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Review

γ′ Fibrinogen as a novel marker of thrombotic disease

David H. Farrell*

Department of Medicine, Division of Cardiovascular Medicine, Oregon Health and Science University, Portland, OR, USA

Abstract

γ′ Fibrinogen is an isoform of fibrinogen that normally constitutes about 7% of total plasma fibrinogen, and arises from an alternative processing event in the γ chain mRNA. γ′ Fibrinogen is a newly-emerging cardiovascular disease (CVD) risk factor that appears to have an independent association with CVD from that of total fibrinogen, which is itself a well-established CVD risk factor. γ′ Fibrinogen shows a significant association with coronary artery disease and myocardial infarction in at least four case-control studies, including the Stockholm Coronary Artery Risk Factor study and the Framingham Heart Study. γ′ Fibrinogen is also significantly associated with stroke, as shown in the Erasmus Stroke Study and others. The role of genetic polymorphisms in the association between γ′ fibrinogen and CVD is under active investigation. γ′ Fibrinogen increases during inflammation, and is differentially regulated from total fibrinogen under pathologic conditions, as demonstrated in the Periodontitis and Vascular Events study. The association between γ′ fibrinogen and venous thromboembolism remains unclear, however, with some studies showing an inverse association with γ′ fibrinogen levels and other studies showing the opposite.

Keywords: cardiovascular disease; γ′ fibrinogen; inflammation; risk marker; thromboembolism; thrombosis.

Introduction

The association between total fibrinogen levels and cardiovascular disease (CVD) is well established by many epidemiologic studies which show that fibrinogen is a significant independent risk factor for CVD (1–3). However, possible mechanisms by which fibrin(ogen) may contribute to CVD continue to be a subject of controversy. Elevated levels of fibrinogen have been proposed to promote fibrin formation, increase platelet aggregation, increase plasma viscosity, or simply reflect an inflammatory state (4), but a mechanistic role for elevated fibrinogen in CVD remains elusive. Studies have shown that a first-order relationship exists between fibrinogen concentration and fibrinolysis rate (5), suggesting that elevated fibrinogen levels may actually cause thrombosis by slowing fibrinolysis rates (6).

γ′ Fibrinogen

Fibrinogen is a six-chain molecule containing two copies each of the Aα, Bβ, and γ chains, and different combinations of altered chains can be assembled, especially in fibrinogens resulting from heterozygous polymorphisms or mutations (7, 8). The fibrinogen γ chain, in particular, has two isoforms, the γA (also known as γ^{50} or simply γ) isoform and the γ′ (also known as $\gamma^{57.5}$ or γB) isoform that arise from alternative mRNA processing (9, 10). The current hypothesis is that competition between spliceosome cleavage of intron 9, which removes the intron and generates the γA mRNA, vs. polyadenylation within intron 9 at the AAUAAA site that cleaves off the 3′ end of the pre-mRNA to generate the γ′ mRNA, regulates the ratio of γA to γ′ mRNA. The γ′ chain is usually paired with the more abundant γA chain. γ′ Fibrinogen typically constitutes approximately 7% of total fibrinogen in plasma (11–13), although this percentage can vary widely among individuals, particularly in pathological conditions (14–16).

γ′ Fibrinogen is a newly-emerging risk marker for thrombotic disease. γ′ Fibrinogen has several biochemical and biophysical properties that are different from the more common isoform containing only γA chains. Some of these properties may actually contribute mechanistically to thrombosis. Clots made from γ′ fibrinogen in the presence of coagulation factor XIII (also known as "fibrin-stabilizing factor" in earlier nomenclature) are highly resistant to fibrinolysis (6, 17, 18), which may allow the formation of more stable thrombi. The γ′ chain is also reported to contain a binding site for zymogen factor XIII (19, 20). In addition, the γ′ chain contains a binding site for thrombin (21–24), and clots made from γ′ fibrinogen have an altered clot architecture (18, 25, 26). The γ′ chain protects thrombin from inactivation by antithrombin (27), which appears to contribute to the heparin resistance of clot-bound thrombin (28, 29). This could increase the length of time that thrombin remains active on the clot surface, potentially causing increased thrombus formation. Possibly as a

*Corresponding author: David H. Farrell, PhD, Division of Cardiovascular Medicine, Department of Medicine, School of Medicine, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97239-3098, USA
Phone: +1 503 494 8602, Fax: +1 503 222 2306,
E-mail: farrelld@ohsu.edu
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result of these properties, epidemiologic studies suggest that γ′ fibrinogen may be a risk factor for CVD (11–16, 30–33).

γ′ Fibrinogen has certain inhibitory properties towards hemostasis, which have lead to the opposite hypothesis that low levels of γ′ fibrinogen may predispose towards thrombosis (34, 35). For example, thrombin binding to the γ′ chain inhibits cleavage of factor VIII (36), which slows the rate of coagulation. In addition, thrombin binding to the γ′ chain inhibits thrombin activation of platelets through PAR-1 (37, 38), which would inhibit hemostasis. The effect of the γ′ chain on thrombin cleavage of fibrinopeptides A and B has been controversial, with three different results reported from three different laboratories (18, 25, 26). However, the inhibitory properties of the γ′ chain towards hemostasis have been proposed as a protective mechanism against venous thrombosis (39).

γ′ Fibrinogen and arterial thrombosis

The altered biochemical and biophysical properties of γ′ fibrinogen compared to total fibrinogen have prompted a series of investigations into its association with CVD. The first epidemiologic study on γ′ fibrinogen proposed that an elevated ratio of γ′ fibrinogen to total fibrinogen may be a marker of arterial thrombosis (30). The underlying hypothesis for this study came from the observation that the γ′ chain does not bind the platelet receptor $\alpha_{IIb}\beta_3$ (GpIIb-IIIa) during platelet aggregation (40–42). The hypothesis was that platelet activation during thrombosis would preferentially sequester fibrinogen containing γA chains, thereby increasing the ratio of γ′ fibrinogen to γA fibrinogen in plasma. In this study, γ′ fibrinogen was measured in control subjects aged 18–45 years (n=200) and control subjects aged 65–85 years (n=233) for comparison with peripheral arterial disease (PAD) and ischemic stroke patients (n=179) and PAD, ischemic stroke, and myocardial infarction patients (n=236). Whether the original hypothesis was correct is still unresolved, but nevertheless the study found an increased γ′ fibrinogen/total fibrinogen ratio that was correlated with D-dimer levels. Consistent with this hypothesis, ticlopidine treatment, which inhibits platelet activation through the ADP receptor, lowered the γ′ fibrinogen/total fibrinogen ratio. One limitation of this study was that multivariate analysis was not performed to calculate the odds ratio for the association of γ′ fibrinogen with CVD.

The next study to investigate the association of γ′ fibrinogen with CVD was a small case-control study of coronary artery disease (CAD) (11). In this study (n=133), γ′ fibrinogen levels were measured in patients undergoing elective, outpatient diagnostic cardiac catheterization. Those patients diagnosed with CAD by angiography (n=91) were considered CAD cases, and the remaining patients (n=42) were considered controls. The mean γ′ fibrinogen level in the cases was 0.413±0.016 g/L (mean±SE) compared to 0.299±0.024 g/L in the controls (p<0.0001). This study also determined a mean γ′ fibrinogen level of 0.285 g/L in normal blood donors. The odds ratio comparing the highest to lowest quartile, adjusting only for age and gender, was 7.16 (95% CI 1.82–27.7). There was no significant correlation between γ′ fibrinogen and total fibrinogen levels in this cohort, suggesting that γ′ fibrinogen was not simply a surrogate marker for total fibrinogen. In contrast to the original paper by Drouet et al. (30), there was no significant association between the ratio of γ′ fibrinogen/total fibrinogen and CAD.

A subsequent epidemiologic study on myocardial infarction was performed in the Stockholm Coronary Artery Risk Factor cohort (12). This study examined γ′ fibrinogen levels in younger MI patients, under the age of 60 (n=387), and in population-based sex- and age-matched controls (n=387). The mean γ′ fibrinogen levels were significantly higher in cases (0.28±0.12 g/L; mean±SD) than controls (0.25±0.11 g/L) (p=0.001), but there was no significant difference in the γ′ fibrinogen/total fibrinogen ratio. A weak correlation was found between γ′ fibrinogen levels and total fibrinogen levels in both patients (r=0.29) and controls (r=0.18). γ′ Fibrinogen levels were significantly associated with MI in a multivariate model (OR=1.24; 95% CI 1.01–1.52), after adjustment for age, gender, smoking, alcohol consumption, body mass index (BMI), total fibrinogen, interleukin-6, insulin, triglycerides, and high density lipoprotein (HDL)-cholesterol. Single nucleotide polymorphisms (SNPs) were also investigated for their association with γ′ fibrinogen levels. One interesting allele in particular, *FGG* 9340T>C (rs1049636), occurs in intron 9 between the exon 9/intron 9 splice site and the γ′ mRNA polyadenylation site within intron 9 (Figure 1). *FGG* 9340C was associated with significantly higher γ′ fibrinogen levels, while the *FGA* 2224A (rs2070011) allele was associated with lower γ′ fibrinogen levels. However, although the *FGG* 9340T and *FGA* 2224G alleles were significantly associated with γ′ fibrinogen levels, neither was associated with MI. In addition, since these were haplotype-tagging SNPs, the possibility remained that they were simply proxies for functional SNPs located elsewhere in the fibrinogen gene locus.

The issue of genetic modifiers of γ′ fibrinogen and their association with arterial thrombosis has been further investigated in a pediatric cohort from the German study center Muenster (43). An independent family-based cohort of 268 nuclear families with thromboembolic nonvascular stroke showed a significant association of the FGG-H2 haplotype with stroke. This haplotype includes two SNPs in the intron 9/exon 10 region (Figure 1), *FGG* 9615C>T (rs2066864) and

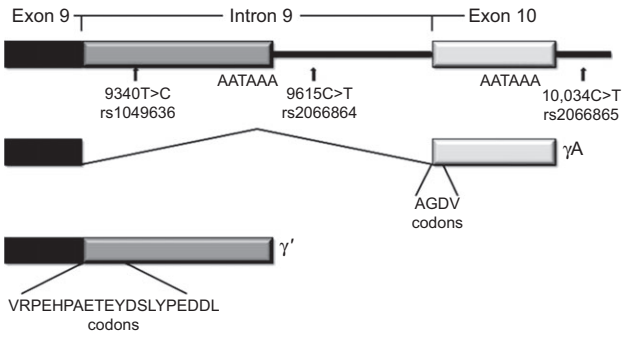

Figure 1 Alternative processing of the γ chain mRNA. Common SNPs are shown at 9340, 9615, and 10,034.

FGG 10,034C>T (rs2066865). The 10,034T allele is located within a GT-rich downstream sequence element in a potential cleavage stimulation factor binding site, and has been shown to decrease the ratio of γ′/γA mRNA in a minigene construct (44). The 10,034T allele has also been associated with decreased γ′ fibrinogen levels and a decreased γ′ fibrinogen/total fibrinogen ratio (34). 10,034T is a haplotype-tagging SNP for the FGG-H2 haplotype. Although carriers of the FGG-H2 haplotype did have decreased γ′ fibrinogen levels and lower γ′ fibrinogen/total fibrinogen ratios, as shown previously (34), the study design precluded any calculations of odds ratios for γ′ fibrinogen levels and stroke (43).

More problematic, the FGG-H2 haplotype is in linkage disequilibrium with an Aα chain polymorphism in the coding region, *FGA* Thr312Ala (rs6050), near the factor XIIIa α-fibrin/α-fibrin cross-linking site at Aα328 (45). The Ala312 variant produces stiffer clots with increased α chain cross-linking, larger fiber diameters, and a lower number of fibers per unit area (46). The rs6050 SNP itself has been associated with stroke risk, and the A2 haplotype containing the minor allele of rs6050 showed a significant association with ischemic stroke (OR 1.4; 95% CI 1.1–1.8) (47). There are therefore at least two functional SNPs in the FGG-H2 haplotype, *FGG* 10,034C>T (rs2066865) that results in decreased γ′ fibrinogen levels, and *FGA* Thr312Ala (rs6050) that results in increased α chain cross-linking, larger fiber diameters, and a lower number of fibers per unit area. Therefore, ascribing decreased γ′ fibrinogen levels as the cause of the association of the FGG-H2 haplotype with increased stroke risk, may be a premature conclusion.

In contrast, another case-control study (n=249) found that an elevated γ′ fibrinogen/total fibrinogen ratio was associated with the acute phase of ischemic stroke, but was lower 3 months after stroke during the convalescent phase (14). The FGG-H2 haplotype, although associated with decreased γ′ fibrinogen levels, was not associated with risk of stroke. In contrast, the FGG-H3 haplotype was associated with reduced risk of stroke, but was not associated with the γ′ fibrinogen/total fibrinogen ratio. The authors concluded that the increase in γ′ fibrinogen levels during the acute phase may actually be a result of the event, rather than the cause, and suggested that the acute phase may affect the alternative splicing events that give rise to the γ′ mRNA. A follow-up study (n=356) by several of these authors demonstrated a significant association between unfavorable stroke outcome (Rankin Scale score >2) and elevated γ′ fibrinogen levels, independent of total fibrinogen, as well as an elevated γ′ fibrinogen/total fibrinogen ratio (33). The odds ratio for an unfavorable outcome per unit increase in γ′ fibrinogen was 1.48 (95% CI 1.15–1.91) after adjustment for age, sex, and time to blood draw. The authors concluded that the absolute levels of γ′ fibrinogen may be more important than the γ′ fibrinogen/total fibrinogen ratio.

The relationship between γ′ fibrinogen levels and known CVD risk factors was investigated in the Framingham Heart Study (13). γ′ Fibrinogen levels were significantly associated with several known CVD risk factors in the Framingham Offspring cohort (n=3300), including age, sex, BMI, smoking, diabetes, blood glucose, and triglycerides, and inversely associated with HDL levels. The reference interval, defined as the 2.5th and 97.5th percentile limits for γ′ fibrinogen in health individuals without CVD, was 0.088–0.551 g/L. The range of γ′ fibrinogen measured in these samples varied nearly 40-fold, from 0.037 g/L to 1.443 g/L.

A subsequent community-based study and a genome-wide association study of γ′ fibrinogen levels were also performed in the Framingham Heart Study (32). Individuals with prevalent CVD had significantly elevated γ′ fibrinogen levels (p=0.002) compared to those without CVD, 0.278±0.006 g/L vs. 0.258±0.002 g/L. Analyzing the relationship between γ′ fibrinogen and CVD, the odds ratio of an event per 0.1 g/L increase in γ′ fibrinogen was 1.12 (95% CI 1.03–1.21) after adjustment for sex, age, BMI, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, HDL cholesterol, and triglycerides. In a multivariate model comparing the prevalence of CVD between the highest and lowest tertiles, the adjusted odds ratio was 1.53 (95% CI 1.14–2.05). The association between γ′ fibrinogen and myocardial infarction was even higher, with an adjusted odds ratio of 1.76 (95% CI 1.06–2.92).

The odds of CVD and myocardial infarction were increased for individuals who were in the highest tertiles of both γ′ fibrinogen and total fibrinogen. Whereas individuals in the highest tertile of γ′ fibrinogen had an adjusted odds ratio of 1.76 for myocardial infarction, and individuals in the highest tertile of total fibrinogen had an adjusted odds ratio of 1.99 (95% CI 1.21–3.28), those in the highest tertiles of both γ′ fibrinogen and total fibrinogen had an adjusted odds ratio of 3.08 (95% CI 1.41–6.72). These results suggest that γ′ and total fibrinogen are not simply surrogate markers for one another, but that each analyte adds predictive power to risk assessment for CVD.

In a genome-wide association study examining 2.5 million SNPs, 54 SNPs in or near the fibrinogen gene locus demonstrated genome-wide significance (32). The top-signal SNP was rs7681423 in the fibrinogen gene locus near *FGG*, although the top two SNPs associated with γ′ fibrinogen levels were not associated with CVD. These results suggest that genetics may not play a major role in the association between γ′ fibrinogen and CVD. These findings contrast those for total fibrinogen levels, which are associated with different genetic loci, particularly *FGB* encoding the Bβ chain of fibrinogen. Since γ′ fibrinogen is an acute phase reactant that increases in response to inflammation, these results suggest that environmental factors may play a greater role than genetic factors in its association with CVD.

γ′ Fibrinogen and inflammation

The association between γ′ fibrinogen and arterial thrombosis may be due, at least in part, to the association between γ′ fibrinogen and inflammation. Inflammation is now a well-established risk factor for arterial thrombosis (48), and γ′ fibrinogen has shown a strong association with inflammation in several published studies. In the case-control stroke study mentioned above (14), γ′ fibrinogen levels were increased

during the acute phase of stroke when C-reactive protein (CRP) levels were elevated, and decreased during the convalescent phase when CRP levels decreased. Furthermore, the ratio of γ′ fibrinogen/total fibrinogen was also increased during the acute phase compared to the convalescent phase.

Subsequent studies showed that γ′ fibrinogen was highly associated with C-reactive protein (CRP) levels, and may represent a new marker for inflammation (16). γ′ Fibrinogen levels were measured in patients from the Periodontitis and Vascular Events study (n=284) (16). These patients had a history of recent CVD within the last 3 years, as well as periodontal disease. The mean γ′ fibrinogen level in this cohort appeared to be highly elevated compared to historical controls, 0.622±0.017 g/L (mean±SE), although no formal comparison to controls was presented, and the percentage of γ′ fibrinogen was 18.6%±0.62% of total fibrinogen, compared to historical controls of 7.2% (11) and 6.8% (13).

γ′ Fibrinogen showed a significant association with CRP levels (p=0.006 after adjustment for age, gender, smoking status, BMI, type 2 diabetes, and total fibrinogen), and a one unit increase in CRP was associated with a 1.9% increase in γ′ fibrinogen (16). In addition, the γ′ fibrinogen level was highly associated (p<0.001) with a high-risk CRP level of >3 mg/L. Each 0.1 g/L increase in γ′ fibrinogen increased the odds of being in this high-risk group by 20% (p<0.001) after adjustment for BMI and total fibrinogen. In contrast, total fibrinogen levels were relatively normal in this cohort, 3.70 g/L, suggesting that γ′ fibrinogen may display differential regulation from total fibrinogen in the setting of combined CVD history and periodontal inflammation. Further support for inflammatory regulation of γ′ fibrinogen was provided by a case-control study (n=211) of patients with intracerebral hemorrhage (49) in which the authors proposed that the absolute rise in γ′ fibrinogen observed in their study is an acute phase response.

γ′ Fibrinogen and venous thrombosis

The association between γ′ fibrinogen and venous thromboembolism (VTE) is much more controversial. The first publication on this subject, a case-control study (n=948) from the Leiden Thrombophilia Study, showed a significant association between the FGG-H2 haplotype and VTE (34). This association was replicated in a subsequent study case-control study of VTE (n=712) using samples from the Medical University Graz (50). The FGG-H2 haplotype contains the *FGG* 10,034C>T SNP (rs2066865), which is associated with decreased γ′ fibrinogen levels. However, as mentioned earlier, this SNP is in linkage disequilibrium with the *FGA* Thr312Ala (rs6050) polymorphism that results in stiffer clots with increased Aα chain cross-linking, larger fiber diameters, and a lower number of fibers per unit area (46). The authors' hypothesis was that γ′ fibrinogen was protective against VTE by virtue of its "antithrombin I" activity (39). This is an activity described over half a century ago as an inhibitory activity of fibrin towards thrombin (51). The hypothesis proposed that the γ′ chain high affinity binding site for thrombin sequestered active thrombin. As a result, less free thrombin was available to cleave its substrates. These findings lead to a model in which low γ′ fibrinogen levels caused reduced sequestration of thrombin, resulting in a hypercoagulable state and VTE (31). This mechanism was also proposed to explain the association between low γ′ fibrinogen levels and thrombotic microangiopathy in a case-control study (n=87) (35).

There is biochemical support for this hypothesis as well. Binding of the γ′ chain to thrombin allosterically modulates the active site (52). This antithrombin I activity inhibits thrombin activation of coagulation under static conditions by inhibiting factor VIII cleavage (36). In addition, this activity inhibits thrombin activation of platelets by inhibiting PAR1 cleavage (37, 38). Studies with transgenic mice containing the human fibrinogen thrombin-binding γ′ chain sequence showed a decrease in thrombosis (53).

However, a more recent publication showed results that are opposite to those found in Uitte de Willige et al. (34). In a cross-sectional study of newly-diagnosed pulmonary embolism (PE) patients (n=29), γ′ fibrinogen levels showed the highest levels of γ′ fibrinogen ever published in a clinical study, 0.79±0.33 g/L (mean±SD) during the acute phase of PE (15).

A competing hypothesis of the role of the γ′ chain may provide an alternative explanation for these results. Binding of thrombin to the γ′ chain in humans changes thrombin's cleavage of its substrates in a manner analogous to the binding of thrombin to thrombomodulin (54) or platelet glycoprotein Ibα (GPIbα) (55). For example, activation of factor VIII in the intrinsic pathway by thrombin cleavage is impaired by the γ′ chain, and the activated partial thromboplastin time is prolonged (36). In contrast, binding of the γ′ chain to thrombin does not inhibit the cleavage of small peptide substrates, nor completely inhibit cleavage of fibrinogen (36), but may modulate the kinetics of fibrinopeptide A and B release (18, 25, 26). A competing hypothesis is that thrombin bound to the γ′ chain continues to convert more fibrin at the site of clot formation, although it restricts thrombin cleavage of other substrates. The γ′ chain also protects thrombin from inactivation by antithrombin (27). This would potentially allow continued clot formation at the site where fibrin is being deposited, while preventing diffusion of thrombin due to blood flow. In this model, γ′ fibrinogen, rather than being an antithrombin, would serve as a reservoir for active thrombin at the growing clot surface.

Another potential explanation for the discrepancies between the inverse and positive associations with VTE and γ′ fibrinogen in studies by Uitte de Willige et al. (34) and Cheung et al. (15), respectively, may be in the experimental design. The study by Uitte de Willige et al. (34) was case-control in nature, using age- and gender-matched controls for VTE cases. However, no adjustment was made for warfarin use in this study. Since γ′ fibrinogen is an inflammatory marker, and warfarin is known to decrease systemic inflammation, it is possible that the VTE cases who were exposed to warfarin had decreased inflammation and therefore decreased γ′ fibrinogen levels in the blood samples taken after the event. In contrast, in the study by Cheung et al. (15), blood samples were taken

at the time of the VTE event prior to warfarin exposure and compared to normal population controls (n=173), although the controls were not matched to the cases. A more definitive way to control for warfarin exposure would be to conduct a prospective study with a nested case/cohort design. However, since no such prospective studies have yet been performed the association between γ′ fibrinogen and VTE remains controversial. Further studies will be necessary to definitively address the association between γ′ fibrinogen and VTE.

Conclusions and perspectives

γ′ Fibrinogen is a newly-emerging risk factor for CVD that is upregulated during inflammation. The biochemical properties of γ′ fibrinogen provide support for the hypothesis that, unlike many CVD risk biomarkers, γ′ fibrinogen may play a mechanistic role in thrombosis. The increased fibrinolytic resistance and altered clot architecture of γ′ fibrinogen provide potential mechanisms to explain the association with thrombosis. In addition, the presence of a high-affinity thrombin binding site on the γ′ chain, and the consequent resistance of the bound thrombin to antithrombin inhibition, may also play a mechanistic role in facilitating thrombosis. In contrast, the inhibitory properties of γ′ fibrinogen towards hemostasis, particularly the inhibition of factor VIII cleavage and PAR-1 cleavage by thrombin, present a plausible mechanism for an inverse association with VTE. However, the epidemiologic association of γ′ fibrinogen and VTE is still unresolved.

Conflict of interest statement

Author's conflict of interest disclosure: OHSU and Dr. David Farrell have a significant interest in Gamma Therapeutics, a company that may have a commercial interest in the results of this research and technology. This potential individual and institutional conflict of interest has been reviewed and managed by OHSU, and played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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David H. Farrell, PhD, is a Professor of Medicine in the Division of Cardiovascular Medicine at the Oregon Health and Science University, and has appointments in the Department of Molecular and Medical Genetics and the Department of Physiology and Pharmacology. He is also Founder and Chief Scientific Officer of Gamma Therapeutics, Inc., an Oregon-based early stage biotechnology venture developing a novel class of biopharmaceutical and diagnostic test solutions for the cardiovascular disease industry. Prof. Farrell is currently Co-Chair of the Thrombosis 2 Committee of the American Heart Association and on the Editorial Board of the open access journal *Thrombosis*. His primary research interests are in the biochemistry, genetics, regulation, and epidemiology of the blood clotting factor fibrinogen.