THROMBOSIS AND HEMOSTASIS

Evidence that fibrinogen γ′ regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans

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Key Points

- This paper describes the effect of fibrinogen γ′ on clot structure in plasma (previously shown in purified systems).
- This paper also describes the respective roles of total fibrinogen, fibrinogen γ′ concentration, and ratio on clot structure and lysis rates.

Fibrinogen γ′ is known to influence fibrin clot structure in purified experimental models, but little is known regarding its influence on clot structure in plasma. Furthermore, the environmental and biological factors that affect its concentration are poorly described. We analyzed fibrinogen γ′, total fibrinogen concentration, and fibrin clot structure in 2010 apparently healthy black South Africans and related them to traditional cardiovascular disease (CVD) risk factors. Fibrinogen γ′ generally increased with increasing fibrinogen concentration, but a decreased γ′/total fibrinogen ratio was found at the highest total fibrinogen concentrations. Clot maximum absorbance increased with total fibrinogen and fibrinogen γ′, but decreased with γ′/total fibrinogen ratio. Clot lysis time showed a stronger relationship with fibrinogen γ′ than with total fibrinogen, whereby increased fibrinogen γ′ delayed clot lysis. CVD risk factors (excluding fibrinogen) explained 20% and 3%, respectively, of the variance in fibrinogen γ′ and the γ′/total fibrinogen ratio, with C-reactive protein making the biggest contribution. More than 50% of the variance in fibrinogen γ′ and γ′/total fibrinogen ratio is explained by factors other than total fibrinogen or other traditional CVD risk factors. Our data show that fibrinogen γ′ modulates plasma clot structure and fibrinolysis and is also influenced by factors other than fibrinogen. (*Blood*. 2013;121(16):3254-3260)

Introduction

Fibrinogen γ prime (γ′; previously also called γB or γ57.5) arises from a splice variant of the γ-chain messenger RNA resulting from an alternative polyadenylation signal in intron 9.[1,2] The alternative polyadenylation leads to the translation of a unique 20-amino-acid C-terminal extension encoded by intron 9, which substitutes the 4 γA amino acids of exon 10.[2-4] Approximately 8% to 15% of total fibrinogen is composed of γ′ fibrinogen, of which the majority is in the heterodimeric γA/γ′ form.[3] Fibrinogen γ′ is associated with both venous[5] and arterial thrombosis.[6-10] This association with different thrombotic disorders has been ascribed in part to the effects of γ′ on clot structure, crosslinking by factor XIIIa, thrombin activity, or fibrinolysis.[11]

The relationship between γ′ fibrinogen and clot structure/function has been investigated mainly using in vitro experimental models with purified fibrinogen.[12-14] Although all of these studies agree that γ′ fibrinogen influences clot structure, some differences were observed in the type of changes, possibly due to differences in experimental designs, source material (ie, fibrinogen purified from plasma or recombinant fibrinogen), and selective copurification of other plasma proteins such as FXIII with plasma-derived fibrinogen. However, some of these discrepancies may also be the result of a heterogeneous, nonuniform clot structure that was observed in clots containing γ′ fibrinogen in subsequent in vitro studies.[15,16] Although the use of purified fibrinogen and fixed protein concentrations allows for the detailed study of mechanisms underpinning the effect of γ′ fibrinogen on clot structure, data on the effect of γ′ fibrinogen in varying plasma concentrations in the presence of varying total fibrinogen concentrations on clot structure are needed to provide insight into the in vivo relationships between these variables.

Furthermore, the factors that contribute to γ′ fibrinogen formation and to γ′/total fibrinogen ratio in vivo are poorly understood. Although a constituent of total fibrinogen, variation in γ′ fibrinogen concentration is not merely a reflection of changes in total fibrinogen levels but is also a result of independent control mechanisms.[17,18] For example, in a study comparing ischemic stroke patients in the acute phase with healthy controls, both γ′ fibrinogen and γ′/total fibrinogen ratio were increased,[9] whereas absolute γ′ fibrinogen levels, but not the γ′/total fibrinogen ratio, were increased in intracerebral hemorrhage patients.[19] Fibrinogen γ′ is thought to be related to cardiovascular disease (CVD) risk independent of total fibrinogen concentration.[11,20] Therefore, not only factors that affect γ′ fibrinogen levels but also factors that could potentially influence the γ′/total fibrinogen ratio should be determined.

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Table 1. Correlation between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and measures of clot structure

	γ′ fibrinogen		γ′/total fibrinogen ratio		Total fibrinogen	
Variable	*r*	*P*	*r*	*P*	*r*	*P*
Lag time (min)	0.0517	.04	−0.0245	.32	0.08	.002
Slope	0.1712	<.0001	−0.1829	<.0001	0.3651	<.0001
Maximum absorbance	0.3623	<.0001	−0.2091	<.0001	0.5802	<.0001
Clot lysis time	0.3568	<.0001	0.1720	<.0001	0.1631	<.0001
γ′ fibrinogen	—	—	0.5319	<.0001	0.403	<.0001
γ′/total fibrinogen ratio	0.5319	<.0001	—	—	−0.561	<.0001
Total fibrinogen	0.403	<.0001	−0.561	<.0001	—	—

Our aim was to analyze whether γ′ fibrinogen, total fibrinogen, and γ′/total fibrinogen ratio relate to plasma clot structure in a large population-based study of apparently healthy black South Africans. For this, we used the South African arm of the international Prospective Urban and Rural Epidemiology (PURE) study.[21,22] We also aimed to analyze the relationship between γ′ fibrinogen and cardiovascular risk factors in this well-characterized epidemiological study.

Materials and methods

Study cohort

PURE is a large-scale cohort study that tracks changing lifestyles, risk factors, and chronic disease in rural and urban areas of 17 countries in transition over 12 years. The data reported here are from the baseline data of 2010 randomly selected participants (1260 women and 750 men) from well-established rural (n = 1006, living under tribal law) and urban (n = 1004, living in informal and formal settlements surrounding cities) communities in the North West Province of South Africa collected over a 12-week period in 2005. These participants were recruited from 6000 randomly selected households from the two communities based on representativeness and feasibility for long-term follow-up, according to the guidelines stipulated in the overarching PURE study.[21,22] Apparently healthy black South Africans between the ages of 35 and 65 years were eligible to participate. Exclusion criteria were use of chronic medication for noncommunicable diseases and/or any self-reported acute illness. The study was approved by the ethics committee of the North-West University, South Africa, and subjects signed informed consent before taking part in the study in accordance with the Declaration of Helsinki. All data were treated confidentially and all analyses were performed with coded data.

Blood processing

Fasting blood samples were collected with minimum stasis from the antecubital veins of participants between 7:00 and 11:00 AM. For the analysis of lipids and C-reactive protein (CRP), blood was collected in tubes without anticoagulant. Blood was collected in EDTA tubes for the determination of homocysteine and glycosylated hemoglobin (hemoglobin A1c [HbA_{1c}]) and in fluoride tubes for glucose measurements. For the analysis of γ′ fibrinogen, total fibrinogen, and turbidimetric measurement of clot formation and lysis, blood was collected into citrate tubes and kept on ice until centrifugation (<30 minutes). This procedure did not significantly influence plasma fibrinogen level (data not shown). Samples were centrifuged at 2000*g* for 15 minutes at 10°C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at −18°C, and then after 2 to 4 days stored at −82°C until analysis.

Laboratory and clot structure/fibrinolysis analysis

Details regarding methods used to analyze CRP, serum lipids, total homocysteine, glucose, and HbA_{1c} were published previously.[23] Fibrinogen was measured using a modified Clauss method (Multifibrin U-test, BCS coagulation analyzer; Dade Behring, Deerfield, IL). Turbidimetric analysis (A405 nm) was used to determine plasma fibrinolytic potential of tissue-factor–induced clots and lysed by exogenous tissue plasminogen activator (tPA) with the method of Lisman et al[24] with slightly modified tissue factor and tPA concentrations in order to obtain comparable clot lysis times (CLTs) of about 60 minutes (intra-assay coefficient of variance [CV] = 3.6%, between-plate CV = 4.5%). Final concentrations were tissue factor (125× diluted to an estimated final concentration of 59 pM according to Duckers et al[25]; Dade Innovin, Siemens, Marburg, Germany), $CaCl_2$ (17 mmol/L), tPA (100 ng/mL; Actilyse, Boehringer Ingelheim, Ingelheim, Germany), and phospholipid vesicles (10 μmol/L; Rossix, Mölndal, Sweden). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.[24] Kinetics of clot formation, lag time, slope, and maximum absorbance were additionally calculated from the turbidity curves (supplemental Figure 1). When clotting plasma with thrombin, lag time represents the time required for fibrin fibers to grow sufficiently to allow lateral aggregation. Because we clotted our plasma with tissue factor, lag time additionally included the time it took for activation of the coagulation cascade and was taken at the point where absorbance increased 0.015 from baseline. The slope, calculated at half maximum absorbance, represents the rate of lateral aggregation and the maximum absorbance, calculated as the maximum absorbance at plateau minus the baseline, is an indication of average fiber size. Fibrinogen γ′ was measured according to the method of Uitte de Willige,[5] with an enzyme-linked immunosorbent assay using a 2.G2.H9 mouse monoclonal coating antibody against the human γ′ sequence from Santa Cruz Biotechnology (Santa Cruz, CA) for antigen capture and a goat polyclonal horseradish peroxidase–conjugated antibody against human fibrinogen from Abcam for development (Cambridge, MA). The CV for all assays was <10%.

Statistical analysis

The computer software package Statistica (Statsoft, Tulsa, OK) was used for statistical analyses. A P value ≤ .05 was regarded as statistically significant. Normally distributed variables are reported as mean (95% confidence interval). Nonparametric data were log transformed to improve normality and reported as median (25^{th}-75^{th} percentile]. Student *t* tests for independent samples for parametric data and the Mann-Whitney *U* test for nonparametric data were used for comparison between 2 groups. Analysis of variance with Tukey honest significant difference post hoc test was used for comparison among 3 or more groups. Analysis of covariance was used when comparison between groups required adjustment for confounders. Pearson correlations and univariate regression were used to determine associations between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and markers of clot structure using normally distributed data or log-transformed data for nonparametric variables. Forward stepwise multiple regression analysis was used to determine the main contributors to the variance in γ′ fibrinogen and γ′/total fibrinogen ratio using parametric and log-transformed data.

Results

The mean total fibrinogen concentration of the population was 3.7 g/L (±2.18). The mean γ′ fibrinogen and γ′/total fibrinogen ratio of the population was 0.38 g/L (±0.27) and 12.1% (±8.11), respectively. Table 1 presents the correlation between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and markers of clot formation and structure in plasma. Lag time did not show noteworthy correlations with any of the fibrinogen variables. Slope showed the strongest correlation with total fibrinogen (r = 0.37). Maximum absorbance showed a positive correlation with total fibrinogen (r = 0.58) and γ′ fibrinogen (r = 0.36) and a negative correlation with γ′/total fibrinogen ratio (r = −0.21). The correlation between

Table 2. Univariate regression results of γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen with measures of clot structure

	Lag time (min)			Slope			Maximum absorbance			Clot lysis time (min)		
Univariate regression	**β**	**95% CI**	**%***	**β**	**95% CI**	**%***	**β**	**95% CI**	**%***	**β**	**95% CI**	**%***
γ′ fibrinogen	0.25	0.08; 0.42	1	0.06	0.04; 0.08	2.5	0.11	0.09; 0.12	13.5	7.30	6.37; 8.24	12
γ′/total fibrinogen ratio	−0.11	−0.26; 0.05	<1	−0.06	−0.08; −0.05	3.5	−0.06	−0.07; −0.04	4.5	3.35	2.45; 4.25	3
Total fibrinogen	0.32	0.16; 0.49	1	0.13	0.12; 0.15	14	0.17	0.15; 0.18	33	3.28	2.31; 4.25	2.5

CI, confidence interval.
*Percent variance explained ($R^2 \times 100$).

γ′ fibrinogen and maximum absorbance, although less strong, remained significant after adjustment for total fibrinogen ($r = 0.17$, $P < .0001$; data not shown). CLT showed the strongest correlation with γ′ fibrinogen ($r = 0.36$). Fibrinogen γ′ correlated positively with both γ′/total fibrinogen ratio ($r = 0.53$) and total fibrinogen ($r = 0.40$) whereas γ′/total fibrinogen ratio correlated negatively with total fibrinogen ($r = -0.56$).

The contribution of fibrinogen variables to the variance in the markers of clot formation and structure was analyzed using univariate regression models (Table 2). The results of the regression models are in agreement with the correlations observed. None of the fibrinogen variables contributed significantly to the variance in lag time (all 1% or less). Total fibrinogen was the largest contributor to variance in slope, explaining 14% of the variance. Total fibrinogen was also the largest contributor to the variance in maximum absorbance, explaining 33% of the variance, with γ′ fibrinogen explaining 13.5%. The γ′/total fibrinogen ratio additionally explained 4.5% of the variance in maximum absorbance, with an increase in the ratio being associated with a decreased maximum absorbance. Fibrinogen γ′ was the largest contributor to CLT, explaining 12% of its variance.

The association of γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen with CVD risk markers was analyzed by stratification of the CVD risk markers and comparison of the distribution of the fibrinogen variables between the strata (Table 3 and supplemental Table 1). If total fibrinogen differed between the strata, then an analysis of covariance was performed for γ′ fibrinogen and γ′/total fibrinogen ratio adjusting for total fibrinogen. All 3 fibrinogen variables increased significantly with abdominal obesity and increasing body mass index (BMI) and CRP categories, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio remained after adjustment for total fibrinogen (Table 3). Individuals with low high-density lipoprotein (HDL) cholesterol had higher levels of all fibrinogen variables, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio also remained after adjustment for total fibrinogen. Participants with the metabolic syndrome (using the criteria recommended by Alberti et al[26]) also had higher levels of all fibrinogen variables than those without metabolic syndrome, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio remained after adjustment for total fibrinogen. The same pattern was observed for increasing HbA_{1c} categories (supplemental Table 1). Women had higher γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen

Table 3. The association between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and traditional CVD risk factors

CVD risk factor	**γ′ fibrinogen (mg/mL)**	**γ′/total fibrinogen ratio (%)**	**Total fibrinogen (mg/mL)**
BMI, kg/m²			
<18.5	0.28 (0.20-0.42) (n = 303)	9.24 (9.06-13.4) (n = 288)	2.95 (2.20-4.80) (n = 306)
18.5-24.9	0.28 (0.21-0.41) (n = 732)	10.3 (7.19-14.7) (n = 712)	2.60 (2.10-4.10) (n = 741)
25-29.9	0.35 (0.24-0.46) (n = 298)	10.6 (7.39-15.3) (n = 285)	2.90 (2.30-5.00) (n = 302)
≥30	0.38 (.029-0.56) (n = 345)	10.4 (7.33-15.1) (n = 327)	3.70 (2.60-6.20) (n = 338)
Unadjusted *P* value	<.0001	.001	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
Waist circumference, cm			
Normal	0.28 (0.21-0.40) (n = 1150)	9.98 (7.12-14.3) (n = 1112)	2.60 (2.10-4.10) (n = 1167)
Abdominal obesity	0.38 (0.28-0.53) (n = 605)	10.4 (7.28-15.2) (n = 574)	3.30 (2.50-5.90) (n = 559)
Unadjusted *P* value	<.0001	.067	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
HDL cholesterol, mmol/L			
Normal (≥1.0)	0.29 (0.22-0.43) (n = 1224)	9.76 (6.84-14.2) (n = 1175)	2.80 (2.20-4.80) (n = 1228)
Low (<1.0)	0.35 (0.26-0.51) (n = 514)	11.1 (8.03-15.3) (n = 496)	3.00 (2.30-5.50) (n = 521)
Unadjusted *P* value	<.0001	<.0001	.009
P value adjusted for Fbg	<.0001	<.0001	—
Metabolic syndrome			
Yes	0.38 (0.27-0.52) (n = 425)	10.4 (7.54-15.1) (n = 397)	3.30 (2.50-5.80) (n = 415)
No	0.29 (0.22-0.42) (n = 1290)	10.0 (7.08-14.5) (n = 1251)	2.80 (2.20-4.50) (n = 1309)
Unadjusted *P* value	<.0001	.06	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
CRP, mg/L			
≤3	0.27 (0.21-0.37) (n = 822)	10.5 (7.77-14.8) (n = 797)	2.50 (2.00-3.20) (n = 832)
>3	0.36 (0.26-0.52) (n = 907)	9.73 (6.77-14.3) (n = 866)	3.60 (2.50-6.10) (n = 909)
Unadjusted *P* value	<.0001	.007	<.0001
P value adjusted for Fbg	<.0001	<.0001	—

Fbg, fibrinogen.

Table 4. Multiple regression results for γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and traditional CVD risk factors

	Percent variance explained		
Variable	Model 1: CVD risk factors plus total fibrinogen	Model 2: CVD risk factors plus γ′ fibrinogen	Model 3: CVD risk factors
γ′ fibrinogen			
Total variance explained	26.3		19.9
Risk factors			
Total fibrinogen	16		Not included
CRP	5		13.5
HDL cholesterol	3		3
Gender	2		2
HbA_{1c}	1		1
Homocysteine	1		0.5
γ′/total fibrinogen ratio			
Total variance explained	39.3	37	3
Risk factors			
γ′ fibrinogen	Not included	28.8	Not included
Total fibrinogen	31	Not included	Not included
CRP	4	7	0.5
HDL cholesterol	2	—	2
Gender	1	0.5	—
Homocysteine	0.5	—	1
HbA_{1c}	0.5	—	—
Age	—	1	0.5

Variables that did not enter forward stepwise model are indicated by —.

than men. Total fibrinogen increased with 10-year age categories, whereas γ′ fibrinogen did not. Individuals with increased total cholesterol had increased total fibrinogen but not γ′ fibrinogen. Individuals with hyperhomocysteinemia had lower γ′ fibrinogen and γ′/total fibrinogen ratio but similar total fibrinogen compared with individuals with normal homocysteine levels. Total fibrinogen was moderately increased in individuals with hypertension whereas no difference was observed for γ′ fibrinogen.

To determine the respective contribution of the CVD risk factors to the variance in γ′ fibrinogen and the γ′/total fibrinogen ratio, the CVD risk factors presented in Table 3 and supplemental Table 1 with and without total fibrinogen and or γ′ fibrinogen were entered into forward stepwise regression models (Table 4). The CVD risk factors together with fibrinogen explained 26.3% of the variance in γ′ fibrinogen, with total fibrinogen explaining 16%, followed by CRP (5%), HDL cholesterol (3%), gender (2%), HbA_{1c} (1%), and homocysteine (1%). When fibrinogen was excluded from the model, the CVD risk factors alone explained 19.9% of the variance in γ′ fibrinogen, with CRP now explaining 13.5% and little change in the contribution of the other CVD risk factors. When total fibrinogen or γ′ fibrinogen was entered together with the CVD risk factors, the models explained 39.3% and 37%, respectively, of the variance in γ′/total fibrinogen ratio. The CVD risk factors alone explained only 3% of the variance, of which CRP and HDL cholesterol made the largest contribution.

Discussion

Our data provide evidence that γ′ fibrinogen influences fibrin clot structure in plasma. It has been shown that γ′ fibrinogen changes fibrin structure in vitro in purified systems at levels of 100%[12-16]; however, whether such effects would also occur at physiological levels of 10% to 15% γ′ fibrinogen and in the presence of other plasma proteins was hitherto unknown. We used a large, well-characterized, population-based study to show that this is indeed the case. We found associations of γ′/total fibrinogen ratio with lower maximum absorbency and prolongation of lysis time in agreement with previously published in vitro effects. We also found that γ′ fibrinogen levels associate with other cardiovascular risk factors and not only with total fibrinogen levels.

Fibrinogen γ′ levels correlated positively with total fibrinogen, although the γ′/total fibrinogen ratio decreased with increasing fibrinogen concentration, suggesting that while γ′ fibrinogen increases as total fibrinogen increases, it does so to a relatively lesser extent (ie, γ′ levels were not a constant fraction of total fibrinogen levels throughout the entire range of fibrinogen concentrations), resulting in the decreased ratio at higher total fibrinogen levels. The median γ′ fibrinogen concentration in this black African population is higher to what was previously reported for whites from the Framingham Offspring study (0.38 vs 0.23 g/L). This is in agreement with the relatively high total fibrinogen concentration (3.7 g/L) found in this and other studies investigating fibrinogen concentration in black Africans.[23,27]

Turbidity curves are often used as a measure of clot formation and structure in large data sets due to its high-throughput methodology.[28] Its relevance is further supported by the fact that final clot structure is to a large extent kinetically controlled and information on clot formation is therefore critical in determining clot structure.[29,30] None of the fibrinogen variables showed noteworthy associations with lag time. This is likely due to the fact that the samples were clotted with tissue factor, so lag time represented the time required not only for the formation and growth of protofibrils from monomers but also for activation of the coagulation cascade. Tissue factor rather than thrombin was used to clot the samples because it allows for comprehensive analysis of plasma fibrinolytic potential (reported as CLT), including all coagulation and lysis components in plasma. Additionally, it allows for better comparison of the different variables because clot formation variables and lysis times were determined in the same experiment. In vitro experiments using purified fibrinogen also found no difference in lag time between clots made from γ′ or γA fibrinogen.[12,15]

Of the fibrinogen variables, total fibrinogen correlated best with slope (rate of lateral aggregation), although the fibrinogen variables explained only a small percentage of the variance observed in the slope. These results indicate an increased rate of lateral aggregation with increasing fibrinogen concentration. This is in agreement with a kinetic model developed by Weisel et al[29] that indicates an increase in the maximum rate of protofibril addition per fiber with increasing fibrinogen concentration. An increase in γ′ fibrinogen was associated with a small increase in rate of lateral aggregation, while an increase in the γ′/total fibrinogen ratio was associated with a small decrease. When adjusting for difference in total fibrinogen, the association between γ′ fibrinogen and slope disappeared, suggesting that in vivo (at least in the plasma setting) γ′ fibrinogen does not significantly affect rate of lateral aggregation despite decreased rates observed in purified models for clots made of γ′ compared with γA fibrinogen.[12,15,16] It may be that the effect of γ′ fibrinogen in plasma is relatively small compared with the effect of increasing fibrinogen concentration in this setting.

The variance in maximum absorbance was explained to a much larger extent (>30%) by the fibrinogen variables than by either lag time or slope. An increase in both γ′ fibrinogen and total fibrinogen

was associated with an increased maximum absorbance, with total fibrinogen contributing to a larger extent than γ′ fibrinogen. An increase in the γ′/total fibrinogen ratio was, however, associated with a decrease in maximum absorbance. In agreement with this, turbidity curves obtained from purified fibrinogen models showed decreased maximum absorbance in clots containing γ′ compared with γA fibrinogen, indicating that the γ′/total fibrinogen ratio is indeed an accurate indicator for the relative amount of γ′ in the plasma clot.[12,15,16] Reduced maximum absorbance from turbidity analysis is an indicator of thinner fibrin fibers at constant fibrinogen concentration. The effect of γ′ fibrinogen on fiber diameter as measured by scanning electron microscopy in vitro is generally in agreement with this. Several studies showed that clots containing γ′ fibrinogen have thinner fibers.[12,14-16] Others, however, using a recombinant homodimer form of fibrinogen γ′, found no difference in fiber diameter.[13] Several studies showed that fibrinogen γ′ fibrinogen produced clots with increased branching.[14,16] Additionally it was found that clots containing γ′ fibrinogen were nonhomogenously arranged into tight interconnecting bundles with tighter pores with bundled fibers and large open pores in other areas of the clot.[15,16] Although the use of plasma samples is of immense value in determining the in vivo relationship among γ′ fibrinogen, total fibrinogen, and clot structure, the interpretation of experimental results, especially from an indirect technique such as turbidity, is complicated due to the complex nature of plasma and the interplay of factors that determine maximum absorbency, including fibrinogen concentration and average fibrin fiber diameter. Because of the high-throughput design of turbidity analysis and the time-consuming nature of more direct methods (such as microscopy), it can, however, play a significant role in providing information regarding clot structure in an epidemiological study setting with large subject numbers.

There has been only 1 previous study that investigated the effect of γ′ fibrinogen concentration on clot structure in plasma. Mannila et al[8] investigated the effect of γ′ fibrinogen concentration on plasma permeability (Ks) and fiber mass-length ratio (μ calculated from Ks) in 60 control patients from the Stockholm Coronary Artery Risk Factor study. Fibrinogen γ′ concentration was not found to be associated with Ks or μ. However, the authors did not analyze γ′/total fibrinogen ratio and the overall study size was probably too small to detect the relatively moderate effects of physiological levels of γ′ fibrinogen on clot structure. Our current study is much larger in size and showed significant effects of γ′ fibrinogen on plasma clot structure. In particular, the γ′/total fibrinogen ratio showed similar effects on clot structure as previous in vitro studies, with an association of increased γ′ fibrinogen content with thinner fibers (lower maximum absorbance) and prolonged fibrinolysis.

Although CLT correlated positively with both γ′ fibrinogen and total fibrinogen, fibrinogen γ′ had the strongest association with CLT, explaining 12% of its variance. This suggests that γ′ fibrinogen has a larger effect on CLT than total fibrinogen. An increased CLT indicates a decreased lysis rate, which is in agreement with results from studies using purified fibrinogen that showed decreased lysis rates for clots containing γ′ compared with γA fibrinogen.[13,14,31] Falls and Farrell[31] additionally found decreased lysis rates in afibrinogenemic plasma to which γA/γ′ fibrinogen was added compared with plasma containing an equal concentration of γA/γA fibrinogen. They ascribed the decreased fibrinolysis rate to increased FXIII binding to γ′ fibrinogen and subsequent increased crosslinking. Allan et al,[16] however, found no difference in γ-chain crosslinking between γA/γA- and γA/γ′-containing clots and decreased α-chain crosslinking in γA/γ' clots, indicating that other factors likely play a role in the decrease of fibrinolysis rates by γ′ fibrinogen. Our current data provide clear evidence for a role of γ′ fibrinogen in the regulation of fibrinolysis rates also in plasma obtained from a large number of subjects with varying γ′ fibrinogen levels. As γ′ fibrinogen level increases in individuals, CLT also increases, and γ′ fibrinogen has a larger effect on CLT than total fibrinogen. The results furthermore suggest that this association between γ′ fibrinogen and CLT is not only the result of a denser clot network formed in the presence of higher γ′ fibrinogen concentration because total fibrinogen had a larger impact on maximum absorbance than γ′ fibrinogen and maximum absorbance did not correlate with CLT (data not shown). The mechanism by which γ′ fibrinogen influences fibrinolysis rates should be further investigated using experimental models of clot formation and fibrinolysis.

In general, the association of γ′ fibrinogen with CVD risk factors followed the same trend as that of fibrinogen. Increased levels were observed in women compared with men, subjects with increased BMI, increased waist circumference, elevated CRP, and HbA_{1c} categories and in individuals with metabolic syndrome as well as in individuals with low HDL cholesterol. The association between γ′ fibrinogen and these CVD risk factors remained significant after adjustment for total fibrinogen and is also accompanied by an increase in the γ′/total fibrinogen ratio, suggesting that these associations are not merely reflecting the association of the CVD risk factors with total fibrinogen and that they are likely independent relationships. These results are in agreement with the study of Lovely et al[32] who also found significant associations between γ′ fibrinogen and BMI, (decreased) HDL cholesterol, diabetes, blood glucose, and gender in whites. In two other studies, γ′ fibrinogen did not, however, differ between genders,[7,33] and while we and others[7,33] found no association between γ′ fibrinogen and age, a positive association was found by another group.[32] In this study population, total fibrinogen increased over 10-year age categories and in individuals with increased total cholesterol (>5.2 mmol/L) while no increase was observed for γ′ fibrinogen, resulting in a decreased γ′/total fibrinogen ratio. In hyperhomocysteinemic individuals, on the other hand, decreased γ′ fibrinogen was observed with no change in total fibrinogen consequently also decreasing the γ′/total fibrinogen ratio.

Multiple regression analysis indicated that fibrinogen concentration explained the largest percentage of the variation in γ′ fibrinogen and that the CVD risk factors alone, excluding total fibrinogen, explained 20% and 3% of the variances of γ′ fibrinogen and γ′/total fibrinogen ratio, respectively, suggesting that the γ′/total fibrinogen ratio in apparently healthy black Africans is not strongly affected by CVD risk factors other than γ′ fibrinogen and total fibrinogen. The γ′/total fibrinogen ratio was, however, found to be different between several CVD patient groups and white controls.[5,9,34] Of the other CVD risk factors, CRP was found to be the biggest determinant of both γ′ fibrinogen and γ′/total fibrinogen ratio. These results are in agreement with a case-control study by Cheung et al[34] who found a significant association between CRP and γ′/total fibrinogen ratio in the acute phase of ischemic stroke. These authors hypothesized that messenger RNA processing of γ′ fibrinogen may be altered during the acute phase reaction.

In conclusion, γ′ fibrinogen levels increase as total fibrinogen increases, although to a lesser extent, resulting in a decrease in γ′/total fibrinogen ratio at high fibrinogen concentrations in this apparently healthy black African population. Increases in both γ′ fibrinogen and total fibrinogen were associated with increased maximum absorbance, in agreement with the formation of clots composed of increased fibrin material. However, the γ′/total fibrinogen ratio was associated with decreased maximum absorbance, in agreement with clots made of thinner fibrin fibers. Increased γ′ fibrinogen levels

were associated with prolonged clot lysis. Traditional CVD risk factors (excluding fibrinogen) explained 20% and 3%, respectively, of the variance in γ′ fibrinogen and γ′/total fibrinogen ratio, with CRP making the largest contribution. These data show that physiological levels of γ′ fibrinogen influence fibrin clot structure in plasma and that factors other than fibrinogen, likely involved in the inflammatory response, regulate plasma γ′ fibrinogen concentration. Our findings support future studies of the role of γ′ fibrinogen in thrombosis.

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Authorship

Contribution: M.P. performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; R.C.K. analyzed and interpreted data and performed statistical analysis; J.C.J. and A.K. designed the research and collected data; and R.A.S.A. analyzed and interpreted data and critically reviewed the manuscript.

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