there is an acellular, lipid-rich, necrotic core likely produced by the recruitment and subsequent death of foam cells (46).

Atherosclerosis can eventually result in the thrombotic occlusion of an artery, potentially causing a heart attack or stroke. Thrombosis is usually caused by plaque rupture due to degradation of the fibrous capsule surrounding the lesion. Inflammatory cytokines, proteases, reactive oxygen species and other molecules produced by immune cells can weaken the cap by reducing collagen production by smooth muscle cells and by breaking down the extracellular matrix (33, 47). Rupture of the plaque's fibrous cap exposes the lipid core, which contains procoagulant proteins such as tissue factor. When the circulating blood comes into contact with the ruptured plaque, thrombus formation is initiated. This can quickly lead to occlusion of the artery and loss of blood flow to the tissue it serves, causing injury or death.

1.3.2 Inflammation as a Cardiovascular Risk Factor

Inflammation plays a major role in the pathogenesis of atherosclerosis, so it is perhaps unsurprising that chronic inflammatory conditions are increasingly recognized as contributors to cardiovascular risk. Many autoimmune diseases are associated with an elevated risk of CVD. Individuals with rheumatoid arthritis, one of the most common and well-studied inflammatory autoimmune disease, are known to have an 2- to 3-fold increased risk of heart attack and a 2-fold increased

mortality rate, with 40% of the deaths attributable to CAD (48). Psoriasis, psoriatic arthritis, and inflammatory bowel disease are also associated with increased risk of CVD (49). Often, the increased risk in individuals with autoimmune inflammatory diseases cannot simply be attributed to corresponding increases in traditional cardiovascular risk factors (48), a finding which strengthens the hypothesis that the elevated risk is due mainly to the systemic inflammation that characterizes these conditions.

There is some evidence that chronic infections, particularly periodontal infections, may also contribute to atherosclerotic CVD. These infections represent one of the most common sources of chronic inflammation in humans, with gingivitis present in more that 80% of adults in the US (50), and severe periodontitis estimated to affect 5-15% of individuals in the US and 5-20% of adult populations globally (51-53). As pathogenic bacteria accumulate and form dental plaques, there is an aggressive immune response characterized by the infiltration of large numbers of lymphocytes and monocytes to the affected gum tissue. These cells become activated and secrete proinflammatory cytokines, both locally and systemically, as well as reactive oxygen species and proteases that breakdown the connective tissue surrounding the teeth. Thus, the main features of the disease, pocketing of the gums and tooth attachment loss, are not actually caused directly by the infectious bacteria but by the persistent inflammatory response. This heightened level of inflammation could contribute to the development of atherosclerosis. There is a great deal of epidemiologic data linking periodontitis and CVD, though it has been difficult to determine whether

periodontal infections play a causative role in atherosclerosis due to the overlap in risk factors for the two diseases (54, 55).

As our understanding of the inflammatory nature of cardiovascular disease has grown, there has been a push to indentify markers of inflammation that can be used to predict cardiovascular risk. In fact, many of what we consider to be important traditional cardiovascular risk factors, such as high cholesterol levels, elevated blood pressure, diabetes, obesity and smoking, may raise risk by promoting systemic or vascular inflammation. If, in fact, the impact of these traditional risk factors on cardiovascular health is mediated by inflammatory mechanisms, then markers of this process may have more predictive power than the risk factors themselves.

Many inflammatory molecules have been studied as potential indicators of CVD risk. These include the cytokines TNF-α and IL-6, circulating adhesion proteins, and so-called "acute-phase" proteins produced in the liver that increase or decrease in response to inflammatory stimuli (56). Fibrinogen, serum amyloid A, and C-reactive protein (CRP) are among the acute-phase proteins that increase during inflammation that have been studied as CVD risk markers. Of the many markers that have been investigated, fibrinogen and CRP are two of the best studied and provide similar risk information (57).

CRP is the marker on which most recent studies of inflammation and CVD risk have focused. For more than 80 years, CRP has been recognized as an acute phase reactant that increases dramatically in response to inflammatory stimuli. Its

synthesis in the liver is stimulated by cytokines, mainly IL-6. CRP correlates with many traditional CVD risk factors, especially abdominal adiposity (58), and in many studies, a CRP level above 3 mg/L have been shown to be an independent CVD risk predictor (59). Whether CRP plays a causal role in the pathogenesis of CVD or is simply a bystander marker remains a subject of ongoing debate, with several lines of evidence supporting each position (60, 61).

Although CRP has been shown to be an important risk marker for heart disease in numerous large epidemiological studies, its clinical use in individual risk prediction is also a subject of debate. The results from JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) indicate that statin use reduces cardiovascular events in individuals with elevated CRP (≥2 mg/L) but without elevated cholesterol levels (62), but the interpretation of the study has been controversial. A 2009 systematic review and meta-analysis determined that while CRP can improve risk stratification among individuals currently classified as intermediate risk, the viability of using CRP in addition to traditional risk factors is uncertain (63).

One of the obstacles to its clinical use is that CRP levels can fluctuate quite dramatically due to any infection, injury or inflammation, so any elevation in CRP is not necessarily specific to ongoing vascular disease (64). In addition, there can be a great deal of within-individual variation in measured CRP levels, which can necessitate taking multiple measurements in order to determine a patient's baseline

(65, 66). Nonetheless, CRP continues to be a focus of research and clinical testing, as researchers work to improve cardiovascular risk prediction.

1.3.3 Fibrinogen in Inflammation and Cardiovascular Disease

Fibrinogen represents an important link between inflammation and cardiovascular disease. Like CRP, it is produced in the liver and upregulated in response to inflammation through IL-6 signaling. Unlike CRP, however, which can jump up to 1000-fold during the acute phase, increases in fibrinogen are generally 2 to 20-fold (67). Higher fibrinogen levels are associated numerous CVD risk factors, including smoking, obesity, high cholesterol, hypertension and oral contraception (68, 69).

There is a wealth of evidence linking plasma fibrinogen levels to CVD. The Gothenburg and Framingham studies found that fibrinogen levels were strongly associated with heart attack and stroke (70, 71). Other large epidemiological studies have identified fibrinogen as a risk factor for coronary heart disease, sudden cardiac death, and peripheral artery disease, caused by atherosclerosis in peripheral arteries (72). In addition to being a marker of prevalent CVD, fibrinogen has also been shown to be predictive of future coronary events in individuals with CAD and an independent predictor of mortality (73).

Several studies have investigated whether plasma fibrinogen is increased in early atherosclerosis, not just clinically apparent CVD. In this research, fibrinogen was often significantly correlated with carotid artery intima media thickness, a commonly used measure of pre-clinical vascular disease, and with the presence of

carotid artery plaques (74-78). Interestingly, the association with carotid plaques seemed to be stronger than that with intima media thickness in the studies that examined both (76, 77).

It is not surprising that fibrinogen is associated with CVD, given its role in coagulation and inflammation, but there is still some uncertainty as to whether this association is causal. Evidence that fibrinogen is involved the progression of atherosclerosis can be found by examining the contribution of fibrinogen and fibrin to plaque development. As an atherosclerotic plaque is forming, fibrinogen can enter the intima and be converted to fibrin and, as it is broken down by proteases, fibrin degradation products. Many plaques that show no signs of rupture contain large quantities of fibrin (79). Fibrin(ogen) and fibrin(ogen) degradation products appear to promote the growth of a plaque through a variety of mechanisms, such as increasing smooth muscle cell migration and proliferation, altering endothelial permeability, and promoting extracellular accumulation of LDL (80). Additionally, fibrinogen increases leukocyte and platelet adhesion to plaques through interaction with intracellular adhesion molecule-1 (ICAM-1) on leukocytes and endothelial cells and the integrin Mac-1 on leukocytes (72).

A case could also be made for a causal role for fibrinogen in the development of CVD based on its influence on blood viscosity. Blood viscosity is a measure of the blood's "thickness," or resistance to flow, and is a strong predictor of cardiovascular events (81). It is determined by a number of factors, including hematocrit, which is a measure of red blood cell concentration, and fibrinogen content. Higher fibrinogen

concentration leads to higher blood viscosity, which in turn raises blood flow shear stress, causing activation of endothelial cells and platelets, thereby contributing to vascular dysfunction and disease (82).

Finally, elevated fibrinogen could exacerbate thrombotic CVD through its effects on fibrin clot structure, which determines clot mechanical properties and stability. As fibrinogen concentration increases, fibrinopeptide A cleavage by thrombin is enhanced and this promotes the formation of a denser fibrin network (83). Clots formed with higher fibrinogen concentrations are mechanically stiffer and less porous (84, 85), and this decrease in porosity causes the clots to be resistant to fibrinolysis. Thus, elevated fibrinogen could predispose an individual to thrombosis via the formation of rigid blood clots that are resistant to enzymatic breakdown (86).

1.4 The γ' Fibrinogen Variant

1.4.1 γ' Fibrinogen Structure

The term γ' fibrinogen is used to describe a fibrinogen molecule that contains a splice variant of the γ chain known as the γ' chain. The fibrinogen γ chain gene, *FGG*, consists of 10 exons. The most common form of the γ chain, γA, is produced when intron 9 is spliced from the RNA and exons 9 and 10 are joined before translation. Production of the γ' chain occurs when a polyadenylation sequence within intron 9 is recognized and exon 10 is cleaved from the RNA. As a result of this alternative splicing, these chains differ at their carboxyterminal sequences, with the γ' chain having 20 residues (VRPEHPAETEYDSLYPEDDL) in place of the last 4 (AGDV) of the γA chain (Figure 1.6) (87, 88). The two Tyr residues in the γ' chain C-terminus are sulfated posttranslationally, adding to the overall negative charge of this region. This difference in charge allows the γ' fibrinogen to be separated from γA/γA fibrinogen on an ion exchange column, typically using a diethylaminoethyl (DEAE) resin.

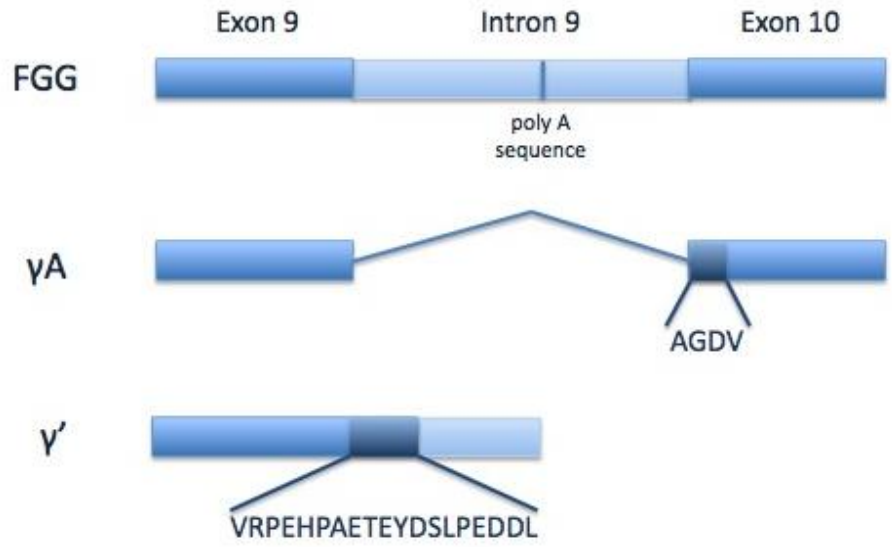

Figure 1.6. Alternative splicing of the fibrinogen γ chain mRNA.

Removal of intron 9 leads to the production of the γA chain, while utilization of a polyadenylation sequence with in intron 9 results in the γ' chain. These isoforms differ in their C-termini, with the differing residues shown above.

It is worth emphasizing that the γ' chain is a splice variant that appears to be present in all humans and is not caused by a mutation. There is evidence that the γ' sequence evolved from a primitive, unspliced version of the fibrinogen gamma chain gene, and similar sequences can be found in other mammals, including mice, rats, and chimpanzees (89). In humans, approximately 10% of circulating fibrinogen contains a γ' chain, though this varies between individuals (90). Because they are in the minority, nearly all γ' chains are incorporated into fibrinogen molecules along with a γA chain, forming heterodimers of γA/γ' fibrinogen (91).

1.4.2 Unique properties of γ' fibrinogen

Because of the altered C-terminus of the γ' chain, γ' fibrinogen functionally differs from γA/γA fibrinogen. One major difference is the ability of γ' fibrinogen to bind to thrombin with relatively high affinity. Thrombin has two positively charged regions near its active site, called exosites I and II. Exosite I interacts with the fibrin(ogen) E region of both γA/γA and γ' fibrinogen with low affinity, while exosite II binds to the γ' chain C-terminus with high affinity (92). Both of these regions can bind to γ' fibrinogen simultaneously, providing a higher-affinity interaction than either region alone (93).

The binding of thrombin to the γ' chain has complex functional impacts on thrombin activity. In general, γ' fibrinogen acts as an inhibitor of thrombin, an effect that was noted over 60 years ago and led to its early designation as antithrombin I (94, 95). Thrombin-catalyzed polymerization of γ' fibrinogen is significantly slower than that of γA/γA fibrinogen (96). Exosite occupancy is known to regulate thrombin specificity, and experiments using a peptide based on the γ' chain C-terminus indicate that it inhibits the intrinsic coagulation pathway (97) and reduces thrombin-induced platelet aggregation (98). However, thrombin bound to γ' fibrinogen retains its catalytic activity, and while this interaction reduces factor VIII activation, its effects on fibrinopeptide release are unclear (96, 97). In addition, γ' fibrinogen protects thrombin from heparin-mediated inactivation by serpins (99). It has been speculated that the reversible binding to γ' fibrinogen may serve to localize thrombin to the site of the growing clot (100).

The decrease in thrombin-induced platelet aggregation in the presence of the γ' chain is only partially mediated by γ' binding to thrombin. The γ' chain lacks the AGDV sequence found at the γA C-terminus which is responsible for binding platelets via integrin αIIbβ3. Thus, γ' fibrinogen does not support platelet aggregation to the same extent as γA/γA fibrinogen. Interestingly, while platelets contain fibrinogen in their α-granules, all of it is γA/γA fibrinogen (101). The reason for this is not known, but it may indicate an internalization mechanism for fibrinogen through vesicles containing αIIbβ3 that excludes γ' fibrinogen.

Another novel property of γ' fibrinogen is its ability to interact with FXIII, the transglutaminase responsible for stabilizing clots by crosslinking fibrin monomers. In 1963, it was reported that FXIII from plasma copurified with fibrinogen and that it eluted with γ' fibrinogen from a DEAE column (102). A mixture of purified FXIII and γ' fibrinogen will also coelute from this type of column, while a γA/γA fibrinogen/FXIII mixture elutes in two peaks (103). Ultracentrifugation studies have also shown binding between γ' fibrinogen and FXIII, which can be competitively inhibited with a γ' C-terminal peptide, indicating that this region is involved in the interaction (104). In 2009, it was reported that platelet binding of FXIII is mediated by γ' fibrinogen (105). Demonstration of direct binding between these two proteins *in vitro* has proven somewhat challenging, however. An attempt to look at this using recombinant fibrinogens found similar FXIII binding for γA/γA, γA/γ', and γ'/γ' isoforms (106), indicating that more work is needed to understand the nature of this interaction.

Fibrin clots formed with γ' fibrinogen appear to have altered clot architecture and FXIIIa cross-linking when compared with γA/γA fibrin clots, though there are some discrepancies between the findings of different research groups. Cooper *et al*., using fibrinogen purified from plasma, found that γA/γ' fibrin clots had a dense network with thinner fibers and smaller pores than γA/γA clots (91). They also noted the slower release of fibrinopeptide B from γA/γ' fibrinogen, which might influence fiber thickness. Siebenlist *et al*. had similar findings with regards to clot architecture and fibrinopeptide B cleavage, but they also found delayed fibrinopeptide A release as well (107).

To address the issue of heterogeneity in fibrinogen purified from plasma, which likely contributes to the disparate results, some groups have used recombinant fibrinogens. Collet *et al.* compared recombinant γA/γA and γ'/γ' homodimers and found that the γ'/γ' fibrinogen formed clots that were 25% less dense with slightly larger fiber diameters (108). More dramatically, they found that the cross-linked γ'/γ 'fibrin clots were 3-times stiffer than their γA/γA counterparts and that their lysis rate was 10-times slower, in agreement with an earlier finding by Falls and Farrell, who found that plasma-purified γA/γ' fibrin clots were highly resistant to fibrinolysis after cross-linking (109). More recently, Gersh *et al*. were able to recombinantly express all three fibrinogen types: γA/γA, γA/γ', and γ'/γ' (96). They found faster fibrinopeptide B release from γ'/γ' fibrinogen than the other types, but similar rates of fibrinopeptide A release. Overall, the γ' chain appeared to inhibit fibrin polymerization, with the γ'/γ' isoform having the slowest rate, followed by the

γ'/γA heterodimer, which in turn was slower than the γA/γA homodimer. Electron microscopy of these non-cross-linked clots demonstrated thinner, more tightly packed fibers for γ'/γ' clots, as compared with γA/γA clots, while γA/γ' clots were markedly non-uniform, with areas resembling dense γ'/γ' clot interspersed with larger pores. Further studies will be needed to understand the contribution of these fibrinogen isoforms to the structure of more physiologically relevant clots, containing platelets and red blood cells, and formed in flowing blood.

1.4.3 The Association of γ' Fibrinogen with Cardiovascular Disease

If indeed γ' fibrinogen forms clots that are denser, stiffer, and more resistant to fibrinolysis than γA/γA fibrinogen, it is easy to imagine that a high level of this isoform may confer a prothrombotic phenotype. One of the first groups to look at this, Drouet *et al*. in 1999, found that the ratio of γ' fibrinogen to total fibrinogen levels was increased in individuals with peripheral arterial disease, myocardial infarction (MI), or ischemic stroke compared with controls (110). A subsequent study by Lovely *et al.* demonstrated that γ' fibrinogen levels were significantly elevated in subjects with CAD, with an odds ratio of 7.16 (95% CI = 1.82 – 27.7) per γ' fibrinogen quartile (90). Interestingly, this study found no significant association between γ' fibrinogen and total fibrinogen levels, supporting the idea that γ' fibrinogen may be an independent risk marker. Research by Mannila *et al*., utilizing samples from the Stockholm Coronary Artery Risk Factor study found a similar link between elevated γ' fibrinogen and past MI, with weak correlation between γ' and total fibrinogen (111). More recently, γ' fibrinogen was measured in 3300 subjects

from the Framingham Offspring Study (112). In this study γ' fibrinogen was significantly associated with many traditional CVD risk factors, including age, body mass index, smoking, diabetes, triglycerides, LDL cholesterol and blood glucose, and inversely associated with high-density lipoprotein (HDL) cholesterol. Subjects in this cohort with prevalent CVD had higher levels of γ' fibrinogen, and the association remained significant after adjustment for sex, age, BMI, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, HDL cholesterol, and triglycerides (113).

While many studies have shown a clear association between elevated γ' fibrinogen levels and arterial thrombosis, other work indicates that venous thrombosis may be linked to reduced levels of this isoform. In the Leiden Thrombophilia Study, γ' fibrinogen levels in the lowest quartile were associated with deep venous thrombosis, with an odds ratio of 3.6 after adjustment for other risk factors (114). To investigate this relationship, Mosesson *et al.* created a transgenic mouse expressing humanized γ' fibrinogen chains (115). They found no difference in thrombosis between the human-γ'-expressing and wild-type mice in a venous injury model except in the presence of the pro-thrombotic factor V Leiden, where the human γ' chain appeared to decrease thrombus volume. Unfortunately, differing levels of fibrinogen expression in these mouse models confound interpretation of this study's results. Decreased γ' fibrinogen levels were also found to be associated with thrombotic microangiopathy, a rare, life-threatening condition characterized by systemic microvascular thrombosis (116).

Little is known about the mechanisms that regulate the expression of γ' fibrinogen, but there is evidence that both genetic and environmental factors play a role. A recent genome-wide association study identified several single nucleotide polymorphisms (SNPs) associated with γ' fibrinogen levels (113). The strongest evidence of association was found for rs7681423, located in the *PLRG1* gene, which codes for a component of the spliceosome, approximately 8.35 kb upstream of *FGG*. Other associated SNPs, particularly rs1049636 and rs2066864, both in intron 9, rs2066861 in intron 8, and rs2066865 in the *FGG* 3' untranslated region have shown association in previous studies (111, 114). With the exception of rs1049636, the SNPs listed above are linked to decreased γ' fibrinogen levels. These studies indicate that certain SNP genotypes associated with increased γ' fibrinogen may also correlate with elevated MI risk in individuals with high levels of both γ' and total fibrinogen (111), while genotypes associated with decreased γ' fibrinogen levels appear to increase the risk of DVT (114).

There is emerging evidence that, in addition to genetic regulation, γ' fibrinogen levels may be influenced by inflammation. A recent study by Cheung *et al.* demonstrated both significantly increased γ' fibrinogen levels and γ'/ total fibrinogen ratios during the acute phase of ischemic stroke as compared with the convalescent stage in the same individuals (117). Another study showed that γ' fibrinogen levels were higher in blood samples drawn from subjects within 7 days of the onset of stroke symptoms, compared to samples drawn at 8 or more days from symptom onset in different subjects (118). Similarly, γ' fibrinogen levels are higher

in the acute phase of pulmonary embolism than in the nonacute phase, and higher in subjects with refractory unstable angina than those with stabilized angina (119). This study also found correlation between γ' fibrinogen and CRP in the acute phase. These findings indicate that γ' fibrinogen is preferentially increased over γA/γA fibrinogen during acute inflammation, which may be due to altered mRNA processing during the acute phase reaction.

1.5 Thesis Research

The focus of this thesis research is the further characterization of γ' fibrinogen as a cardiovascular risk marker. This includes investigating the nature of the biophysical interaction between the γ' C-terminus and thrombin, studying links between γ' fibrinogen and inflammatory markers, and examining the variability of this molecule in healthy individuals.

Project I: Surface plasmon resonance technology (described in Chapter 2) is used to study the role electrostatic interactions in thrombin binding to the γ' chain. It is expected that the interaction between the highly negatively-charged γ' chain C-terminus and the positively-charged exosite II of thrombin is due in large part to the electrostatic complementarity of these regions. Some studies, however, have indicated that certain charged residues on the γ' chain may be particularly important for this binding. The contributions of each of the charged residues is examined using a series of γ' chain-based peptides in which individual charged residues are replaced with uncharged analog residues (e.g. Glu⇒Gln). The affinities of these peptides for thrombin are determined using a Biacore T100. The details of this project are presented in Chapter 3.

Project II: This project focuses on the link between γ' fibrinogen and inflammation in subjects from the Periodontitis And Vascular Events (PAVE) study. The PAVE study was a pilot study to determine the feasibility of a multi-center trial designed to test whether aggressive periodontitis treatment could reduce the risk of cardiovascular events in individuals at high risk. While the larger scale study was

never performed, baseline data and blood samples were collected from the approximately 300 subjects enrolled. These data include demographic information, CRP measurements, and comprehensive dental exam documentation. An enzyme-linked immunosorbent assay (described in Chapter 2) is used to measure the levels of γ' fibrinogen in plasma samples from these subjects. Associations between γ' fibrinogen and several markers of inflammation are examined. This research is described in Chapter 4.

Project III: Chapter 5 discusses a study on the variability of γ' fibrinogen in healthy individuals. Blood samples were taken from 15 adult subjects at varying intervals over the course of a year. These samples are analyzed for γ' fibrinogen along with more established cardiovascular risk factors, including HDL and LDL cholesterol, triglycerides, fasting glucose and CRP. In addition, a comprehensive metabolic panel was run for each sample to confirm the healthy status of the subjects throughout the study. Both the between-subject (inter-individual) and within-subject (intra-individual) variabilities are calculated for γ' fibrinogen and other risk markers. Correlations between the markers are also examined. The goal of this research is to assess the potential utility of γ' fibrinogen measurement in a clinical setting, with respect to its variance in healthy subjects.

Chapter 2. Methods Background and Details

2.1 Biacore Surface Plasmon Resonance Binding Experiments

Biacore devices utilize surface plasmon resonance (SPR) technology to measure molecular binding. This technology relies upon the phenomenon of evanescent wave production, which occurs when there is total internal reflection of light waves at the interface of two materials of differing refractive indices, in this case, between a glass surface and an aqueous buffer. A thin coating of gold at this interface allows the evanescent wave to excite surface plasmons, electromagnetic waves that are present along the surface where a metallic and a dielectric (insulator) surface meet. When monochromatic, p-polarized light hits this surface at a particular angle there is resonant energy transfer from the wave to the surface plasmons, and the intensity of the light reflected at that angle is reduced (Figure 2.1). This resonance angle is exquisitely sensitive to the conditions at the metal/dielectric interface, a property upon which SPR technology depends.

Biacore instruments measure interactions with SPR in four flow cells on the surface of a sensor chip. Each chip has a glass surface coated with a very thin layer of gold. For most applications, this gold is covered with a dextran matrix that can be coupled to molecules of interest, thus immobilizing these molecules on the chip surface. As a sample passes through a flow cell over the chip, the binding or dissociation of molecules from the chip surface will result in a change in the resonance angle proportionate to the mass of the bound sample (120).

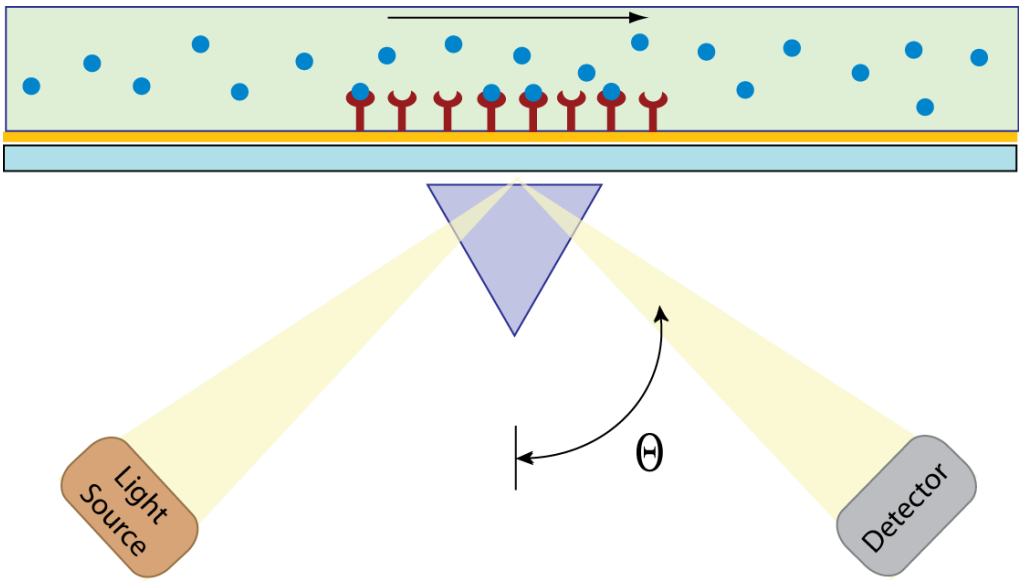

Figure 2.1. Illustration of surface plasmon resonance. The arrow indicates the flow of buffer. Θ is the resonance angle.

This information is recorded on a sensorgram, in which the angle shift, or response, is plotted in "resonance units" (RU) over time (Figure 2.2). The flow cell system allows for one of the flow cells to be used as a reference cell, with either a non-binding control molecule or no material beyond the dextran matrix bound to the chip surface for that cell. As the sample solution is passed over the flow cells in series, the response of the reference flow cell can then be subtracted from the responses from the experimental flow cells to correct for background variation in the refractive index of the solutions.

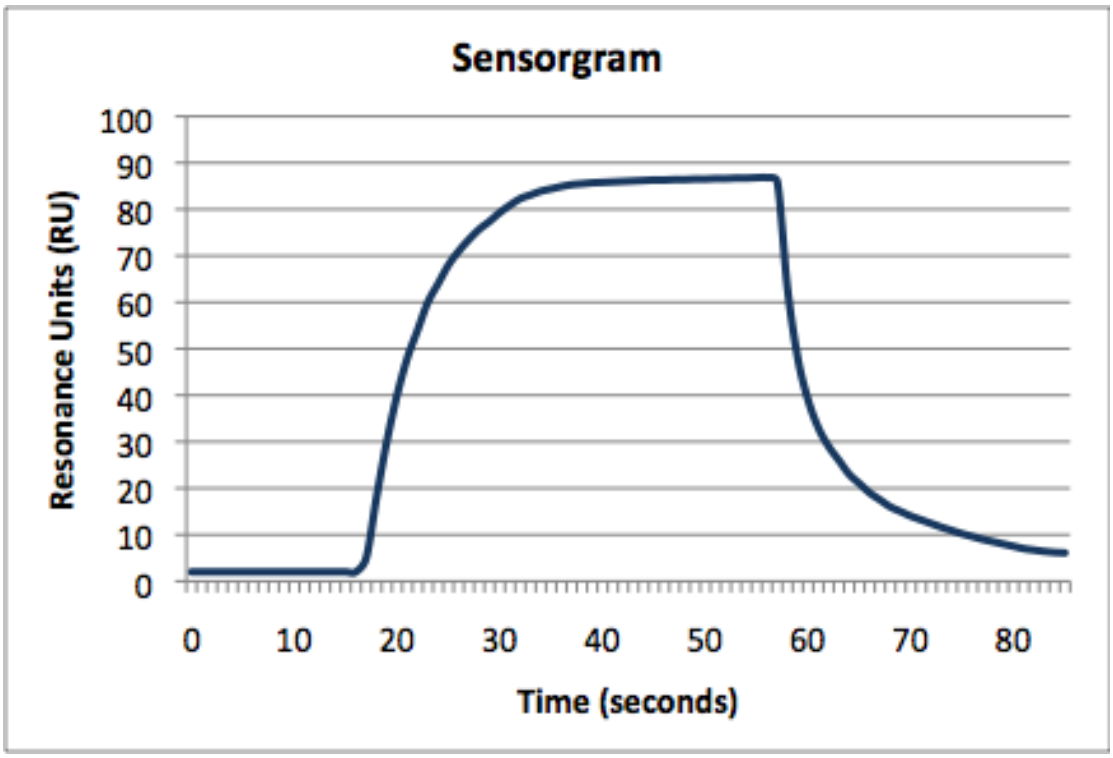

Figure 2.2. An example of an SPR sensorgram. The binding of molecules flowing over the surface of a chip is plotted as an increase in resonance units over time. A decrease in the signal is seen as the analytes dissociate and are carried away.

For the experiments described in Chapter 3 of this dissertation, a Biacore T100 instrument was used with SA chips. These chips have streptavidin pre-immobilized on the dextran matrix, which allowed the experimental peptides to be bound to the chip through an N-terminal biotin molecule, a very stable interaction. More information on these methods can be found in Chapter 3, section 3.3.

2.2 Measurement of γ' Fibrinogen Levels in Plasma

The research projects described in Chapters 4 and 5 required the measurement of γ' fibrinogen levels in human plasma. For the study investigating the association between γ' fibrinogen levels and inflammation (Chapter 4), an enzyme-linked immunosorbent assay was used to quantify γ' fibrinogen, while the study on the variability of γ' fibrinogen and other CVD risk markers (Chapter 5) utilized Luminex technology. In all cases, these measurements were performed with plasma from blood samples collected into tubes containing citrate.

2.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA is a plate-based biochemical assay used to detect and quantify a particular molecule of interest. This analyte of interest is immobilized to the wells of a plate, either through direct binding or, more often, via an antibody pre-bound to the wells. Standards with known concentration of the analyte are assayed along with unknown samples to allow quantitation. After a wash step to remove unbound analyte, a specific detection antibody is then added to the wells to form a complex with the analyte. This antibody may be conjugated to a dye molecule or an enzyme, or an enzyme- or dye-bound secondary molecule may be used to bind the detection antibody. Typically, after another wash step, a colorimetric substrate for the enzyme is added next, followed by a solution to quench the reaction after the color has developed. The measurement of the color signal produced by this process for

the samples and the standards can then be used to generate a curve equation used to quantitate the original concentration of the analyte.

The ELISA assay used to measure γ' fibrinogen was developed by Lovely *et al.*, and an evaluation of this assay was published in 2010 (112).

2.2.2 Luminex Assay

Similar to the ELISA, Luminex xMAP® bioassays rely upon capture and detection antibodies to measure analyte concentration. Unlike the ELISA, these Luminex assays are bead-based, utilizing color-coded microspheres to bind the analyte of interest. These 5.6-micron polystyrene microspheres are coupled to a capture molecule, such as an antibody, and then incubated with standards and unknown samples in the wells of a 96-well plate (121). Unbound analyte is washed from the beads and a detection molecule is added, often an antibody either directly coupled to a fluorescent dye, or coupled through a secondary molecule. After removal of unbound detection molecules, the beads from each well are passed through a fluidics system, similar to a flow cytometer, which measures the signal from each individual bead. Readings from many beads from each well are averaged together and the results from the standard wells are used to generate a curve equation for unknown sample quantitation, as in an ELISA. In general, Luminex assays yield more reproducible results than ELISAs, with less background signal. More information on this technology can be found at the Luminex website: www.luminexcorp.com.

For the γ' fibrinogen Luminex assay, the monoclonal γ' chain antibody, 2.G2.H9, was covalently coupled to the microspheres for γ' fibrinogen capture. These microspheres were incubated with plasma samples diluted 1:1000 and standard controls of known γ' fibrinogen concentration. For detection of the bead-bound fibrinogen, a biotinylated polyclonal anti-fibrinogen antibody was used along with a streptavidin-phycoerythrin conjugate. The streptavidin binds tightly to the biotinylated antibody, and the phycoerythrin fluorophore allows detection of the bound fibrinogen. The details of this method can be found in Chapter 5, section 5.3.

Chapter 3. The Role of Electrostatic Interactions in Thrombin Binding to the Fibrinogen γ' Chain

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Author contributions:

Kristine Alexander contributed to the experimental design, the peptide-thrombin Biacore binding assays, statistical analysis, and the writing of the paper.

Michael Fried contributed to the analysis of the effect of salt concentration on peptide-thrombin binding and the writing of the paper.

David Farrell contributed to the experimental design, research funding, and writing of the paper.

3.1 Abstract

Thrombin binds to the highly anionic fibrinogen γ' chain through anion-binding exosite II. This binding profoundly alters thrombin's ability to cleave substrates, including fibrinogen, factor VIII, and PAR1. However, it is unknown whether this interaction is due mainly to general electrostatic complementarity between the γ' chain and exosite II or if there are critical charged γ' chain residues involved. We therefore systematically determined the contribution of negatively charged amino acids in the γ' chain, both individually and collectively, to thrombin binding affinity. Surface plasmon resonance binding experiments were performed using immobilized γ' chain peptides with charged-to-uncharged amino acid substitutions; i.e., Asp to Asn, Glu to Gln, and pTyr to Tyr. Individually, the substitution of uncharged for charged amino acids resulted in only minor changes in binding affinity, with a maximum change in K_d from 0.440 M to 0.705 M for the Asp419Asn substitution. However, substitution of all three charged amino acids in a conserved β-turn that is predicted to contact thrombin, pTyr418Tyr, Asp419Asn, and pTyr422Tyr, resulted in the loss of measurable binding, as did substitution of all the flanking charged amino acids. In addition, the binding of the γ' chain to thrombin was reduced in a dose-dependent manner by increasing [NaCl], resulting in a net loss of 3-4 ion pairs between thrombin and the γ' chain. Therefore, although each of the individual charges in the γ' chain contribute only incrementally to the overall binding affinity, the ensemble of the combined charges play a profound role in the thrombin-γ' chain interactions.

3.2 Introduction

Thrombin, a trypsin-like serine protease, plays a central role in blood coagulation (20). Thrombin cleavage is responsible for activating several of the coagulation factors, including fibrinogen, which is then converted to the insoluble fibrin fibers that make up the protein meshwork of the blood clot. On its surface, adjacent to its active site, thrombin contains two basic regions, exosites I and II. These anion-binding exosites function to modulate thrombin's activity and specificity (122-124). Their proximity to the active site allows for the direct involvement in the recognition of thrombin substrates, and cofactors and anticoagulant drugs utilize these exosites to alter thrombin–substrate interactions. Fibrin(ogen) contains two sites that interact with these thrombin exosites: a low-affinity site in the fibrinogen central E domain and a higher-affinity site in the carboxy terminus of the γ′ fibrinogen variant (92).

Fibrinogen is a dimeric molecule, containing two each of three types of polypeptide chains: Aα, Bβ, and γ. The γ′ fibrinogen isoform contains a γ′ chain, produced through alternative splicing of the γ chain mRNA (87, 88). The γ′ chain differs from the more abundant γA at the carboxy terminus, where it has 20 residues (^{408}VRPEHPAETE$_s$YDSL$_s$YPEDDL^{427}) in place of the last four residues (^{408}AGDV^{411}) of the γA chain. This extended C-terminus provides γ′ fibrinogen with unique biochemical properties compared to those of the more common isoform, particularly the high-affinity binding site for thrombin (125), and studies suggest that the γ′ fibrinogen may also serve as a carrier for factor XIII (103, 104).

Competitive binding experiments have demonstrated that γ′ fibrinogen binds to thrombin anion-binding exosite II, the same region that binds to heparin (126). One hypothesis is that γ′ fibrin may function to retain thrombin at the site of the clot, as fibrin-bound thrombin remains active (93) and thrombin dissociates more slowly from clots containing γ′ fibrin than from clots made with γA fibrin alone (99). Additionally, clot-bound thrombin is resistant to inhibition by antithrombin (99). There is also evidence that binding to the γ′ fibrin(ogen) carboxy terminus inhibits thrombin's cleavage of factor VIII, alters fibrin clot formation (97), and inhibits thrombin cleavage of the platelet protease-activated receptor 1 (PAR1) (98, 127).

A number of studies have investigated the interaction between thrombin and γ′ fibrinogen using γ′ carboxy-terminal peptides. The γ′ carboxy terminus is highly anionic, containing four glutamic acid residues, three aspartic acid residues, and two sulfotyrosine residues (128, 129), while the corresponding binding site on thrombin, exosite II, is highly positively charged. It is therefore expected that the binding of the γ′ 410–427 peptide to thrombin is at least partially due to this electrostatic complementarity. This cannot be the entire basis for the interaction, however, as experiments using a reversed sequence of the 414–427 peptide demonstrated greatly reduced affinity (125).

Studies using deleted peptides have indicated that residues 411–427 are involved in this binding and that further truncation at the amino or carboxy terminus significantly weakens or abrogates the interaction (125, 126). Sulfation (125) or phosphorylation (126, 130) of the peptide's two tyrosine residues also appears to

be necessary for maximal binding, with a considerable decrease in affinity for peptides with only a single modified tyrosine. There is evidence that the negative charge of Tyr422 may be more important than Tyr418 (125, 130).

The crystal structure of the thrombin–γ′ peptide complex determined by Pineda *et al.* (131) confirms many of the findings from other studies. The structure showed that the interaction of the γ′ peptide with thrombin was similar to that of heparin, with numerous electrostatic interactions between the cationic residues of exosite II and the anionic residues of the γ′ peptide. The peptide's phosphotyrosines are closely associated with positively charged residues in exosite II, with pTyr418 interacting with Arg126, Lys235, and Lys236, and pTyr422 with Lys240. Interestingly, this structure shows the peptide in contact with two thrombin molecules, but the significance of this finding is uncertain and may represent a crystal packing artifact.

While the crystal structure and other studies have implicated regions of the peptide important for thrombin binding, the individual contribution of each of the peptide's charged residues to this binding has not been determined. In this paper, we test the predictions of the crystal structure by examining these contributions systematically, using peptides with charged-to-uncharged substitutions, and measuring the thrombin binding affinities using surface plasmon resonance. These experiments are designed to determine whether the γ′ chain–thrombin interaction is due mainly to cooperative binding of the ensemble of charge residues or if there are critical charged residues in the peptide that are required for binding.

3.3 Materials and Methods

Materials. N-Terminally biotinylated γ′ peptide was synthesized by Abgent (San Diego, CA). N-Terminally biotinylated analogue peptides were synthesized by Abgent and Anaspec (Fremont, CA). See Table 3.1 for peptide sequences. All peptides were >95% pure, with the peptide purity verified by high-performance liquid chromatography and mass spectrometry. FPRck-inhibited thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Series S Sensor Chips SA and HBS-EP+ [10 mM HEPES (pH 7.4), 150mM NaCl, 0.05% P20 surfactant, and 3 mM EDTA] buffer were purchased from GE Healthcare Biosciences Corp. (Piscataway, NJ).

Surface Plasmon Resonance. Surface plasmon resonance experiments were performed with a Biacore T100 instrument (GE Healthcare). Biotinylated peptides were immobilized on streptavidin chips following the manufacturer's instructions. Briefly, flow cell surfaces were prepared for immobilization with three 60 s injections of 1 M NaCl and 50 mM NaOH. Biotinylated peptides were immobilized to sensor chip flow cells at a level of 150 response units (RU). Reference flow cells without immobilized peptide were used as controls.

All peptide–thrombin binding assays were performed at 25°C. HBS-EP+ was used as both running and binding buffer. Various concentrations of FPRck-thrombin were injected over flow cells bound with wild-type and analogue peptides until equilibrium binding was reached. Kinetic injections were performed for 20 s at a flow rate of 50 μL/min, followed by a 30 s dissociation time. No regeneration of the

flow cells following injections was necessary. Buffer-only injections were included for each condition, and these responses were subtracted from the binding signals. Responses from control flow cells were similarly subtracted to account for changes in the refractive index. BIAevaluation was used to calculate affinity constants (K_d) based on a 1:1 Langmuir binding model. K_d determinations for each analogue peptide were based on three replicate experiments using freshly prepared thrombin dilutions.

For experiments with NaCl concentrations of 200–350 mM, calculated K_d values were greater than the highest concentration of thrombin used. Because the equilibrium affinity analysis calculates K_d as the concentration of analyte at which half-maximal binding occurs, K_d values outside the range of analyte concentrations used cannot be accurately determined. To address this problem, the maximal binding response for the flow cells used for these experiments was measured in binding experiments with buffer containing 150 mM NaCl, and this value was used to correct the calculated K_d for the higher-salt experiments.

The salt concentration-dependent peptide–thrombin interaction can be written as

$$\mathrm{P} + \mathrm{T} \overset{K_a}{\rightleftharpoons} \mathrm{P{\cdot}T} + n(\mathrm{M^+} + \mathrm{X^-})$$

where P and T represent peptide and thrombin (with associated counterions), respectively, P·T is the peptide–thrombin complex (with associated counterions) (132), M^+ and X^- are cations and anions bound or released on complex formation,

and n is the net ion stoichiometry (net ion release is indicated by n > 0; net ion uptake is indicated by n < 0). The equilibrium association constant is given by

$$K_a = \frac{[P \cdot T][MX]^n}{[P][T]}$$

Thus, a graph of ln K_{obs} as a function of ln[MX] should have a slope of −n.

3.4 Results

Kinetics of Binding of Thrombin to the γ′ Peptide. To test the contributions of the individual charged residues in the γ′ carboxy-terminal peptide to thrombin binding, we performed binding studies using the wild-type peptide as well as analogue peptides in which negatively charged residues were replaced individually with uncharged residues with a similar side chain size, Glu-to-Gln, Asp-to-Asn, and pTyr-to-Tyr (see Table 3.1 for peptide sequences). Figure 3.1 shows the locations of the negatively charged residues of the γ′ peptide in complex with thrombin according to the crystal structure of Pineda *et al*. (131).

Table 3.1. Binding Affinities of the Charged-to-Uncharged γ′ Peptides[a]

Peptide	Sequence	K_d (μM) (mean ± standard error of the mean)	P value (compared to wild-type)*
Wild-type	PEHPAETE$_o$YDSL$_o$YPEDDL	0.440 ± 0.011	–
#1	P**Q**HPAETE$_o$YDSL$_o$YPEDDL	0.441 ± 0.016	1.00
#2	PEHPA**Q**TE$_o$YDSL$_o$YPEDDL	0.485 ± 0.011	0.46
#3	PEHPAET**Q**$_o$YDSL$_o$YPEDDL	0.541 ± 0.010	0.025
#4	PEHPAETE **Y**DSL$_o$YPEDDL	0.583 ± 0.011	0.008
#5	PEHPAETE$_o$Y**N**SL$_o$YPEDDL	0.705 ± 0.011	<0.001
#6	PEHPAETE$_o$YDSL **Y**PEDDL	0.702 ± 0.021	0.018
#7	PEHPAETE$_o$YDSL$_o$YP**Q**DDL	0.582 ± 0.018	0.073
#8	PEHPAETE$_o$YDSL$_o$YPE**N**DL	0.608 ± 0.019	0.053
#9	PEHPAETE$_o$YDSL$_o$YPED**N**L	0.465 ± 0.010	1.00
#10	PEHPAETE **YN**SL **Y**PEDDL	–	–
#11	P**Q**HPA**Q**T**Q**$_o$YDSL$_o$YP**QNN**L	–	–
#12	P**Q**HPA**Q**T**Q**$_o$YDSL$_o$YPEDDL	1.968 ± 0.025	<0.001
#13	PEHPAETE$_o$YDSL$_o$YP**QNN**L	3.189 ± 0.034	0.002

[a]BIAevaluation was used to calculate affinity constants (K_d) based on a 1:1 Langmuir binding model. The standard error of the mean was calculated from triplicate determinations. *P values were adjusted for multiple comparisons using Bonferroni correction.

Interactions between these peptides and thrombin were studied by surface plasmon resonance (SPR) using a Biacore T100 instrument. The peptides were immobilized to a chip surface, and solutions containing thrombin were passed over the chip until equilibrium binding was achieved. The kinetics of binding demonstrated a rapid k_{on} rate as well as a rapid k_{off} rate, resulting in square wave-like binding isotherms (Figure 3.2). Kinetic quantitation of these interactions was not possible, as both the association and dissociation were extremely rapid and, in the case of the dissociation rate, outside the limits of quantification for the instrument. For this reason, equilibrium affinity analyses were performed to measure the K_d for the binding interaction.

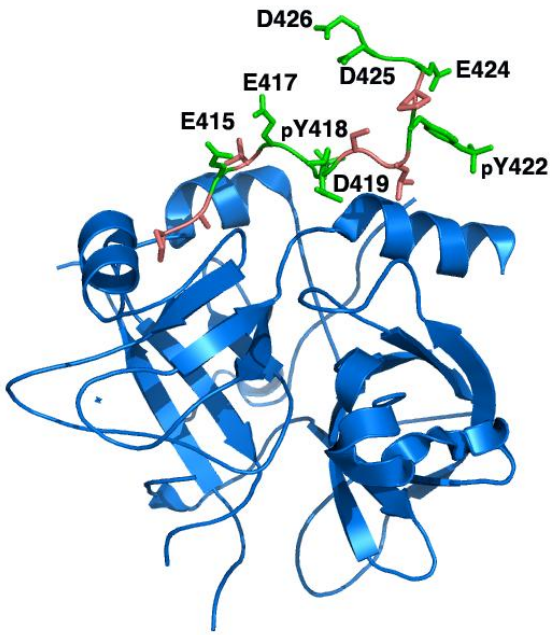

Figure 3.1. Crystal structure of thrombin in complex with the γ′ peptide. Residues substituted in our experiments are colored green. Note that the structure does not predict the positions of the N-terminal residues Pro410–His412; these residues, including the Glu411 altered in peptide 1, are not shown.

The wild-type γ′ peptide bound thrombin with a K_d (± standard error) of 0.440 ± 0.011 μmol/L, which is similar to the affinity of 0.63 μmol/L found previously by fluorescence polarization (126) and the K_d of 0.20 μmol/L measured for the binding of thrombin to γ′ fibrinogen (125). The rapid k_{on} and k_{off} rates are apparent in the binding isotherms for these experiments (Figure 3.2).

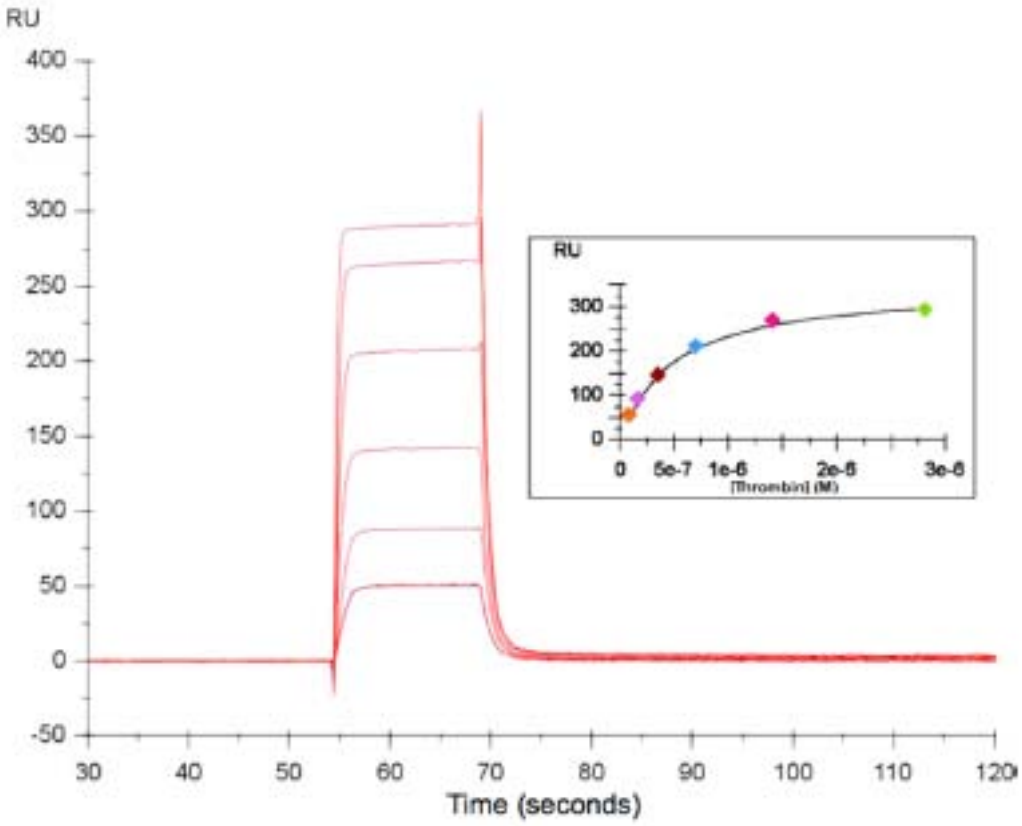

Figure 3.2. Binding isotherms for binding of thrombin to the immobilized wild-type γ′ peptide using surface plasmon resonance. Increasing concentrations of thrombin were bound to the wild-type γ′ peptide in a Biacore instrument at 25 °C until equilibrium binding was reached, and the resonance units were quantitated with time. Kinetic injections were performed for 20 s, followed by a 30 s dissociation time. The inset shows the maximal binding as measured in resonance units as a function of thrombin concentration. BIAevaluation was used to calculate affinity constants (K_d) based on a 1:1 Langmuir binding model.

Binding of Thrombin to Analogue γ′ Peptides. The single-residue charged-to-uncharged analogue peptides bound thrombin with affinities ranging from 0.441 to 0.705 μmol/L (Table 3.1). The analogue peptides with substitutions in the first two or last four charged residues (peptides 1, 2, and 7–9) did not differ from the wild-type peptide with respect to thrombin affinity, while the other analogue peptides exhibited significantly lower affinities. In particular, peptides 5 and 6, corresponding to changes of Asp419Asn and pTyr422Tyr, respectively, bound thrombin with the lowest affinities. These residues have been shown by two-dimensional nuclear magnetic resonance (NMR) to assume a β-turn conformation (130) and contact thrombin exosite II (131).

Sulfation (or phosphorylation) of the γ′ peptide's two Tyr residues has been shown to be important for thrombin binding (125, 126), and our results confirm and extend these findings, with a significant decrease in affinity seen with either monophosphorylated peptides, pTyr418Tyr and pTyr422Tyr. We found that removal of the negative charge on Tyr422 reduced the thrombin affinity more than removal of the Tyr418 charge, in agreement with other reports (125, 130).

In addition to pTyr418 and pTyr422, the Pineda crystal structure (131) predicts a number of contacts between thrombin exosite II and Asp419 of the γ′ peptide, located between the phosphotyrosines. These three residues, along with Glu415, are the only charged residues predicted to interact with exosite II on a single thrombin molecule. This is an important feature of the crystal structure model, because the co-crystal depicts two thrombin molecules bound to a single γ′ chain

(131). We therefore sought to determine whether these three residues were required for thrombin interaction, and if they were able to mediate this binding in the absence of the other anionic residues. SPR studies using a peptide with uncharged replacements pTyr418Tyr, Asp419Asn, and pTyr422Tyr (peptide 10) demonstrated a complete absence of measurable binding (Figure 3.3). Conversely, experiments with a peptide in which all other negatively charged residues were substituted instead (peptide 11) also showed no measurable interaction with thrombin (Figure 3.3), indicating that these three charged residues, while necessary for thrombin binding, are not sufficient in the absence of the other negatively charged residues. To further investigate this, we performed binding experiments using peptides that had either the amino-terminal three charges (peptide 12) or the carboxyl-terminal three charges (peptide 13) substituted with uncharged residues. These peptides demonstrated substantially reduced binding affinities, as compared with those of the singly substituted peptides, but unlike peptide 10 did have measurable binding.

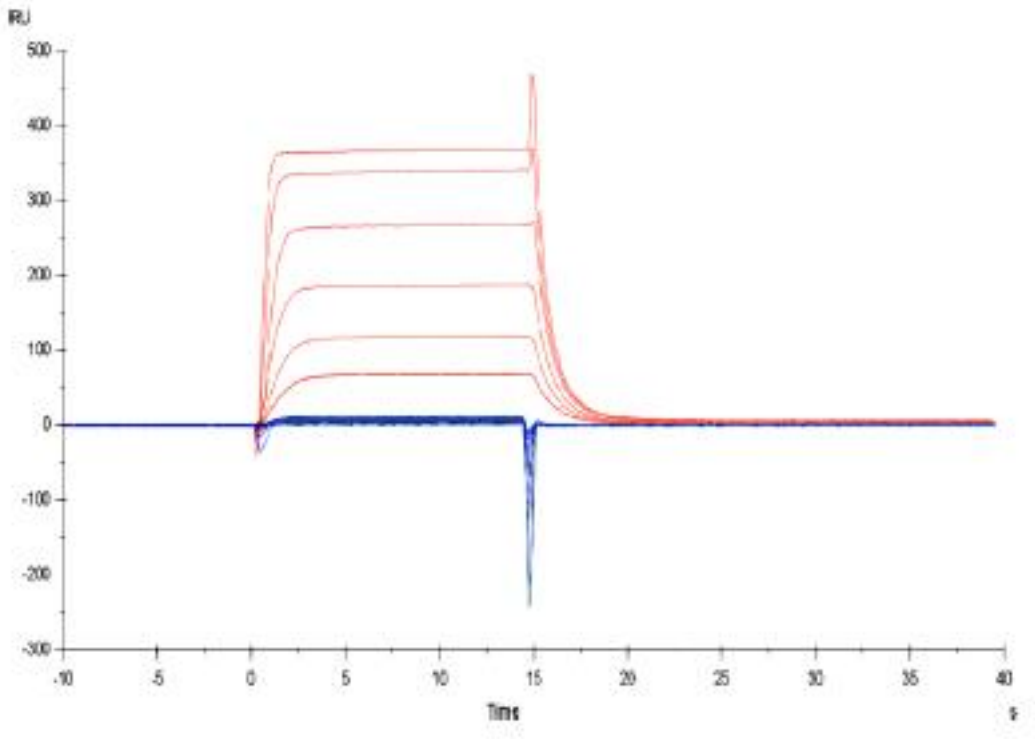

Figure 3.3. Thrombin binding curves for the wild-type γ′ peptide (red) and analogue peptides 10 and 11 (blue and green, respectively). The data show that neither peptide 10 with the central three charged-to-uncharged amino acid changes (pTyr418Tyr, Asp419Asn, and pTyr422Tyr) nor peptide #11 with flanking charged-to-uncharged amino acid changes (Glu411Gln, Glu415Gln, Glu417Gln, Glu424Gln, Asp425Asn, and Asp426Asn) displayed measurable binding.

Effect of NaCl Concentration on Thrombin−γ′ Peptide Affinity. To further investigate the nature of thrombin−γ′ peptide interactions, we determined the dependency of the binding on ionic strength. Binding studies were performed in the presence of increasing concentrations of salt at 150, 200, 250, and 350 mM NaCl

(Table 3.2). These experiments demonstrated a strong salt dependence of the binding, with an increase in NaCl concentration from 150 to 200 mM associated with a 12-fold decrease in affinity, from 0.488 to 6.05 μmol/L, respectively. Increasing the NaCl concentration to 250 mM resulted in an additional 3.1-fold decrease in affinity to 18.6 μmol/L. Further increasing the salt concentration to 350 mM resulted in an additional 6.5-fold decrease in affinity to 120 μmol/L, a greater than 240-fold decrease in affinity compared to the thrombin–γ′ peptide interaction.

Table 3.2. Dependence of thrombin-γ′ peptide binding on NaCl concentration

[NaCl] (mM)	K_d (μM) (mean ± standard error of the mean)
150	0.488 ± 0.042
200	6.05 ± 0.64
250	18.6 ± 0.98
300	120 ± 2.1

To quantitate the release of counterions during the binding of thrombin to the γ′ peptide, the dependence of the natural logarithm of the K_a on the natural logarithm of the NaCl concentration was investigated (Figure 3.4). The slope indicates that there was a net release of 7.6 ± 0.6 ions in the binding reaction. Assuming a 1:1 stoichiometry, there may be three or four ion pairs formed between the γ′ peptide and thrombin, with net release to solvent of the associated counterions.

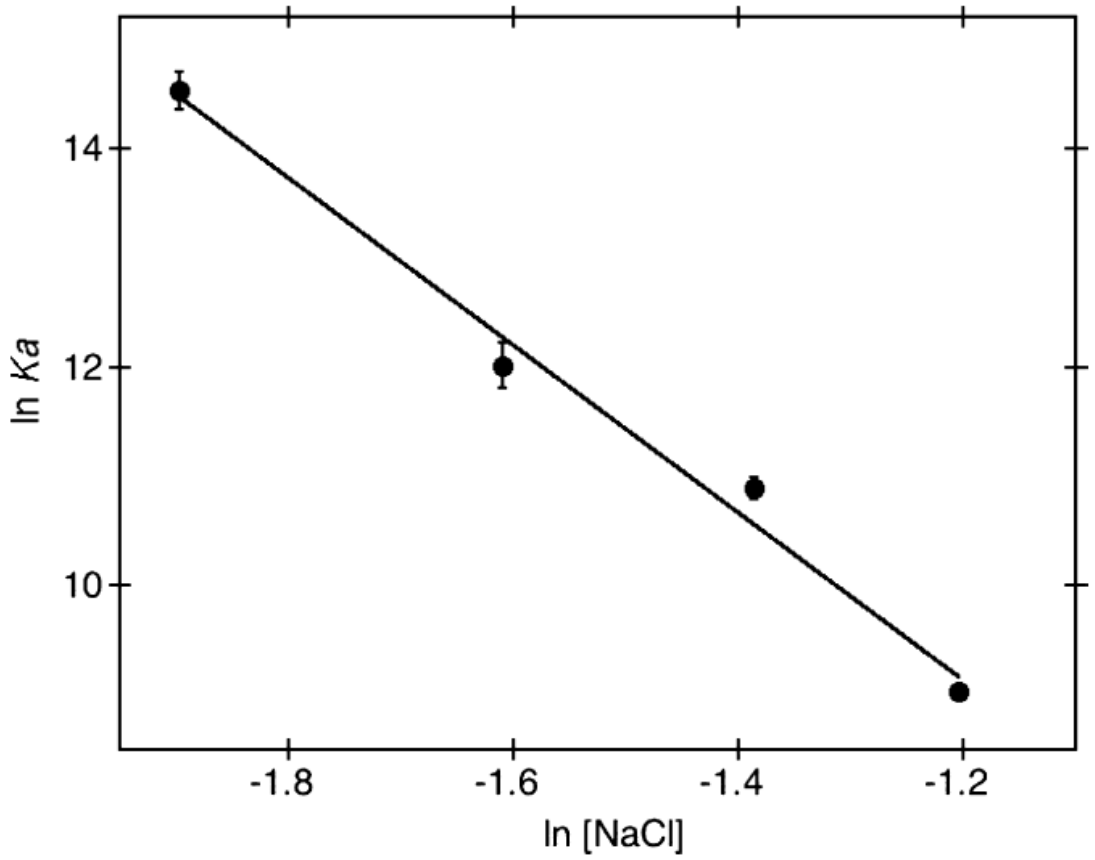

Figure 3.4. Linkage plot. The slope indicates a net release of 7.6 ± 0.6 ions for the binding of thrombin to the γ′ peptide. Assuming that counterions are released from protein groups forming ion pairs, the data indicate that three or four ion pairs formed between thrombin and the γ′ peptide, with net release to solvent of associated counterions.

3.5 Discussion

It has been shown previously that the highly anionic carboxy terminus of the γ′ fibrinogen chain binds to exosite II of thrombin (126) and modulates the activity of thrombin toward several of its substrates (97-99, 127). The crystal structure of thrombin in complex with the γ′ carboxy-terminal peptide, along with binding studies, has implicated several residues and regions of the peptide as being important for this binding. In this work, we have further examined the binding contributions made by each of the peptide's negatively charged residues and investigated the salt dependence of this interaction using SPR.

The k_{on} and k_{off} rates for the binding of thrombin to the γ′ peptide were quite rapid. It is tempting to speculate that such rapid binding may serve to increase the local thrombin concentration surrounding the growing fibrin clot in the presence of rapidly flowing blood. A similar strategy is employed in the catch bonds between platelet glycoprotein Ib and von Willebrand factor and allows rapidly moving platelets to be slowed by sequential tethering events to von Willebrand factor coated on injured subendothelium (133). The γ′ chain−thrombin exosite II interaction by itself would result in both a rapid association and a rapid dissociation. However, during blood coagulation, thrombin also interacts with intact fibrin through exosite I (134). The additional interaction between exosite I and fibrin may further stabilize the initial complex between exosite II and the γ′ chain. Thrombin has been visualized on the growing clot surface using intravital microscopy (135), although the cognate receptor for thrombin on the clot remains

unclear. Thrombin bound to fibrin is also protected from inactivation by antithrombin III (99).

Altering the charged residues in the γ′ chain had differential effects on thrombin binding, depending upon their location. Substituting many of the charged residues, including Gln411 for Glu, appeared to have no effect, although several previous studies have indicated that amino-terminal truncation of the peptide from Pro410 or Glu411 to Ala414 results in a significant decrease in affinity (125, 126, 130). However, the reduced affinity seen with the truncated peptides likely has less to do with the loss of the charge on Glu411 and more to do with other interactions of the other side chains with thrombin. This hypothesis is supported both by our results and by the Pineda crystal structure, which predicts interactions between Pro413 of the peptide and Val163 and Arg165 of thrombin (131), as well as NMR data by Sabo *et al*. that indicate a lack of contact between thrombin and residues Pro410–His412 (130).

Both the crystal structure and the NMR study by Sabo *et al*. predict a β-turn conformation in the region that includes Tyr422 and Asp425 (130, 131). In the crystal structure, this β-turn bridges exosite II of a second, symmetry-related thrombin molecule and predicts interactions between the peptide's Asp426 and Arg93 on thrombin. In our experiments, the replacement of Asp426 on the γ′ peptide had no affect on thrombin binding. Many of the charged γ′ peptide residues that are implicated in thrombin binding in the crystal structure (131) appeared to influence binding affinity in our study. These include pTyr418, Asp419, and

pTyr422. These data tend to support the Pineda model of two thrombin molecules binding to a single γ′ chain, although the lack of an effect of the Asp426Asn substitution on binding affinity is puzzling. The structure also did not contain electron density for residues 410−412, which, along with our data showing that Glu411 does not contribute to binding and the results of N-terminal deletion studies, indicates that this region does not have any important interactions with exosite II.

Taken together, these results demonstrate the significant role of electrostatic interactions between thrombin anion-binding exosite II and the fibrinogen γ′ chain. However, while each of the individual charges contributes only incrementally to the overall binding affinity, the ensemble of the combined charges plays a profound role in the thrombin−γ′ chain interactions.

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Chapter 4. Association Between γ' Fibrinogen Levels and Inflammation

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Author contributions:

Kristine Alexander contributed to the experimental design, the measurement of γ' fibrinogen, statistical analysis, and the writing of the paper.

Theresa Madden contributed to accessing the previously collected data and experimental design.

David Farrell contributed to the experimental design, research funding, and editing of the paper.

4.1 Abstract

The γ' fibrinogen isoform produces clots that are stiffer and more resistant to breakdown than the more common fibrinogen isoform, γA. Increased levels of γ' fibrinogen are associated with several forms of cardiovascular disease. The purpose of this cross-sectional study was to investigate the relationship between γ' fibrinogen, an emerging risk factor for cardiovascular disease, and inflammatory markers in subjects with a chronic inflammatory state. The 284 subjects for this study came from the Periodontitis And Vascular Events (PAVE) study, and γ' fibrinogen and total fibrinogen in plasma were measured by ELISA. Information on patient demographics and health status, as well as levels of C-reactive protein (CRP), an inflammatory marker, have previously been collected for this study. The mean (SE) γ' fibrinogen level in the subjects was 0.622 (0.017) mg/ml. Levels of γ' fibrinogen were correlated with CRP ($p = 0.006$), with a one unit increase in CRP associated with a 1.9% increase in γ' fibrinogen, after adjustment for potential confounders. Total fibrinogen was not correlated with γ' fibrinogen in these subjects. The number of dental sites with evidence of tissue inflammation was also significantly associated with γ' fibrinogen levels. These results provide an important step in the evolution of γ' fibrinogen not only as a general risk factor for cardiovascular disease, but as a potentially useful biomarker for assessing a patient's inflammatory state and associated cardiovascular disease risk.

4.2 Introduction

Fibrinogen is a large (340 kD) protein with a vital role in haemostasis. It has a dimeric structure, with each half of the fibrinogen molecule made up of three polypeptide chains: Aα, Bβ and γ. During coagulation, thrombin converts fibrinogen to fibrin, which polymerizes and forms the protein meshwork of the growing blood clot. The fibrin clot is stabilized through the action of FXIIIa, a transglutaminase that crosslinks adjacent fibrin molecules. The γ' isoform of fibrinogen is formed by the incorporation of a splice variant of the fibrinogen γ chain called the γ' chain. This alternative splicing event results in the extended carboxyl terminus of the γ' chain, which contains 20 residues in place of the four carboxylterminal residues on the more common γA chain (87, 88). The γ' fibrinogen isoform makes up approximately 10% of circulating fibrinogen, although this value can vary widely between individuals (90, 111, 112). The unique sequence of the γ' chain contains a high affinity binding site for thrombin (92, 93, 126, 130, 131). Clots formed with γ' fibrin are more resistant to fibrinolysis (108, 109) and have altered clot architecture compared with γA/γA fibrin clots (91, 96). Because of these characteristics, γ' fibrinogen has been studied as a possible risk factor for thrombosis (100). Research by our laboratory and others has linked levels of γA/γ' fibrinogen with cardiovascular disease (CVD), including coronary artery disease (CAD) (90), heart attack (110, 111), and stroke (117).

The pathogenesis of cardiovascular disease has a number of inflammatory components. While it is well established that total fibrinogen is elevated during

inflammation, it is not known whether γ' fibrinogen is similarly increased. A recent report shows elevated γ' fibrinogen levels in the acute phase of ischemic stroke and pulmonary embolism relative to the non-acute phase (119). To date, however, it is not known how γ' fibrinogen levels relate to the presence of chronic, low-level inflammation.

In this cross-sectional study, we examined γ' fibrinogen levels in subjects with both periodontal and cardiovascular disease to gain a better understanding of the relationship between γ' fibrinogen and inflammation. We hypothesized that elevated γ' fibrinogen levels would be associated with higher levels of the inflammatory marker high-sensitivity C-reactive protein (hsCRP), and would similarly correlate with the degree of periodontal disease-associated inflammation.

4.3 Materials and Methods

Study subjects

The 284 subjects in this study were from the Periodontitis And Vascular Events (PAVE) cohort. These subjects had both periodontal disease and history of recent cardiovascular disease. The study was approved by the relevant institutional review boards, and all participants gave written informed consent. The PAVE pilot study, conducted from January 2003 to June 2005, was originally designed to determine the feasibility of a randomized secondary prevention trial to investigate the effects of periodontal treatment on inflammation and risk of future cardiovascular events. This was a multicenter trial conducted at the University at Buffalo, the University of North Carolina at Chapel Hill, Boston University, Kaiser Permanente Center for Health Research/Oregon Health and Science University, and the University of Maryland. Written informed consent was obtained from subjects prior to enrollment in the PAVE study. Complete information on the PAVE pilot study can be found in several previous publications (136-138).

Inclusion criteria for the PAVE study included mild to moderate periodontitis and recent evidence of coronary heart disease (136). Mild to moderate periodontitis was defined as having a minimum of six natural teeth with at least three teeth having a pocket depth of ≥ 4 mm, at least two teeth having interproximal attachment loss of ≥ 2 mm, and at least 10% of dental sites exhibiting bleeding on probing. Cardiovascular criteria were 50% or more blockage of a coronary artery or a history of a coronary event within three to 36 months prior to enrollment. Coronary events

included myocardial infarction, coronary artery bypass graft surgery, and coronary transluminal angioplasty with or without a stent. Participation was limited to those aged 75 and below.

Data collection

All data and laboratory samples used in this study were collected at baseline, prior to dental treatment. As a part of the PAVE study, serum levels of hsCRP were measured using latex-enhanced nephelometry as described (138). This automated assay has been approved for use in assessing cardiovascular risk by the United States Food and Drug Administration.

Measures of periodontal inflammation

Thorough periodontal examinations were performed on PAVE subjects as previously described (138). Periodontal data was missing for three subjects. Estimates of periodontal disease severity used for these analyses came from the PAVE dataset and included extent of pocket depth greater than or equal to 5 mm, extent of bleeding on probing, and number of sites with evidence of gingivitis, as indicated by a Gingival Index score of 1 or more.

We examined whether γ' fibrinogen level was associated with the severity of the periodontal disease, particularly the inflammatory component, using the clinical data collected for the PAVE study. A study by Beck and Offenbacher indicates that the useful clinical variables for estimating systemic inflammatory exposure relevant to cardiovascular disease include the extent of bleeding on probing and an extent of

pocket depth ≥ 5 mm (139). In addition to these variables, we examined whether the number of dental sites with tissue inflammation, identified as sites with a gingival index > 0, was associated with γ' fibrinogen or CRP.

Total and γ' fibrinogen measurements

Plasma γ' fibrinogen levels were measured by enzyme-linked immunosorbent assay using a modification of the method described by Lovely *et al*. (112). Briefly, the γ' fibrinogen in subject plasma samples and standards were captured in 96-well plates using the 2.G2.H9 monoclonal antibody and detected using horseradish peroxidase (HRP)-conjugated sheep anti-human fibrinogen (Accurate Chemical, Westbury, NY, USA) along with high sensitivity HRP colorimetric substrate and stop solution (BioFX Laboratories, Owings Mills, MD, USA). Total fibrinogen levels were measured in a similar fashion as previously described (90).

Statistical analysis

Statistical analyses were performed using SPSS (17.0). CRP, γ' fibrinogen, and total fibrinogen levels were log transformed when analyzed as dependent variables by linear regression. Differences in mean γ' fibrinogen levels between groups were assessed by t-test or ANOVA after log-transformation. The relationship between γ' fibrinogen and CRP was assessed using a multivariate linear regression model. Further analyses were performed using logistic regression with CRP as a dichotomized variable, with a cut-off of > 3 mg/l defined as "high CRP" according to the three-level definition by Ridker (140), with adjustment for body mass index (BMI) and total fibrinogen levels. Correlations between γ' fibrinogen, CRP, or total

fibrinogen, and periodontal disease variables were examined using linear regression. Missing data were very limited, at less than 2.2% for all analyzed variables, and were excluded from analyses.

4.4 Results

We measured plasma levels of γ' fibrinogen in a group of 284 subjects from the PAVE study with both periodontitis and CVD. The average age of the subjects was 59 and they were predominantly white and male (Table 4.1). There were relatively large proportions of current (16.2%) and former (52.7%) smokers, which is not unexpected as smoking is a major risk factor for both cardiovascular and periodontal disease.

Table 4.1. Characteristics of the PAVE cohort.

Characteristic	Value
Age (n=284)	59.2 ± 8.9 years
Gender (n=284)	
Female	78 (27.5%)
Male	206 (72.5%)
Ethnicity (n=281)	
Non-Hispanic	275 (96.8%)
Hispanic	6 (2.1%)
Race (n=282)	
White	238 (84.4%)
Black	33 (11.7%)
Other	11 (3.9%)
Smoking (n=283)	
Current smoker	46 (16.2%)
Past smoker	149 (52.7%)
Never smoked	88 (31.1%)

The mean (SE) plasma γ' fibrinogen level in the PAVE subjects was 0.622 (0.017) mg/ml, with females having a slightly higher level than males, 0.690 (0.036) vs. 0.597 (0.019) mg/ml, respectively (p=0.022). The mean (SE) plasma total fibrinogen level was 3.70 (0.074) mg/ml. In this cohort, γ' fibrinogen represented on average 18.6 (0.62) % of total plasma fibrinogen.

We investigated the relationship between γ' fibrinogen, total fibrinogen, and several cardiovascular risk factors previously measured in this cohort, including hsCRP. The γ' fibrinogen concentration was significantly correlated with CRP level, while there was no significant association between γ' fibrinogen and total fibrinogen, BMI, age, smoking status, or type II diabetes in this model or when evaluated separately (Table 4.2). The Pearson correlation coefficient between γ' fibrinogen and total fibrinogen was 0.098 ($p = 0.099$) after log transformation of both variables.

Further evaluation of the relationship between γ' fibrinogen and hsCRP showed significant correlation ($p = 0.006$) after adjustment for age, gender, smoking status, BMI, type 2 diabetes, and total fibrinogen, with a one unit increase in CRP associated with a 1.9% increase in γ' fibrinogen. Additionally, the γ' fibrinogen level was highly associated ($p < 0.001$) with having a high CRP level of > 3 mg/l, which indicates an elevated risk for future cardiovascular events (140). A 0.1 mg/ml increase in γ' fibrinogen raised the odds of being in this high-risk group by 20% ($p<0.001$, OR 95% CI: 1.086 – 1.321) in a model that included BMI and total fibrinogen.

Table 4.2. Relationship between γ' fibrinogen and cardiovascular disease risk factors.

Risk factor	Mean γ' fibrinogen concentration (SD)	P-value*
Gender		0.022
Female [n=78]	0.690 (0.320)	
Male [n=206]	0.597 (0.270)	
Age		0.631
≤ 55 [n=92]	0.629 (0.287)	
56 – 64 [n=104]	0.613 (0.310)	
≥ 65 [n=88]	0.627 (0.262)	
BMI		0.391
< 25 [n=38]	0.554 (0.201)	
25 – 29.99 [n=118]	0.614 (0.266)	
≥ 30 [n=122]	0.651 (0.326)	
Smoking status		0.394
Nonsmoker [n=88]	0.595 (0.257)	
Former smoker [n=149]	0.620 (0.282)	
Current smoker [n=46]	0.686 (0.351)	
CRP (mg/dl)		<0.001
< 1 [n=94]	0.583 (0.282)	
1 – 3 [n=95]	0.552 (0.228)	
> 3 [n=90]	0.731 (0.323)	
Total fibrinogen (mg/ml)		0.091
< 3.077 [n=94]	0.575 (0.261)	
3.077 – 4.050 [n=96]	0.627 (0.279)	
> 4.050 [n=94]	0.665 (0.287)	
Type 2 diabetes		0.937
Absent [n=221]	0.623 (0.285)	
Present [n=59]	0.628 (0.301)	

*P-value for group differences in mean log-transformed γ' fibrinogen. All variables were assessed independently.

Analysis of the relationship between these markers and clinical measures of periodontitis demonstrated that the extent of pocket depth ≥ 5 mm was associated with CRP (p = 0.042) but not with γ' fibrinogen, while the number of sites with gum inflammation was highly associated with γ' fibrinogen (p = 0.004) but not CRP (Table 4.3). There was no significant association between total fibrinogen and any of the disease variables.

Table 4.3. Relationships between periodontal disease variables and log-transformed CRP, γ' fibrinogen and total fibrinogen by univariate regression.

	Periodontal disease variable (n = 281)	Para-meter estimate	SE	P-value
γ' fibrinogen	Extent of PD ≥ 5mm	0.002	0.002	0.383
	Extent of BOP	–0.002	0.002	0.266
	# sites with gingivitis	0.004	0.001	0.004
CRP	Extent of PD ≥ 5mm	0.012	0.006	0.042
	Extent of BOP	0.003	0.005	0.558
	# of sites with gingivitis	–0.002	0.003	0.577
Total fibrinogen	Extent of PD ≥ 5mm	0.000	0.002	0.931
	Extent of BOP	–0.002	0.002	0.224
	# of sites with gingivitis	0.000	0.001	0.516

4.5 Discussion

Little is known about the relationship between γ' fibrinogen, an emerging CVD risk factor, and inflammation. Therefore, we measured γ' and total fibrinogen levels in a study sample consisting of 284 subjects from the PAVE study cohort, all with mild to moderate periodontal disease and a recent history of coronary artery disease or CVD event. Our results demonstrate a link between γ' fibrinogen and inflammation as represented by hsCRP and gingivitis.

The mean γ' fibrinogen level in these subjects, 0.622 mg/ml, was the highest seen in any published study to date with the exception of a study by Cheung *et al*. that noted a mean of 0.79 mg/ml in a group of 16 acute phase pulmonary embolism patients (119). Our finding, in conjunction with the relatively normal mean total fibrinogen of 3.70 mg/ml, supports the concept that γ' fibrinogen is particularly elevated in the setting of chronic inflammation. A striking finding is that γ' fibrinogen represented, on average, 18.6% of total plasma fibrinogen in this population. This is over twice the normal percentage of γ' fibrinogen in healthy blood donors, in which γ' fibrinogen represents only 7.2% of total plasma fibrinogen (90), and in individuals in the Framingham Offspring Study, in which γ' fibrinogen represents 6.8% of total plasma fibrinogen (112). These results suggest that γ' fibrinogen may display differential regulation from total fibrinogen in the presence of combined cardiovascular and periodontal disease.

While γ' fibrinogen was significantly associated with hsCRP as both a continuous and a dichotomized variable, no significant correlation was found with other

cardiovascular risk factors measured. This contrasts with the findings of our recent study of γ' fibrinogen in the Framingham Offspring cohort, in which γ' fibrinogen was associated with a number of traditional cardiovascular risk factors, including age, gender, BMI, smoking status, diabetes and total fibrinogen (112). These disparate results are likely due to the major differences between the PAVE cohort and the community-based Framingham Offspring cohort, particularly the fact that all of the PAVE subjects had significant cardiovascular and periodontal disease at the time of data collection, while only a relatively small fraction of community subjects would be expected to have similar conditions.

Our investigation into the relationship between γ' fibrinogen and periodontal disease-associated inflammation was hampered by a lack of validated measures of this inflammation. Periodontal disease has been the subject of extensive study, but until fairly recently this research has focused on the disease as an outcome, and not as a risk factor for other conditions, such as cardiovascular disease. Beck and Offenbacher attempted to address this problem by looking at correlation between dental variables and markers of cardiovascular risk (139). They saw an association between the extent of bleeding on probing and serum soluble intercellular adhesion marker, and between extent of pocket depth ≥ 5 mm and serum CRP level. In our study we also examined correlations between the number of sites with gingivitis and the analytes CRP, total and γ' fibrinogen. We did this with the idea that the number of dental sites with tissue inflammation may be a better marker of current (and not historic) inflammatory burden. Our findings replicated the association

between the extent of pocket depth ≥ 5 mm and CRP levels, and additionally we saw correlation between γ' fibrinogen and the number of sites with gingivitis.

One of the main limitations of the present study is the lack of a control group of subjects. This makes it impossible to assess the individual contributions of CVD and periodontitis on the variables measured. Another major difficulty is the lack of validated clinical measures of periodontal disease severity that are known to associate with cardiovascular risk. Additionally, the discontinuation of the PAVE pilot study has prevented analysis of the effect of periodontal treatment on inflammation and γ' fibrinogen levels, and assessment of the relationship between these variables and future cardiovascular events.

In conclusion, the link between γ' fibrinogen and inflammation is intriguing, particularly in light of the growing literature associating γ' fibrinogen with thrombotic disease (90, 110-112, 114, 116, 117, 119, 141, 142). The lack of association between γ' fibrinogen and total fibrinogen levels seen in this study and a previous case-control study of CAD (90) contrasts with the correlation between both fibrinogen isoforms seen in a large community-based cohort (112), and this may indicate a dysregulation of these isoforms under pathological conditions. Further work will be needed to determine whether γ' fibrinogen is indeed regulated independently of total fibrinogen in an inflammatory setting, and, if so, what mechanisms are involved.

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Chapter 5. Biologic Variability in Biochemical Markers of Cardiovascular Risk

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Jon Oberdorf contributed to the measurement of γ' fibrinogen and the writing of the paper.

David Farrell contributed to the experimental design, research funding, and editing of the paper.

5.1 Abstract

Context: A variety of biochemical markers are commonly measured to help predict the risk of cardiovascular disease. Unfortunately, these markers often fail to identify many of the individuals that will go on to experience a cardiovascular event. As a result, the search continues for other markers of cardiovascular disease demonstrating greater predictive value for risk assessment. The clinical utility of any marker used as a risk assessment tool is dependent upon the long and short-term biologic variability that the marker shows in different individuals.

Objective: To assess the sources of variance in the measurement of commonly used biochemical cardiovascular risk markers, including total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, high-sensitivity C reactive protein (hsCRP), fibrinogen, and the γ' isoform of fibrinogen, recently shown to display good predictive utility for cardiovascular risk.

Design: In this study we measured a panel of currently used biochemical cardiovascular risk markers and γ' fibrinogen in blood samples collected from healthy individuals over the course of one year.

Setting: Oregon Health & Science University, an academic medical center in Portland, Oregon.

Participants: 15 apparently healthy individuals ranging in ages from 21 - 54.

Outcome Measure: We estimated the within- and between-subject variability for each of these markers. These estimates were used to calculate intra-class correlation, reliability, and indices of individuality for the markers.

Results: HDL cholesterol demonstrated the best utility for risk assessment as it had the lowest variability profile, with an intra-class correlation (ICC) of 0.84. hsCRP showed the highest levels of both within- and between-subject variability, with an ICC of 0.37. Stated differently, it would require eight separate measurements of hsCRP performed on blood collected over multiple days to provide the risk assessment information provided by a single sampling and measurement of HDL cholesterol.

Conclusions: The variability profile of γ' fibrinogen was very similar to that of LDL cholesterol, indicating that a limited number of measurements are necessary to establish an individuals' baseline concentration for assessing risk. hsCRP did not perform well in our study as this marker showed very high biological variability. A single measurement of hsCRP lacks sufficient clinical utility to justify routine measurement.

5.2 Introduction

Clinical laboratory tests provide information for a variety of purposes, including diagnosis, monitoring of disease, and risk assessment. With respect to the latter, a number of biochemical markers have been proposed for assessment of the risk of cardiovascular disease (CVD). The prognostic utilities of markers of risk assessment are often based on epidemiologic studies involving thousands or tens of thousands of individuals. However, in order for any marker of risk assessment to have good clinical utility in an individual subject, the biological variability of the marker must be low enough to enable appropriate risk stratification for each individual using as few serially-collected blood samples as possible.

The level of variability that is acceptable for an analyte depends primarily on two parameters; the analytical variation that is observed in the measurement of the analyte, and the within-subject and between-subject biologic variation that the analyte shows over time. In order for an analyte to have good clinical utility for risk assessment in an individual, the variability associated with measurement of the analyte should be relatively small compared to the within- and between-subject variance. Analytes with a large between-subject (inter-individual) variability relative to the other sources of variance are generally considered to be more reliable, and allow better estimation of an association of the marker with disease (143).

Serum high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol, non-HDL cholesterol, triglycerides, fibrinogen,

and basal hsCRP concentrations have been identified as risk markers for CVD in numerous large-scale epidemiological studies. However, some of these analytes show significant differences in biologic variability, which adversely impacts the prognostic utility when a single measurement of the analyte is performed in an individual patient.

In an effort to assess the impact of biologic variability of various cardiac risk markers on their prognostic utility, we examined the short and long-term biologic variability of several markers of cardiovascular risk in a cohort of 15 healthy adults. We measured the concentrations of serum HDL, LDL, total cholesterol, triglycerides, hsCRP and total fibrinogen over the course of one year in order to determine the biologic variability of each of these markers.

In addition to the currently used risk markers described above, we also measured γ' fibrinogen concentrations. The γ' isoform of fibrinogen has been proposed as a cardiovascular risk marker independent of total fibrinogen. This isoform, which represents approximately 10% of an individual's circulating fibrinogen, has a high affinity thrombin-binding site that may mediate its effect on thrombosis risk. Studies have linked it to coronary artery disease (90), myocardial infarction (111, 113), and ischemic stroke (117). In this study we examine the biologic variability of this analyte for the first time, and compare it to biomarkers currently used for assessment of CVD risk.

5.3 Materials and Methods

Study subjects

We recruited 15 apparently healthy adults (9 women and 6 men) to participate in a 12-month study designed to quantify the magnitude of changes in commonly measured risk factors for CVD. Subjects ranged in age from 21 to 54 years at the time of recruitment. Information was collected on each participant's general health and medication use. Individuals taking cholesterol-lowering medications were excluded. The institutional review board of the Oregon Health & Science University approved the study, and all subjects provided written informed consent.

Blood sample collection

Venous blood samples were collected from each participant between 0700 and 1000 following an overnight fast. Participants were encouraged to reschedule blood collection if they felt ill. Samples were collected on days 1, 3, 5, 7, 14, 21 and 28, and then monthly for one year. Thus, a total of 18 samples were collected over one year for subjects who completed the protocol.

Blood was collected into plain evacuated serum tubes, EDTA tubes, and citrate tubes depending on the analyte to be measured. Blood samples were processed within 45 minutes of collection, and serum and plasma were aliquoted into 1.5 ml screw-cap tubes for processing and storage. Samples were stored at 4°C for measurements made within 24 hours, and stored long term at -70 °C.

Measurement of cardiac risk factors

Analysis of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and hsCRP was performed on serum samples within 24 hours of blood collection at the Oregon Health & Science University clinical chemistry laboratory. Aliquots of samples were stored at -70 °C until measurement of other markers. Measurement of total fibrinogen and γ' fibrinogen was performed in batch mode using previously frozen citrate plasma. Fibrinogen is stable under these storage conditions (144, 145). Non-HDL cholesterol was calculated as total cholesterol – HDL cholesterol.

In addition to the measurement of, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and hsCRP within 24 hours following collection, these same analytes were also measured again in batch mode from all study participants following conclusion of the study. This batch analysis was performed in order to ascertain that there was no systematic degradation in any of the analytes that would contribute to the observed biological variation

Analysis of γ' fibrinogen was performed using a microsphere-based bioassay. The capture antibody 2.G2.H9, specific for the fibrinogen γ' chain, was covalently coupled to Microplex™ Microspheres (Luminex Corp.). Approximately 2,500 of these microspheres/well were then pre-loaded onto 96 well filter plates (Millipore #MABVN1250) and excess buffer was removed by vacuum. 100 μl samples of diluted plasma (1:1000 dilution in assay buffer: PBS + 0.1%BSA/ 0.1% Tween/0.05% sodium azide/ 1mM EDTA) were then added to each well. Filter plates were incubated for 2 hours at room temperature with gentle shaking, and

washed 3 times with assay buffer. Microsphere samples then underwent two sequential 30 minute incubations with 100 μl of 4 μg/ml biotinylated anti-fibrinogen polyclonal antibody (Innovative IASHFBGN-GF-BIO) followed by 4 μg/ml Streptavidin-R-Phycoerythrin (Life Technologies S-866). The filter plates were then washed 5 times, the beads resuspended in 100 μl/well of assay buffer, and median R-Phycoerythrin fluorescence/microsphere was determined by flow cell fluorometry using a Luminex™ 200 system. The γ' fibrinogen concentrations of plasma samples were determined by comparing their values to those of duplicate calibrators run with each plate. Curve fitting of calibrators and back calculation of plasma values were performed using Luminex Xponent™ software (weighted 5-parameter logistic analysis).

Statistical analysis

Between-subject (s_G^2) and within-subject, or biologic, (s_I^2) variance components were estimated using a random effects analysis of variance fit with a maximum likelihood estimation method using SPSS (v20). For this study, it was assumed that there was no systematic within-subject error, and that the within-subject variance remained constant at differing analyte concentrations. The within-subject variance estimated included method-dependent analytical error. Measured values of hsCRP >10 mg/L were excluded from analysis, as these likely represented transient inflammation due to illness.

To allow for comparisons between tests, the variance values were expressed as intra-individual (CV_I), and inter-individual (CV_G) coefficients of variation (CV) for

this population. The CV was calculated as 100% x (standard deviation / mean). The variance estimations were used to calculate the intra-class correlation coefficient, a reliability measure defined as the proportion of the total variance that can be attributed to between-subject variance: $s_G^2 / (s_I^2 + s_G^2)$. The Index of Individuality, defined as the ratio of the within-subject variance to the between subject variance (CV_I / CV_G) was also calculated.

The validity coefficient, which represents the difference between a measured value and the true value due to variability, can be used to generate another useful indicator of a marker's clinical utility. The validity coefficient was calculated as $(1 / (1 + (s_I^2 / ks_G^2)))^{1/2}$, with k = number of measurements performed on an individual. Thus, using a set value for the validity coefficient, the minimum number of measurements required to reach this value was determined.

5.4 Results

The mean age of the study subjects was 39.9 years and the median age was 39 years. Two participants were unable to complete the protocol; one moved away from the study site and another was disqualified after beginning treatment with statins for high cholesterol. The mean number of blood samples per subject was 16.9, and the lowest number of samples from any subject was 11.

The mean, standard deviation, and coefficients of variation for each of the cardiovascular biomarkers are shown in Table 1. Total cholesterol had the lowest within-subject variance, with a CV_I of 7.6, followed by non-HDL cholesterol (CV_I =9.4) and total fibrinogen (CV_I = 10.8). The low within-subject variance for non-HDL cholesterol is not surprising, given the low values for this source of variance seen in both total and HDL cholesterol, from which it is calculated. hsCRP exhibited the highest within-subject variance by far, with a CV_I of 71.7. The second highest CV_I of 29.3 was for triglycerides. Between-subject variance showed a similar trend, with the lowest values seen for total fibrinogen and cholesterol, and the highest for hsCRP and triglycerides. The overall variance for hsCRP was much higher than for the other analytes, with a standard deviation nearly equal to the mean value.

Table 5.1. Descriptive statistics for CVD risk markers measured in a cohort of 15 apparently healthy adults over a 1-year period

Biomarker	Mean	SD	CV_I	CV_G
Cholesterol (mg/dL)	191.3	27.9	7.6	12.1
LDL (mg/dL)	126.2	28.1	10.3	19.3
HDL (mg/dL)	47.2	13.0	11.1	25.2
Non-HDL Cholesterol	144.1	29.5	9.4	18.4
Triglycerides (mg/dL)	89.6	40.5	29.3	33.5
CRP (mg/L)	1.62	1.47	71.7	55.5
Fibrinogen (mg/dL)	300.4	47.7	10.8	11.8
γ' Fibrinogen (mg/dL)	22.9	6.8	14.3	27.4

SD = standard deviation, CV_I = within-subject coefficient of variation, CV_G= between-subject coefficient of variation.

The proportions of the total variance attributed to each source are presented in Table 2. The variance data for each of these biomarkers were also used to calculate the Index of Individuality (II) and intra-class correlation coefficient (ICC). The Index

of Individuality provides a measure of the within-subject variability relative to between-subject variability. The ICC is a reliability measure that describes the relative correlation of repeated measurements for a single individual (the $CV_G\%$ = ICC x 100%). By definition, these two indicators are inversely related to one another. In this study, the II ranged from 0.44 for HDL to 1.29 for hsCRP, and the ICCs were between 0.38 (hsCRP) and 0.84 (HDL). The II and ICC for γ' fibrinogen were 0.52 and 0.79, respectively, while these values for total fibrinogen were 0.91 and 0.55, respectively. This reflects the finding that γ' fibrinogen has a reduced proportion of within-individual variance, which is approximately half that of total fibrinogen. Of note, the II and ICC calculated for total fibrinogen were nearly identical to the II of 0.92 (146) and the ICC of 0.56 (147) found in similar studies.

Table 5.2. Index of Individuality, intra-class correlation coefficients, and variance components as a percentage of total variance for CVD risk markers

Biomarker	Index of Individuality	ICC	CV_I %	CV_G %
Cholesterol	0.63	0.72	28.4	71.6
LDL	0.53	0.78	22.0	78.0
HDL	0.44	0.84	16.3	83.7
Non-HDL Cholesterol	0.51	0.80	20.5	79.5
Triglycerides	0.87	0.57	43.4	56.6
CRP	1.29	0.37	62.5	37.5
Fibrinogen	0.91	0.55	45.4	54.6
γ' Fibrinogen	0.52	0.79	21.4	78.6

To gain insight into the impact of these variability data on the clinical utility of these measures, the validity coefficient (VC) for a single measurement of a biomarker was calculated, as well as the number of samples needed for a biomarker with the same VC as HDL cholesterol (0.91), which showed the highest value for this measure. For the other biomarkers, only two measurements were needed to achieve a VC that was similar to HDL. However, for triglycerides, fibrinogen, and hsCRP more than

two measurements were needed, with hsCRP requiring a total of eight separate measurements to achieve the same VC as that provided by a single measurement of HDL cholesterol.

Table 5.3. Validity coefficients (VC) and the number of repeated measurements needed to reach a VC of 0.91 (that of HDL cholesterol) in a study with 15 apparently healthy adults. The number of measurements is rounded up to the nearest integer.

Biomarker	Validity Coefficient	# of sample measurements for VC = 0.91
Cholesterol	0.85	2
LDL	0.88	2
HDL	0.91	1
Non-HDL Cholesterol	0.89	2
Triglycerides	0.75	4
hsCRP	0.61	8
Fibrinogen	0.74	4
γ' Fibrinogen	0.89	2

The use of hsCRP in cardiovascular risk prediction is based upon the categorization of hsCRP values, with concentrations < 1 mg/L considered "low risk", 1 – 3 mg/L "moderate risk", and values greater than 3 up to 10 mg/L considered "high risk" (148). Thus, in addition to examining hsCRP's variability profile, we also evaluated

the likelihood of misclassification of risk of CVD when using a single measurement of hsCRP. We assumed that an individual's mean hsCRP value obtained from all measurements performed over the course of this study (excluding values >10 mg/L) represented the correct risk category. Next, we calculated the number of single hsCRP measurements that would have resulted in incorrect risk classifications for that subject. We found an overall misclassification rate of 25% for hsCRP (see table 4).

Table 5.4. Misclassification of CRP risk category based on individual measurements, with a subject's mean CRP value used to determine correct risk category

Subjects in each CRP risk category (# of measurements)	Number (%) misclassified "low risk"	Number (%) misclassified "moderate risk"	Number (%) misclassified "high risk"
Low risk 6 (100)	*	18 (18.0%)	2 (2.0%)
Moderate risk 8 (118)	15 (12.7%)	*	16 (13.6%)
High risk 1 (13)	3 (23.1%)	3 (23.1%)	*

5.5 Discussion

The estimated direct and indirect costs of cardiovascular disease (CVD) and stroke in 2010 were $503.2 billion (149). While the average cost of a single lipid profile is relatively low when compared to the direct and indirect costs of CVD, the cumulative costs of screening can be substantial, especially if biochemical markers of risk assessment require multiple repeated measurements to establish an individual's true baseline.

The variability profile of a risk marker is an important factor in determining the clinical utility of a single measurement, but this attribute tends to be overlooked, with more focus placed on disease-association levels in large epidemiological studies. These studies are extremely valuable in identifying potential biomarkers of risk assessment, but do not necessarily provide insight into the reliability of a single measurement performed in an individual patient, an important practical consideration (150).

We measured CVD risk markers in a cohort of 15 apparently healthy individuals over the course of a year to directly compare their within- and between-individual variability. Of the risk markers measured, HDL cholesterol had the best variability profile for a prognostic biomarker, with the majority of its variability due to differences between subjects, and a relatively small variance observed within a single individual. These characteristics gave HDL the highest value for both the interclass-correlation and the validity coefficients.

In contrast, hsCRP measurements demonstrated a high level of variability, both between subjects and within individuals. The within-individual variance of hsCRP was nearly identical to that seen by Clark and Fraser, who found a CV_I of 63% (151), but was higher than that found in other studies, even after exclusion of values >10 mg/L (146, 152). This is probably due in some part to the small size and general level of good health in our cohort, as well as the duration of the study and number of blood samples analyzed. Sakkinen et al. saw a marked increase in the intraindividual CV for hsCRP at 24 weeks of follow-up, compared to 6 weeks, demonstrating the strong effect of study duration on this variance parameter (146).

An increasing number of studies have questioned the clinical utility of hsCRP in assessing risk of CVD where measurement of this marker did not improve the CVD risk estimation when added to other established risk factors (153-157). This is due in large part to the high intraindividual variability of this analyte. The within-subject standard deviation for hsCRP in our study was 2.7 mg/L. While others have reported smaller values for this estimate, it is still quite high relative to the cut-off values for risk classification of <1 mg/L, 1-3 mg/L, and >3 mg/L, resulting in a considerable chance of misclassification, and making any assessment of a "real" change in a an individual's baseline hsCRP level nearly impossible (153). We found that it would require 8 replicate measurements of hsCRP to achieve the validity of a single measurement of HDL cholesterol in our cohort, and some have suggested that even more repeated measures might be necessary for establishing an individual's true hsCRP value (158).

The γ' isoform of fibrinogen has been gaining attention as a potential CVD risk factor. Recent research has also suggested a connection between γ' fibrinogen and inflammation (159), with increased concentrations seen in the acute phase of pulmonary embolism and stroke (119), indicating that it may be useful as a marker of inflammatory processes as well. In our study, the variability profile of γ' fibrinogen was quite similar to that of LDL cholesterol, which could make it a more attractive risk marker than total fibrinogen for clinical risk assessment.

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Chapter 6. Summary and Conclusions

Increasingly, γ' fibrinogen is gaining recognition as a potentially valuable risk marker for CVD. This isoform of the blood coagulation protein, fibrinogen, is produced when a splice variant of the fibrinogen γ chain is incorporated into the molecule, and it represents approximately 10% of an individual's circulating fibrinogen (90). The γ' chain extended C-terminus includes a high affinity thrombin binding site lacking in the more common γA chain, and may also mediate binding to FXIII, a fibrin cross-linking enzyme. Evidence from *in vitro* experiments indicates that γ' fibrinogen forms clots with increased stiffness and resistance to enzymatic breakdown, as compared to γA/γA fibrinogen (108, 109), and studies have found associations between increased γ' fibrinogen levels and several forms of CVD, including CAD, ischemic stroke, and MI (90, 110, 111, 117). Recent work by Cheung *et al*. indicates that γ' fibrinogen may be elevated in the setting of acute inflammation (119).

One of the major mechanisms by which γ' fibrinogen is proposed to modify cardiovascular risk is through the binding of thrombin. Thrombin is the main activating enzyme in the coagulation cascade (see figure 1.3) and is also important for activating platelets. While all fibrin(ogen) molecules contain a low-affinity binding site for thrombin, the extended C-terminus of the γ' chain binds to thrombin's exosite II with higher affinity. Previous studies have implicated certain regions of the peptide as important for this binding (125, 126, 130) and a crystal

structure of thrombin in complex with a γ' C-terminal peptide, published in 2007 by Pineda *et al*. identifies possible contacts between the peptide and exosite II (131).

To test the predictions of the crystal structure and more generally investigate the nature of this interaction, the binding of thrombin to peptides corresponding to the γ' chain C-terminus was measured using a Biacore T100 surface plasmon resonance instrument (see Chapter 3). The affinity of thrombin for the γ' C-terminal peptide, PEHPAETEpYDSLpYPEDDL (where pY = phosphotyrosine), and analogue peptides with charged-to-uncharged substitutions was determined. While the Biacore T100 can also be used to study the kinetics of binding, the rapid k_{on} and k_{off} of the γ' peptide-thrombin interaction was beyond the measurement limits for the instrument.

An overall affinity ($K_d \pm SE$) of 0.440 ± 0.011 μM was found for thrombin and the unsubstituted γ' peptide. Experiments using the analogue peptides generally supported the predictions of the crystal structure, indicating that while some of the individual peptide residues contributed more to thrombin binding than others, with the substitution of Asp419Asn and pTyr422Tyr having the most impact, no single-residue substitutions substantially reduced the binding affinity. Multiple substitutions, however, either of the three residues that appear most important for binding (pTyr418, Asp419, and pTyr422), or of all charged residues but these three, completely abrogated the interaction. This demonstrated that while these three residues are required, they are not sufficient for the binding in the absence of the other charges. Thus, the interaction between the γ' C-terminus and thrombin

appears to be mediated by the ensemble of the charges, a product of general electrostatic complementarity. In support of this, experiments measuring the binding between the unsubstituted peptide and thrombin in the presence of increasing salt concentrations showed a significant salt dependence for the interaction, with an estimated 3-4 solvent ion pairs released upon binding.

While this research has helped to clarify the nature of the interaction between the γ' chain C-terminus and thrombin, more work will need to be done to understand the physiological ramifications of this binding. In blood, thrombin binds to the complete fibrin(ogen) molecule, utilizing both the low- and high-affinity binding sites. The kinetics of this binding remains to be explored. In addition, it appears that the binding of the γ' chain to thrombin's exosite II has functional consequences for thrombin, altering its ability to cleave coagulation factor VIII, fibrinogen, and the platelet receptor, PAR1 (97, 98, 127). Fibrinogen-bound thrombin may also be protected from inactivation by antithrombin III (99). The overall impact of γ' fibrinogen-thrombin binding is matter of debate, with some speculating that γ' fibrinogen may help localize thrombin to the growing clot, enhancing coagulation (160), and others proposing that the main consequence of this binding is the sequestration of thrombin, with γ' fibrinogen acting as an anticoagulant (161).

While atherosclerotic CVD is now recognized to be an essentially inflammatory disease, until recently there had been no investigation into the relationship between γ' fibrinogen and inflammation. In 2008, Cheung *et al*. published a report showing elevated γ' fibrinogen levels during the acute phase of stroke (117). At this time, we

were conducting our own research on γ' fibrinogen and inflammation, using data and blood samples collected for the PAVE study.

The PAVE cohort consists of approximately 300 subjects with both coronary heart disease and mild to moderate periodontitis. As part of the PAVE study, the participants' baseline medical and dental data were recorded, plasma samples were taken, and CRP levels were measured. We measured levels of γ' and total fibrinogen in the plasma samples by ELISA, and tested for correlations between γ' fibrinogen, total fibrinogen, CRP, and gum tissue inflammation (see Chapter 4).

The levels of γ' fibrinogen found in this cohort were higher than have previously been reported in any group, with a mean value of 0.622 (0.017) mg/ml. The γ' fibrinogen concentration was significantly correlated with both CRP and the extent of gum tissue inflammation. There was no association between γ' fibrinogen and total fibrinogen in this group, nor was γ' fibrinogen associated with any of the other cardiovascular risk factors measured in this group. This lack of association is likely due to the high CVD-risk status of the cohort, with the presence of both cardiovascular and periodontal diseases blunting correlations between the risk factors.

The high level of γ' fibrinogen found in this group, along with the association between γ' fibrinogen and inflammation, suggest that this isoform may be differentially regulated in the setting of chronic inflammation as well as in the acute phase. This hypothesis is supported by data from studies indicating that γ' fibrinogen levels are correlated with those of total fibrinogen in large, population-

based cohorts (111, 113), but not in smaller groups with more acute disease (118, 119, 159). Additionally, experiments from our lab indicate that IL-6, a major inflammatory cytokine known to up-regulate fibrinogen synthesis, may disproportionately increase the production of γ' fibrinogen (unpublished data). At maximal doses of IL-6, γA mRNA showed a 3.6-fold increase in γA mRNA, but γ' mRNA showed an 8.3-fold increase in liver cells.

While there is currently little known about the regulation of alternative splicing in the γ' chain pre-mRNA, there are a number of potential mechanisms by which the balance of spliceoforms could be shifted. The definition of exons and the recruitment of the spliceosome typically require many proteins binding to enhancer and silencer elements in the pre-mRNA. Although the interactions between these proteins and the RNA are often weak, the actions of multiple proteins binding to other RNA-binding proteins as well as to RNA elements allow for remarkable specificity. Changes is splicing could therefore occur through regulation of splice silencer elements that block access to splice sites or prevent the binding of splice activators, and alteration of the relative balance or positions of splice activators and inhibitors (162). Modification of the phosphorylation status of these proteins is a common regulatory mechanism known to influence alternative splicing. Pre-mRNA editing in which a cytosine or adenosine is deaminated can also alter splicing by changing the nucleotide sequence. This type of RNA editing has been shown to regulate the expression of variants of apolipoprotein B and other proteins (163). In addition, studies have found that simply changing the rate of transcription can affect splicing (164, 165).

The hypothesis that splice-site selection in the γ' chain mRNA is shifted as a result of inflammatory signaling is not without precedent. There is evidence that a number of inflammatory mediators can alter patterns of alternative splicing in many genes. The cytokines TNF-α and interferon-γ (IFN-γ) causes the upregulation of variant isoforms of the receptor CD44 in epithelial and myelomonocytic cells(166), and these cytokines, along with interleukin-1β (IL-1β) and lipopolysaccharide, increase the production of alternatively spliced inducible nitric-oxide synthase in cultured cells (167). Several labs have also shown that cytokines influence the alternative splicing of fibronectin (168-170).

The mechanisms by which inflammatory cytokines modulate splicing are poorly understood, but likely involve changes in the expression and/or the phosphorylation of serine/arginine-rich (SR) proteins, which are involved in regulating the selection of splice sites. Alteration of SR protein function appears to be a common way that splicing modulated in response to cellular signals (171). Eisenreich et al. recently showed that TNF-α-induced alternative splicing occurs, at least partially, through phosphorylation of SR proteins via activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway (172). Similarly, the SR protein alternative splicing factor 2 appears to be downregulated in inflamed muscle through a TNF-α-dependent pathway (173).

Due to the connections between inflammation and CVD, as discussed in section 1.3, the activation of inflammatory signaling pathways likely represents the process by which many traditional cardiovascular risk factors promote disease. These

connections, in conjunction with the ability of inflammatory mediators to influence alternative splicing patterns, provide a plausible mechanism for a disproportionate increase in γ' fibrinogen relative to total fibrinogen in certain pathological conditions. For example, traditional CVD risk factors associated with γ' fibrinogen levels, such as obesity, smoking, and diabetes (112), induce inflammatory responses that increase circulating levels of cytokines, including TNF-α, IL-6, and other inflammatory mediators (174). As previously described, activation of these cytokine signaling pathways can result in changes in the localization and phosphorylation status of SR proteins, which may lead to changes in splice site selection during the production and processing of the fibrinogen γ chain pre-mRNA. Thus, multiple CVD risk factors, through their convergent activation of inflammatory signaling pathways, could cause significant changes in the γ chain alternative splicing pattern.

Therefore, potential mechanisms by which γ' fibrinogen expression could be disproportionately up-regulated by inflammatory mediators include the activation of splice silencer elements that block access to splice sites or prevent the binding of splice activators, pre-mRNA editing that alters splicing by changing the nucleotide sequence, modulation of the rate of transcription, and changes in the localization and phosphorylation status of SR proteins. Any of these mechanisms could be activated through inflammatory signaling initiated by or concurrent with CVD risk factors, though current evidence points toward an SR protein-mediated process.

Future research will be necessary to delineate the exact mechanisms underlying this apparent dysregulation.

Measurement of γ' fibrinogen in other populations with increased inflammation, such as those with rheumatoid arthritis and Crohn's disease, would further contribute to our understanding of its pathophysiology. Studying the effects of anti-inflammatory medications on γ' fibrinogen could also yield valuable information. If these drugs caused a reduction in the γ' fibrinogen levels, this would not only provide evidence of a mechanistic link between inflammation and this isoform, but may indicate the potential use of γ' fibrinogen in assessing the effectiveness of this type of medication.

If γ' fibrinogen is indeed a marker for inflammation, it may be that its links with CVD are simply a result of the inflammatory nature of the disease process, and not reflective of γ' fibrinogen's role in coagulation. Prospective studies, in which subjects are followed over time, would allow better estimation of the CVD risk associated with γ' fibrinogen, but would not definitively demonstrate a causal role for the protein. Currently, our lab is working on a project using morpholino oligomers to alter the splicing efficiency at the last intron of the FGG mRNA, with the goal of either increasing or decreasing relative quantity of the γ' chain variant in a hepatocyte cell line. If this project is successful, it might one day be possible to use similar technology to influence the production of γ' fibrinogen in humans, and to determine whether this isoform is merely a risk marker or directly contributes to disease.

Cardiovascular risk prediction is important for identifying individuals at high risk of having a cardiovascular event, allowing initiation of preventative therapy. Currently, the Framingham risk score is used to stratify patients into low-, intermediate-, and high-risk groups based on their estimated 10-year risk of a major coronary event. While this tool has proven very useful clinically, many of those who will have an event are not identified as high-risk. This has spurred researchers to study emerging risk factors with the goal of improving the sensitivity of these prediction tools, with the particular aim of appropriately reclassifying intermediate-risk individuals that are actually at high risk.

Because of the connection between inflammation and CVD, inflammatory markers, including CRP, fibrinogen, leukocyte count, and periodontal disease have been a focus of this research, as well as markers of subclinical atherosclerosis, such as coronary artery calcium and carotid intima media thickness. While many of these markers have been shown to be associated with increased CVD risk, this association alone is not sufficient to recommend clinical testing for a marker. Other important considerations are the ability of the marker to reclassify intermediate-risk individuals (allowing different management that reduces their risk), and the cost and reliability of the measurement (175). The biologic variability of a potential risk factor, that is the variability of the marker both within and between individuals, is a major determinant of this reliability.

Previous studies have demonstrated associations between γ' fibrinogen and many forms of CVD, but little was known about the variability of this protein. To address

this we measured levels of γ' fibrinogen, along with total fibrinogen, CRP, and lipids, in a cohort of 15 apparently healthy subjects over a one-year period (see Chapter 5). These data were then used to calculate and compare the within-subject and between-subject variabilities of these markers. HDL cholesterol exhibited the lowest proportion of within-subject variability (ICC = 0.84) and CRP had the highest (ICC = 0.37). In general, a lower proportion of this type of variance is desirable for a clinical risk factor, as it requires fewer measurements to establish a baseline level of the marker for an individual. The relative levels of between- and within-individual variance found for γ' fibrinogen were similar to those of LDL cholesterol, with ICC values for these markers of 0.79 and 0.78, respectively.

The variability profile of γ' fibrinogen in this cohort was favorable for potential clinical measurement. CRP, however, demonstrated a low reliability level, supporting the idea that this marker may lack sufficient clinical utility to justify measurement for the purpose of CVD risk prediction (153-156, 175). Further research will need to be done to determine the value of γ' fibrinogen measurement as an addition to currently used risk factors. Additionally, the variance of this marker in relevant disease states, such as diabetes and CAD, should be assessed. If the γ' fibrinogen isoform is differentially regulated in settings of inflammation, it is likely that the variance profile in these conditions could be quite different from that seen in healthy individuals.

In conclusion, the focus of this dissertation has been the characterization of γ' fibrinogen as a potential CVD risk factor. The electrostatic nature of the interaction

between the γ' chain C-terminus and thrombin was investigated using SPR, the relationship between γ' fibrinogen and inflammation was explored in a cohort with both CVD and periodontal disease, and the variance profiles of this isoform and commonly used biochemical CVD risk factors were estimated in 15 healthy subjects. This research and the current body of evidence supports the hypothesis that γ' fibrinogen is a CVD risk factor, and that its association with CVD is likely mediated by an inflammatory mechanism. To justify the clinical use of this marker, more work will need to be done to determine whether γ' fibrinogen levels are predictive of CVD events, and if these levels can be lowered to reduce CVD risk.

Chapter 7. References

1. Henschen A. Disulfide bridges in the middle part of human fibrinogen. Hoppe Seylers Z Physiol Chem. 1978;359:1757-70.

2. Townsend RR, Hilliker E, Li YT, et al. Carbohydrate structure of human fibrinogen. Use of 300-MHz 1H-NMR to characterize glycosidase-treated glycopeptides. J Biol Chem. 1982;257:9704-10.

3. Burton RA, Tsurupa G, Medved L, et al. Identification of an ordered compact structure within the recombinant bovine fibrinogen alphaC-domain fragment by NMR. Biochemistry. 2006;45:2257-66.

4. Gorkun OV, Veklich YI, Medved LV, et al. Role of the alpha C domains of fibrin in clot formation. Biochemistry. 1994;33:6986-97.

5. Weisel JW, Stauffacher CV, Bullitt E, et al. A model for fibrinogen: Domains and sequence. Science. 1985;230:1388-91.

6. Kant JA, Fornace AJ,Jr, Saxe D, et al. Evolution and organization of the fibrinogen locus on chromosome 4: Gene duplication accompanied by transposition and inversion. Proc Natl Acad Sci U S A. 1985;82:2344-8.

7. Fu Y, Grieninger G. Fib420: A normal human variant of fibrinogen with two extended alpha chains. Proc Natl Acad Sci U S A. 1994;91:2625-8.

8. Takeda Y. Studies of the metabolism and distribution of fibrinogen in healthy men with autologous 125-I-labeled fibrinogen. J Clin Invest. 1966;45:103-11.

9. Crabtree GR, Kant JA, Fornace AJ,Jr, et al. Regulation and characterization of the mRNAs for the A alpha, B beta and gamma chains of fibrinogen. Ann N Y Acad Sci. 1983;408:457-68.

10. Fowlkes DM, Mullis NT, Comeau CM, et al. Potential basis for regulation of the coordinately expressed fibrinogen genes: Homology in the 5' flanking regions. Proc Natl Acad Sci U S A. 1984;81:2313-6.

11. Hu CH, Harris JE, Davie EW, et al. Characterization of the 5'-flanking region of the gene for the alpha chain of human fibrinogen. J Biol Chem. 1995;270:28342-9.

12. Mizuguchi J, Hu CH, Cao Z, et al. Characterization of the 5'-flanking region of the gene for the gamma chain of human fibrinogen. J Biol Chem. 1995;270:28350-6.

13. Yu S, Sher B, Kudryk B, et al. Intracellular assembly of human fibrinogen. J Biol Chem. 1983;258:13407-10.

14. Huang S, Mulvihill ER, Farrell DH, et al. Biosynthesis of human fibrinogen. subunit interactions and potential intermediates in the assembly. J Biol Chem. 1993;268:8919-26.

15. Yu S, Sher B, Kudryk B, et al. Fibrinogen precursors. order of assembly of fibrinogen chains. J Biol Chem. 1984;259:10574-81.

16. Roy SN, Mukhopadhyay G, Redman CM. Regulation of fibrinogen assembly. transfection of hep G2 cells with B beta cDNA specifically enhances synthesis of the three component chains of fibrinogen. J Biol Chem. 1990;265:6389-93.

17. Roy S, Overton O, Redman C. Overexpression of any fibrinogen chain by hep G2 cells specifically elevates the expression of the other two chains. J Biol Chem. 1994;269:691-5.

18. Weisel JW. Fibrinogen and fibrin. Adv Protein Chem. 2005;70:247-99.

19. Esmon CT. The protein C pathway. Chest. 2003;124:26S-32S.

20. Di Cera E. Thrombin. Mol Aspects Med. 2008;29:203-54.

21. Stubbs MT, Bode W. A player of many parts: The spotlight falls on thrombin's structure. Thromb Res. 1993;69:1-58.

22. Pechik I, Madrazo J, Mosesson MW, et al. Crystal structure of the complex between thrombin and the central "E" region of fibrin. Proc Natl Acad Sci U S A. 2004;101:2718-23.

23. Lewis SD, Shields PP, Shafer JA. Characterization of the kinetic pathway for liberation of fibrinopeptides during assembly of fibrin. J Biol Chem. 1985;260:10192-9.

24. Mihalyi E. Clotting of bovine fibrinogen. kinetic analysis of the release of fibrinopeptides by thrombin and of the calcium uptake upon clotting at high fibrinogen concentrations. Biochemistry. 1988;27:976-82.

25. Bilezikian SB, Nossel HL, Butler VP,Jr, et al. Radioimmunoassay of human fibrinopeptide B and kinetics of fibrinopeptide cleavage by different enzymes. J Clin Invest. 1975;56:438-45.

26. Hanna LS, Scheraga HA, Francis CW, et al. Comparison of structures of various human fibrinogens and a derivative thereof by a study of the kinetics of release of fibrinopeptides. Biochemistry. 1984;23:4681-7.

27. Blomback B, Hessel B, Hogg D, et al. A two-step fibrinogen--fibrin transition in blood coagulation. Nature. 1978;275:501-5.

28. Weisel JW, Medved L. The structure and function of the alpha C domains of fibrinogen. Ann N Y Acad Sci. 2001;936:312-27.

29. Weisel JW, Nagaswami C, Makowski L. Twisting of fibrin fibers limits their radial growth. Proc Natl Acad Sci U S A. 1987;84:8991-5.

30. Ariens RA, Lai TS, Weisel JW, et al. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood. 2002;100:743-54.

31. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005;129:307-21.

32. Reed GL, Houng AK. The contribution of activated factor XIII to fibrinolytic resistance in experimental pulmonary embolism. Circulation. 1999;99:299-304.

33. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352:1685-95.

34. Mahmoudi M, Curzen N, Gallagher PJ. Atherogenesis: The role of inflammation and infection. Histopathology. 2007;50:535-46.

35. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-26.

36. Massberg S, Brand K, Gruner S, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med. 2002;196:887-96.

37. von Hundelshausen P, Weber KS, Huo Y, et al. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation. 2001;103:1772-7.

38. Bjorkbacka H. Multiple roles of toll-like receptor signaling in atherosclerosis. Curr Opin Lipidol. 2006;17:527-33.

39. Kruth HS, Huang W, Ishii I, et al. Macrophage foam cell formation with native low density lipoprotein. J Biol Chem. 2002;277:34573-80.

40. Jonasson L, Holm J, Skalli O, et al. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis. 1986;6:131-8.

41. Hansson GK, Jonasson L, Seifert PS, et al. Immune mechanisms in atherosclerosis. Arteriosclerosis. 1989;9:567-78.

42. Holman RL, McGill HC,Jr, Strong JP, et al. The natural history of atherosclerosis: The early aortic lesions as seen in new orleans in the middle of the of the 20th century. Am J Pathol. 1958;34:209-35.

43. Strong JP, McGill HC,Jr, Tejada C, et al. The natural history of atherosclerosis; comparison of the early aortic lesions in new orleans, guatemala, and costa rica. Am J Pathol. 1958;34:731-44.

44. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, american heart association. Circulation. 1994;89:2462-78.

45. Vukovic I, Arsenijevic N, Lackovic V, et al. The origin and differentiation potential of smooth muscle cells in coronary atherosclerosis. Exp Clin Cardiol. 2006;11:123-8.

46. Hegyi L, Skepper JN, Cary NR, et al. Foam cell apoptosis and the development of the lipid core of human atherosclerosis. J Pathol. 1996;180:423-9.

47. Hansson GK, Libby P. The immune response in atherosclerosis: A double-edged sword. Nat Rev Immunol. 2006;6:508-19.

48. Wilson PW. Evidence of systemic inflammation and estimation of coronary artery disease risk: A population perspective. Am J Med. 2008;121:S15-20.

49. El-Gabalawy H, Guenther LC, Bernstein CN. Epidemiology of immune-mediated inflammatory diseases: Incidence, prevalence, natural history, and comorbidities. J Rheumatol Suppl. 2010;85:2-10.

50. Burt B, Research, Science and Therapy Committee of the American Academy of Periodontology. Position paper: Epidemiology of periodontal diseases. J Periodontol. 2005;76:1406-19.

51. Petersen PE, Bourgeois D, Ogawa H, et al. The global burden of oral diseases and risks to oral health. Bull World Health Organ. 2005;83:661-9.

52. Oliver RC, Brown LJ, Loe H. Periodontal diseases in the united states population. J Periodontol. 1998;69:269-78.

53. Borrell LN, Burt BA, Taylor GW. Prevalence and trends in periodontitis in the USA: The [corrected] NHANES, 1988 to 2000. J Dent Res. 2005;84:924-30.

54. Moutsopoulos NM, Madianos PN. Low-grade inflammation in chronic infectious diseases: Paradigm of periodontal infections. Ann N Y Acad Sci. 2006;1088:251-64.

55. Beck JD, Offenbacher S. Systemic effects of periodontitis: Epidemiology of periodontal disease and cardiovascular disease. J Periodontol. 2005;76:2089-100.

56. Tracy RP. Emerging relationships of inflammation, cardiovascular disease and chronic diseases of aging. Int J Obes Relat Metab Disord. 2003;27:S29-34.

57. Danesh J, Collins R, Appleby P, et al. Association of fibrinogen, C-reactive protein, albumin, or leukocyte count with coronary heart disease: Meta-analyses of prospective studies. JAMA. 1998;279:1477-82.

58. Brooks GC, Blaha MJ, Blumenthal RS. Relation of C-reactive protein to abdominal adiposity. Am J Cardiol. 2010;106:56-61.

59. Nakou ES, Liberopoulos EN, Milionis HJ, et al. The role of C-reactive protein in atherosclerotic cardiovascular disease: An overview. Curr Vasc Pharmacol. 2008;6:258-70.

60. Anand SS, Yusuf S. C-reactive protein is a bystander of cardiovascular disease. Eur Heart J. 2010;31:2092-6.

61. Bisoendial RJ, Boekholdt SM, Vergeer M, et al. C-reactive protein is a mediator of cardiovascular disease. Eur Heart J. 2010;31:2087-91.

62. Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008;359:2195-207.

63. Buckley DI, Fu R, Freeman M, et al. C-reactive protein as a risk factor for coronary heart disease: A systematic review and meta-analyses for the U.S. preventive services task force. Ann Intern Med. 2009;151:483-95.

64. Pepys MB, Hirschfield GM. C-reactive protein: A critical update. J Clin Invest. 2003;111:1805-12.

65. Kaski JC, Garcia-Moll X. C-reactive protein as a clinical marker of risk. Circulation. 2000;102:E63-4.

66. Platz EA, Sutcliffe S, De Marzo AM, et al. Intra-individual variation in serum C-reactive protein over 4 years: An implication for epidemiologic studies. Cancer Causes Control. 2010;21:847-51.

67. Green F, Humphries S. Control of plasma fibrinogen levels. Baillieres Clin Haematol. 1989;2:945-59.

68. de Moerloose P, Boehlen F, Neerman-Arbez M. Fibrinogen and the risk of thrombosis. Semin Thromb Hemost. 2010;36:7-17.

69. Norris LA, Bonnar J. Haemostatic changes and the oral contraceptive pill. Baillieres Clin Obstet Gynaecol. 1997;11:545-64.

70. Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, et al. Fibrinogen as a risk factor for stroke and myocardial infarction. N Engl J Med. 1984;311:501-5.

71. Kannel WB, Wolf PA, Castelli WP, et al. Fibrinogen and risk of cardiovascular disease. the framingham study. JAMA. 1987;258:1183-6.

72. Stefanadi E, Tousoulis D, Papageorgiou N, et al. Inflammatory biomarkers predicting events in atherosclerosis. Curr Med Chem. 2010;17:1690-707.

73. Papageorgiou N, Tousoulis D, Siasos G, et al. Is fibrinogen a marker of inflammation in coronary artery disease? Hellenic J Cardiol. 2010;51:1-9.

74. Grebe MT, Luu B, Sedding D, et al. Fibrinogen promotes early atherosclerotic changes of the carotid artery in young, healthy adults. J Atheroscler Thromb. 2010;17:1003-8.

75. Lee AJ, Mowbray PI, Lowe GD, et al. Blood viscosity and elevated carotid intima-media thickness in men and women: The edinburgh artery study. Circulation. 1998;97:1467-73.

76. Ebrahim S, Papacosta O, Whincup P, et al. Carotid plaque, intima media thickness, cardiovascular risk factors, and prevalent cardiovascular disease in men and women: The british regional heart study. Stroke. 1999;30:841-50.

77. Tosetto A, Prati P, Baracchini C, et al. Association of plasma fibrinogen, C-reactive protein and G-455>A polymorphism with early atherosclerosis in the VITA project cohort. Thromb Haemost. 2011;105:329-35.

78. Levenson J, Giral P, Razavian M, et al. Fibrinogen and silent atherosclerosis in subjects with cardiovascular risk factors. Arterioscler Thromb Vasc Biol. 1995;15:1263-8.

79. Smith EB. Fibrinogen, fibrin and fibrin degradation products in relation to atherosclerosis. Clin Haematol. 1986;15:355-70.

80. Rabbani LE, Loscalzo J. Recent observations on the role of hemostatic determinants in the development of the atherothrombotic plaque. Atherosclerosis. 1994;105:1-7.

81. Lowe GD, Lee AJ, Rumley A, et al. Blood viscosity and risk of cardiovascular events: The edinburgh artery study. Br J Haematol. 1997;96:168-73.

82. Lominadze D, Dean WL, Tyagi SC, et al. Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. Acta Physiol (Oxf). 2010;198:1-13.

83. Blomback B. Fibrinogen structure, activation, polymerization and fibrin gel structure. Thromb Res. 1994;75:327-8.

84. Carr ME,Jr, Carr SL. Fibrin structure and concentration alter clot elastic modulus but do not alter platelet mediated force development. Blood Coagul Fibrinolysis. 1995;6:79-86.

85. Blomback B, Carlsson K, Hessel B, et al. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. Biochim Biophys Acta. 1989;997:96-110.

86. Kim PY, Stewart RJ, Lipson SM, et al. The relative kinetics of clotting and lysis provide a biochemical rationale for the correlation between elevated fibrinogen and cardiovascular disease. J Thromb Haemost. 2007;5:1250-6.

87. Chung DW, Davie EW. Gamma and gamma' chains of human fibrinogen are produced by alternative mRNA processing. Biochemistry. 1984;23:4232-6.

88. Fornace AJ,Jr, Cummings DE, Comeau CM, et al. Structure of the human gamma-fibrinogen gene. alternate mRNA splicing near the 3' end of the gene produces gamma A and gamma B forms of gamma-fibrinogen. J Biol Chem. 1984;259:12826-30.

89. Doolittle RF, Hong S, Wilcox D. Evolution of the fibrinogen gamma' chain: Implications for the binding of factor XIII, thrombin and platelets. J Thromb Haemost. 2009;7:1431-3.

90. Lovely RS, Falls LA, Al-Mondhiry HA, et al. Association of gammaA/gamma' fibrinogen levels and coronary artery disease. Thromb Haemost. 2002;88:26-31.

91. Cooper AV, Standeven KF, Ariens RA. Fibrinogen gamma-chain splice variant gamma' alters fibrin formation and structure. Blood. 2003;102:535-40.

92. Meh DA, Siebenlist KR, Mosesson MW. Identification and characterization of the thrombin binding sites on fibrin. J Biol Chem. 1996;271:23121-5.

93. Pospisil CH, Stafford AR, Fredenburgh JC, et al. Evidence that both exosites on thrombin participate in its high affinity interaction with fibrin. J Biol Chem. 2003;278:21584-91.

94. Seegers WH, Nieft M, Loomis EC. Note on the adsorption of thrombin on fibrin. Science. 1945;101:520-1.

95. Seegers WH. Multiple protein interactions as exhibited by the blood-clotting mechanism. J Phys Colloid Chem. 1947;51:198-206.

96. Gersh KC, Nagaswami C, Weisel JW, et al. The presence of gamma' chain impairs fibrin polymerization. Thromb Res. 2009;124:356-63.

97. Lovely RS, Boshkov LK, Marzec UM, et al. Fibrinogen gamma' chain carboxy terminal peptide selectively inhibits the intrinsic coagulation pathway. Br J Haematol. 2007;139:494-503.

98. Lovely RS, Rein CM, White TC, et al. gammaA/gamma' fibrinogen inhibits thrombin-induced platelet aggregation. Thromb Haemost. 2008;100:837-46.

99. Fredenburgh JC, Stafford AR, Leslie BA, et al. Bivalent binding to gammaA/gamma'-fibrin engages both exosites of thrombin and protects it from inhibition by the antithrombin-heparin complex. J Biol Chem. 2008;283:2470-7.

100. de Willige SU, Standeven KF, Philippou H, et al. The pleiotropic role of the fibrinogen gamma' chain in hemostasis. Blood. 2009;114:3994-4001.

101. Francis CW, Nachman RL, Marder VJ. Plasma and platelet fibrinogen differ in gamma chain content. Thromb Haemost. 1984;51:84-8.

102. Mosesson MW, Finlayson JS. Biochemical and chromatographic studies of certain activities associated with human fibrinogen preparations. J Clin Invest. 1963;42:747-55.

103. Siebenlist KR, Meh DA, Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing gamma chains. Biochemistry. 1996;35:10448-53.

104. Moaddel M, Farrell DH, Daugherty MA, et al. Interactions of human fibrinogens with factor XIII: Roles of calcium and the gamma' peptide. Biochemistry. 2000;39:6698-705.

105. Nagy B,Jr, Simon Z, Bagoly Z, et al. Binding of plasma factor XIII to thrombin-receptor activated human platelets. Thromb Haemost. 2009;102:83-9.

106. Gersh KC, Lord ST. An investigation of factor XIII binding to recombinant {gamma}'/{gamma}' and {gamma}/{gamma}' fibrinogen. ASH Annual Meeting Abstracts. 2006;108:1705.

107. Siebenlist KR, Mosesson MW, Hernandez I, et al. Studies on the basis for the properties of fibrin produced from fibrinogen-containing gamma' chains. Blood. 2005;106:2730-6.

108. Collet JP, Nagaswami C, Farrell DH, et al. Influence of gamma' fibrinogen splice variant on fibrin physical properties and fibrinolysis rate. Arterioscler Thromb Vasc Biol. 2004;24:382-6.

109. Falls LA, Farrell DH. Resistance of gammaA/gamma' fibrin clots to fibrinolysis. J Biol Chem. 1997;272:14251-6.

110. Drouet L, Paolucci F, Pasqualini N, et al. Plasma gamma'/gamma fibrinogen ratio, a marker of arterial thrombotic activity: A new potential cardiovascular risk factor? Blood Coagul Fibrinolysis. 1999;10 Suppl 1:S35-9.

111. Mannila MN, Lovely RS, Kazmierczak SC, et al. Elevated plasma fibrinogen gamma' concentration is associated with myocardial infarction: Effects of variation in fibrinogen genes and environmental factors. J Thromb Haemost. 2007;5:766-73.

112. Lovely RS, Kazmierczak SC, Massaro JM, et al. {Gamma}' fibrinogen: Evaluation of a new assay for study of associations with cardiovascular disease. Clin Chem. 2010.

113. Lovely RS, Yang Q, Massaro JM, et al. Assessment of genetic determinants of the association of gamma' fibrinogen in relation to cardiovascular disease. Arterioscler Thromb Vasc Biol. 2011;31:2345-52.

114. Uitte de Willige S, de Visser MC, Houwing-Duistermaat JJ, et al. Genetic variation in the fibrinogen gamma gene increases the risk for deep venous thrombosis by reducing plasma fibrinogen gamma' levels. Blood. 2005;106:4176-83.

115. Mosesson MW, Cooley BC, Hernandez I, et al. Thrombosis risk modification in transgenic mice containing the human fibrinogen thrombin-binding gamma' chain sequence. J Thromb Haemost. 2009;7:102-10.

116. Mosesson MW, Hernandez I, Raife TJ, et al. Plasma fibrinogen gamma' chain content in the thrombotic microangiopathy syndrome. J Thromb Haemost. 2007;5:62-9.

117. Cheung EY, de Willige SU, Vos HL, et al. Fibrinogen gamma' in ischemic stroke: A case-control study. Stroke. 2008;39:1033-5.

118. van den Herik EG, Cheung EY, de Lau LM, et al. Gamma'/total fibrinogen ratio is associated with short-term outcome in ischaemic stroke. Thromb Haemost. 2011;105:430-4.

119. Cheung EY, Vos HL, Kruip MJ, et al. Elevated fibrinogen gamma' ratio is associated with cardiovascular diseases and acute phase reaction but not with clinical outcome. Blood. 2009;114:4603,4; author reply 4604-5.

120. GE Healthcare. Technology note 23: Label-free interaction analysis in real-time using surface plasmon resonance. Biacore Systems. 2005 05/2007;2012(3/15):8.

121. Luminex Corporation. **xMAP® technology**. . 2012;2012(04/13). Available from: http://www.luminexcorp.com/TechnologiesScience/xMAPTechnology/index.htm.

122. Ng NM, Quinsey NS, Matthews AY, et al. The effects of exosite occupancy on the substrate specificity of thrombin. Arch Biochem Biophys. 2009;489:48-54.

123. Nimjee SM, Oney S, Volovyk Z, et al. Synergistic effect of aptamers that inhibit exosites 1 and 2 on thrombin. RNA. 2009;15:2105-11.

124. Yang L, Manithody C, Qureshi SH, et al. Contribution of exosite occupancy by heparin to the regulation of coagulation proteases by antithrombin. Thromb Haemost. 2010;103:277-83.

125. Meh DA, Siebenlist KR, Brennan SO, et al. The amino acid sequence in fibrin responsible for high affinity thrombin binding. Thromb Haemost. 2001;85:470-4.

126. Lovely RS, Moaddel M, Farrell DH. Fibrinogen gamma' chain binds thrombin exosite II. J Thromb Haemost. 2003;1:124-31.

127. Lancellotti S, Rutella S, De Filippis V, et al. Fibrinogen-elongated gamma chain inhibits thrombin-induced platelet response, hindering the interaction with different receptors. J Biol Chem. 2008;283:30193-204.

128. Farrell DH, Mulvihill ER, Huang SM, et al. Recombinant human fibrinogen and sulfation of the gamma' chain. Biochemistry. 1991;30:9414-20.

129. Henschen AH. Human fibrinogen--structural variants and functional sites. Thromb Haemost. 1993;70:42-7.

130. Sabo TM, Farrell DH, Maurer MC. Conformational analysis of gamma' peptide (410-427) interactions with thrombin anion binding exosite II. Biochemistry. 2006;45:7434-45.

131. Pineda AO, Chen ZW, Marino F, et al. Crystal structure of thrombin in complex with fibrinogen gamma' peptide. Biophys Chem. 2007;125:556-9.

132. Cantor CR, Schimmel PR. The behavior of biological molecules. In: Biophysical Chemistry Part III. San Francisco: W. H. Freeman and Co.; 1980. p. 871,872, 873.

133. Interlandi G, Thomas W. The catch bond mechanism between von willebrand factor and platelet surface receptors investigated by molecular dynamics simulations. Proteins. 2010;78:2506-22.

134. Kaczmarek E, McDonagh J. Thrombin binding to the A alpha-, B beta-, and gamma-chains of fibrinogen and to their remnants contained in fragment E. J Biol Chem. 1988;263:13896-900.

135. Bellido-Martin L, Chen V, Jasuja R, et al. Imaging fibrin formation and platelet and endothelial cell activation in vivo. Thromb Haemost. 2011;105:776-82.

136. Couper DJ, Beck JD, Falkner KL, et al. The periodontitis and vascular events (PAVE) pilot study: Recruitment, retention, and community care controls. J Periodontol. 2008;79:80-9.

137. Beck JD, Couper DJ, Falkner KL, et al. The periodontitis and vascular events (PAVE) pilot study: Adverse events. J Periodontol. 2008;79:90-6.

138. Offenbacher S, Beck JD, Moss K, et al. Results from the periodontitis and vascular events (PAVE) study: A pilot multicentered, randomized, controlled trial to study effects of periodontal therapy in a secondary prevention model of cardiovascular disease. J Periodontol. 2009;80:190-201.

139. Beck JD, Offenbacher S. Relationships among clinical measures of periodontal disease and their associations with systemic markers. Ann Periodontol. 2002;7:79-89.

140. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation. 2003;107:363-9.

141. Uitte de Willige S, Rietveld IM, De Visser MC, et al. Polymorphism 10034C>T is located in a region regulating polyadenylation of FGG transcripts and influences the fibrinogen gamma'/gammaA mRNA ratio. J Thromb Haemost. 2007;5:1243-9.

142. Uitte de Willige S, Pyle ME, Vos HL, et al. Fibrinogen gamma gene 3'-end polymorphisms and risk of venous thromboembolism in the african-american and caucasian population. Thromb Haemost. 2009;101:1078-84.

143. Fuller WA. Measurement error models. New York: John Wiley & Sons; 1987.

144. Alesci S, Borggrefe M, Dempfle CE. Effect of freezing method and storage at -20 degrees C and -70 degrees C on prothrombin time, aPTT and plasma fibrinogen levels. Thromb Res. 2009;124:121-6.

145. Woodhams B, Girardot O, Blanco MJ, et al. Stability of coagulation proteins in frozen plasma. Blood Coagul Fibrinolysis. 2001;12:229-36.

146. Sakkinen PA, Macy EM, Callas PW, et al. Analytical and biologic variability in measures of hemostasis, fibrinolysis, and inflammation: Assessment and implications for epidemiology. Am J Epidemiol. 1999;149:261-7.

147. De Bacquer D, De Backer G, Braeckman L, et al. Intra-individual variability of fibrinogen levels. J Clin Epidemiol. 1997;50:393-9.

148. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: Application to clinical and public health practice: A statement for healthcare professionals from the centers for disease control and prevention and the american heart association. Circulation. 2003;107:499-511.

149. Lloyd-Jones D, Adams RJ, Brown TM, et al. Executive summary: Heart disease and stroke statistics--2010 update: A report from the american heart association. Circulation. 2010;121:948-54.

150. Hlatky MA, Greenland P, Arnett DK, et al. Criteria for evaluation of novel markers of cardiovascular risk: A scientific statement from the american heart association. Circulation. 2009;119:2408-16.

151. Clark GH, Fraser CG. Biological variation of acute phase proteins. Ann Clin Biochem. 1993;30 (Pt 4):373-6.

152. Ockene IS, Matthews CE, Rifai N, et al. Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. Clin Chem. 2001;47:444-50.

153. McCormack JP, Allan GM. Measuring hsCRP--an important part of a comprehensive risk profile or a clinically redundant practice? PLoS Med. 2010;7:e1000196.

154. Folsom AR, Chambless LE, Ballantyne CM, et al. An assessment of incremental coronary risk prediction using C-reactive protein and other novel risk markers: The atherosclerosis risk in communities study. Arch Intern Med. 2006;166:1368-73.

155. Shah T, Casas JP, Cooper JA, et al. Critical appraisal of CRP measurement for the prediction of coronary heart disease events: New data and systematic review of 31 prospective cohorts. Int J Epidemiol. 2009;38:217-31.

156. Wang TJ, Gona P, Larson MG, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med. 2006;355:2631-9.

157. Kavousi M, Elias-Smale S, Rutten JH, et al. Evaluation of newer risk markers for coronary heart disease risk classification: A cohort study. Ann Intern Med. 2012;156:438-44.

158. Campbell B, Badrick T, Flatman R, et al. Limited clinical utility of high-sensitivity plasma C-reactive protein assays. Ann Clin Biochem. 2002;39:85-8.

159. Alexander KS, Madden TE, Farrell DH. Association between gamma' fibrinogen levels and inflammation. Thromb Haemost. 2011;105:605-9.

160. Farrell DH. Pathophysiologic roles of the fibrinogen gamma chain. Curr Opin Hematol. 2004;11:151-5.

161. Mosesson MW. Update on antithrombin I (fibrin). Thromb Haemost. 2007;98:105-8.

162. Chen M, Manley JL. Mechanisms of alternative splicing regulation: Insights from molecular and genomics approaches. Nat Rev Mol Cell Biol. 2009;10:741-54.

163. Stoilov P, Meshorer E, Gencheva M, et al. Defects in pre-mRNA processing as causes of and predisposition to diseases. DNA Cell Biol. 2002;21:803-18.

164. de la Mata M, Alonso CR, Kadener S, et al. A slow RNA polymerase II affects alternative splicing in vivo. Mol Cell. 2003;12:525-32.

165. Robson-Dixon ND, Garcia-Blanco MA. MAZ elements alter transcription elongation and silencing of the fibroblast growth factor receptor 2 exon IIIb. J Biol Chem. 2004;279:29075-84.

166. Mackay CR, Terpe HJ, Stauder R, et al. Expression and modulation of CD44 variant isoforms in humans. J Cell Biol. 1994;124:71-82.

167. Eissa NT, Strauss AJ, Haggerty CM, et al. Alternative splicing of human inducible nitric-oxide synthase mRNA. tissue-specific regulation and induction by cytokines. J Biol Chem. 1996;271:27184-7.

168. McKay NG, Hunter DJ, Haites NE, et al. Regulation of alternative splicing of the fibronectin IIICS domain by cytokines. Biochem Biophys Res Commun. 1994;199:1005-11.

169. Wang A, Cohen DS, Palmer E, et al. Polarized regulation of fibronectin secretion and alternative splicing by transforming growth factor. J Biol Chem. 1991;266:15598-601.

170. Balza E, Borsi L, Allemanni G, et al. Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett. 1988;228:42-4.

171. Stamm S. Signals and their transduction pathways regulating alternative splicing: A new dimension of the human genome. Hum Mol Genet. 2002;11:2409-16.

172. Eisenreich A, Malz R, Pepke W, et al. Role of the phosphatidylinositol 3-kinase/protein kinase B pathway in regulating alternative splicing of tissue factor mRNA in human endothelial cells. Circ J. 2009;73:1746-52.

173. Xiong Z, Shaibani A, Li YP, et al. Alternative splicing factor ASF/SF2 is down regulated in inflamed muscle. J Clin Pathol. 2006;59:855-61.

174. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444:860-7.

175. Helfand M, Buckley DI, Freeman M, et al. Emerging risk factors for coronary heart disease: A summary of systematic reviews conducted for the U.S. preventive services task force. Ann Intern Med. 2009;151:496-507.