The Role of Fibrinogen Variants in Cardiovascular Diseases and Wound Healing

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The Role of Fibrinogen Variants in Cardiovascular Diseases and Wound Healing

De rol van fibrinogeen varianten in hart-en vaatziekten
en wondgenezing

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學而不思則罔，思而不學則殆。

Learning without thought leads to confusion;
thought without learning ends in danger.

Confucius (551 BC - 479 BC)

To my family

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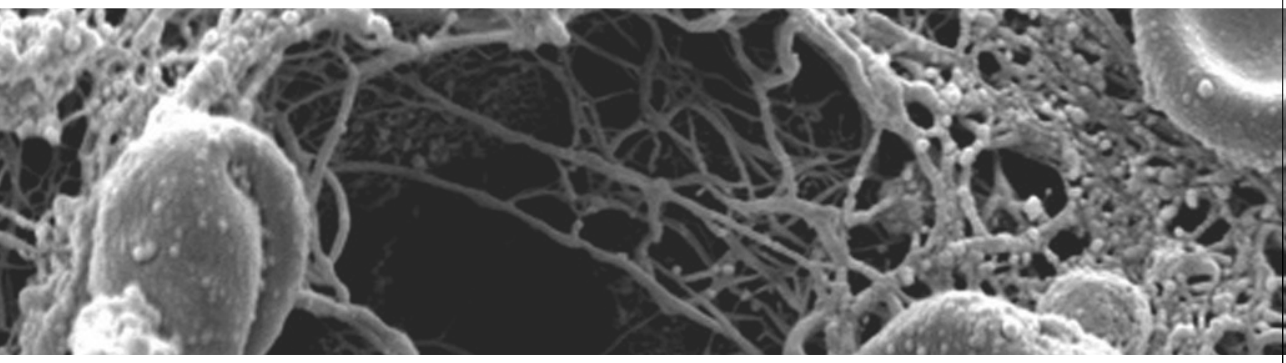

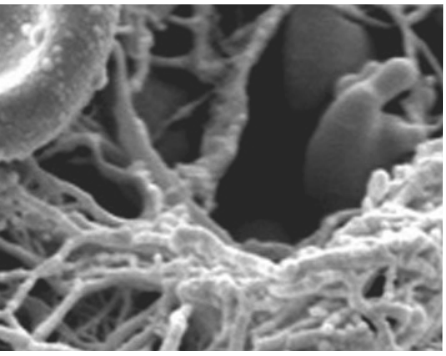

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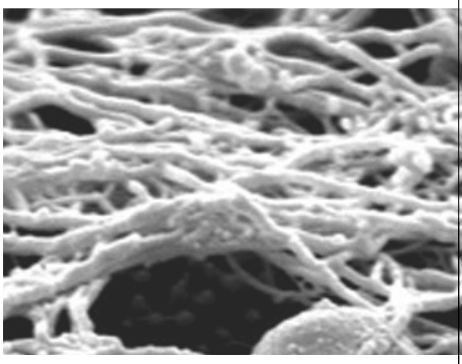


General introduction

HAEMOSTASIS

Maintaining the integrity and patency of the vascular system is essential for the viability of humans. When vascular injury has occurred, fast formation of a thrombus at the site of injury is essential to seal the wound, resulting in haemostasis. Haemostasis is a tightly regulated process, which involves the activation of endothelial cells, platelets, procoagulants and the inhibition of fibrinolytic factors. Haemostasis can be separated in two phases called primary and secondary haemostasis, which occur simultaneously.[1] In primary haemostasis, a platelets plug is rapidly formed at the site of injury, whereas in secondary haemostasis, blood coagulation is initiated, either with negatively charged surfaces (intrinsic pathway) or with tissue factor (extrinsic pathway). The cascade leads to the generation of thrombin and the formation of a fibrin network.[2] The thrombus provides an effective restriction for bleeding. Hence, an imbalance of normal haemostasis caused by pathologic disorders may lead to thrombosis or hemorrhage, which may account for morbidity and mortality.

FIBRINOGEN

Fibrinogen is a central protein in the hemostatic system. At the final stage of the blood coagulation system, thrombin converts the soluble fibrinogen into fibrin monomers, which then polymerize to an insoluble fibrin clot. Furthermore, fibrinogen induces platelet adhesion and aggregation via the $\alpha_{IIb}\beta_3$ integrin, thus promoting blood coagulation.

In addition to their primary function in blood clotting, fibrinogen and fibrin also play a role in various physiological reactions including fibrinolysis, cellular and matrix interactions, wound healing and neoplasia. Moreover, fibrin(ogen) is also involved in platelet aggregation, regulation of Factor (F) XIII activity and inflammatory reactions.[3-4] Fibrinogen is a plasma glycoprotein that is mainly synthesized by hepatocytes in the liver.[5] In plasma, the circulating concentration of fibrinogen is maintained between 2 and 4 mg/mL (6 -12 μM), and its half-life is about 3-4 days.[6] Fibrinogen is an acute phase protein, its levels are up-regulated 2 to10-fold through the action of interleukin 6 (IL-6) and glucocorticoids during the acute-phase response to inflammation, infection and tissue injury.[7-10] In addition, plasma fibrinogen levels are associated with many demographic and environmental factors in healthy individuals. The fibrinogen levels are associated with age, body mass, gender, race, season, smoking, physical exercise, diet and use of several drugs.[11]

Structure of fibrinogen

Fibrinogen molecules are elongated 45 nm structures that consisting of one central E region interconnected by α-helical coiled-coil segments to 2 peripheral D regions. The fibrinogen molecule comprises two sets of identical disulfide-bridged halves, each consisting of three polypeptide chains termed Aα, Bβ and γ.[12] These six polypeptide chains assemble to construct a hexamer $(A\alpha B\beta\gamma)_2$, joined together with 29 disulfide bonds.[13]

The fibrinogen molecule contains at least 12 domains, which are grouped into three major regions named E region, D region and αC region. In the central E region, the amino-termini of the 6 polypeptide chains are connected by five symmetrical disulfide bridges, the nonsymmetrical disulfide bridges formed in this region is called the disulfide ring.[13-15] The two distal D regions store the carboxy-terminals of the Bβ and γ chain and part of the Aα chain. Both D and E regions contains binding sites for fibrin assembly, cross-linking and platelet interactions.[16] The two αC regions, comprising the flexible carboxy-terminal two thirds of the Aα chain (residues 221–610 in human fibrinogen),[17-19] are involved in fibrin assembly,[20] activation of factor XIII,[21] modulation of fibrinolysis[22-23] and cell adhesion.[24-25]

The common human fibrinogen molecule contains 2964 amino acids and has a molecular weight of approximately 340 kilo Dalton (kDa). The Aα, Bβ and γ polypeptide chains consist of 610, 461 and 411 amino acids with molecular weights of 68 kDa, 55 kDa and 49 kDa respectively.[26-28]

The 3 polypeptide chains Aα, Bβ and γ are encoded separately by the fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*) and fibrinogen gamma (*FGG*) genes, which are clustered in a region of approximately 50 kilobases (kb) and located on chromosome 4q23-32. The 8.4 kb-*FGG* gene contains 10 exons and is oriented in tandem with the 5.4 kb-*FGA* gene, which contains 6 exons. The *FGG* gene is located 10 kb upstream of the *FGA* gene. Both genes are transcribed in the direction opposite the 8.2 kb-*FGB* gene, which is located 13 kb downstream of the *FGA* gene and contains 8 exons.[29] Assembly of the 6 separate chains takes place sequentially in the endoplasmic reticulum within 5 minutes of synthesis.[30-31]

Fibrinogen heterogeneity

Fibrinogen has a high degree of heterogeneity which fulfills its multiple functions in healthy individuals. It has been speculated that fibrinogen can occur in more than one million different forms as there are enormous possible combinations of genetic polymorphic site, alternative processing of fibrinogen messenger ribonucleic acids (mRNAs), posttranslational modifications and proteolytic degradation.[32] Moreover, abnormal modifications are associated with diseases but more investigations are needed to clarify they are the real causes or whether they are the consequences of the diseases.

Inherited variations in the fibrinogen genes

Numerous polymorphisms have been identified in the three fibrinogen genes. The majority of them are located in the non-coding regions, except for the Arg448Lys polymorphism in the Bβ gene and the Thr312Ala polymorphism in the Aα gene. An association between the Bβ Arg448Lys polymorphism and variations of clot structure has been suggested,[33] but contradictory results were reported.[34] The Aα Thr312Ala polymorphism is associated with thromboembolic disease.[35-36] The most extensively studied polymorphisms are located in the *FGB* gene, since *in vitro* studies suggested that the synthesis of the Bβ chain is the rate limiting step of the mature fibrinogen production and therefore the *FGB* gene is considered to be involved in determining the fibrinogen plasma levels.[37-38] A consistent association between *FGB* promoter genotypes and elevated plasma levels of fibrinogen plasma concentrations confirmed this hypothesis.[39-41] On the other hand, the variations of *FGA* and *FGG* genes are reported to play a role in regulating fibrin clot structure.[33] Therefore we focused on the variation in the *FGG* and *FGA* genes in this thesis. More recently, the relation between haplotype-tagging polymorphisms in the total fibrinogen gene cluster and fibrinogen plasma levels or the risk of cardiovascular diseases was investigated.[42-45]

Noninherited variations

Posttranslational modifications

In addition to the genetic variations in the fibrinogen genes, many variants in fibrinogen are noninherited. They are the result of alternative splicing, posttranslational modifications and proteolytic degradation. Fibrinogen molecules are modified posttranslationally in different degrees as a result of phosphorylation, glycation, glycosylation, oxidization, sialylation and deamidation. These modifications may affect the characteristics of fibrinogen, and affect proteolysis and polymerization.[32,46]

Proteolytic degradation

At the carboxyl-terminal of the Aα chains, partial proteolytic degradation can occur, which is the main cause of heterogeneity of the fibrinogen molecule (Figure 1). Fibrinogen is mainly synthesized in the high molecular weight (HMW) fibrinogen with two intact carboxyl ends of the Aα chain (molecular weight [MW] 340 kDa, 70% of total fibrinogen). Partial degradation of one Aα chain results in the low molecular weight (LMW) form (MW 305 kDa, 26% of total fibrinogen) and partial degradation of both Aα chains leads to LMW′ fibrinogen (MW 270 kDa, 4% of total fibrinogen).[47-48] The ratio of molecular weight variants varies according to the physiological condition, for example, the percentage of HMW fibrinogen increases during an acute phase reaction.[49-50]

The three molecular weight forms are a mixture of molecules with numerous degradation sites in the carboxyl terminus of the Aα chain. The main termination residues of the degraded Aα chains were Asn-269, Gly-297 and Pro-309.[51] However, no enzyme (included plasmin, gelatinase, trypsin, matrix metalloproteases, and elastase) has been identified that explains the proteolytic cleavage of HMW to LMW and LMW′ fibrinogen.[51] Previous studies showed that the rate of fibrin polymerization, clot stability, fibrin degradation and angiogenesis were influenced in the LMW and LMW′ fibrinogen when compare to HMW fibrinogen.[32,46,52]

Alternative mRNA processing variants

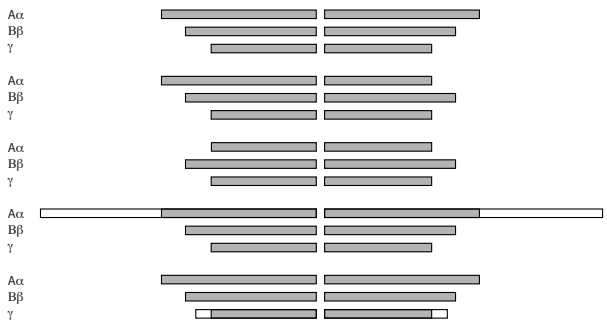
The extended Aα_E variant (α_E, Fib420)

There are two alternative processing variants from the fibrinogen genes (Figure 1). One of them is the result of alternative transcription of the *FGA* gene and includes exon six, which contains additional 236 amino acids. This form results in the generation of a minor form with an extended Aα_E chain (α_E). The carboxy(C)-terminus of both Aα chains has been extended and its molecular weight is 420 kDa, hence it also termed fibrinogen-420.[53-54] Only 1 to 2% of the total fibrinogen molecules is α_E.[55] The α_E levels are three times higher in newborns than in adults.[56] The C-terminus of α_E was reported to have chaperone-like activity,[57] supporting integrin-mediated cell adhesion,[58] mediate leukocyte adhesion and migration via the binding with leukocyte integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$.[59] However, the physiological role of α_E C has not yet been fully elucidated.

The fibrinogen gamma variants (γ′)

Another natural alternative processing variant is the fibrinogen γ′ variant, which comprises 8% to 15% of the total circulating fibrinogen (Figure 1, reviewed in [60]). Fibrinogen γ′ is a result of alternative messenger RNA processing and polyadenylation at the C-terminal of γA. The last 4 amino acids encoded by exon 10 (γA 408-411 AGDV) are replaced by the 20 unique anionic amino acids encoded by intron 9 (γ′ 408-427 VRPEHPAETEYDSLYPEDDL), leads to the formation of γ′.[61] The extended γ′ chain is thus larger than γA chain (MW 51.5 kDa and 49.4 kDa, respectively). In the systemic circulation, γ′ is mostly present as a heterodimer together with the common γA chain (γA/γ′), while less than 1% is present in the homodimeric γ′/γ′ form.[62] Approximately 3% to 34% of the γ′ occur as a shortened version, termed γ^{423P}, which probably arises by post-secretary *in vivo* processing of γ^{427L} fibrinogen chains. The γ^{423P} has impaired thrombin binding potential since the 424–427 sequence (EDDL), which is required for thrombin binding, is not included.

The extension of γ′ fibrinogen contains a high affinity binding site for thrombin, which results in antithrombin I activity.[7] In addition, γ′ fibrinogen contains an extra binding site for the factor XIII (FXIII) B subunit[8, 9] and lost its platelet integrin $\alpha_{IIb}\beta_3$ binding site, which results in reduced platelet-fibrin(ogen) interactions.[10] Several studies reported functional and structural differences between fibrinogen γA and fibrinogen γ′ fibrin matrices, such as slower fibrinopeptide B release,[63-64] slower fibrin polymerization for γ′ fibrinogen,[63,65] thinner fibers and more branch points with scanning electron microscopy on fibrin γ′ fibrin matrices.[63-64] The elevated γ′ levels and γ′/total fibrinogen ratio have been reported in cardiovascular diseases,[66-68] whereas decreased γ′ levels and γ′/total fibrinogen ratio have been associated with venous disease.[69] Furthermore, the *FGG* haplotypes are associated with thrombotic risk.[43,70-71] These associations indicate the γ′ may play a role in cardiovascular events.



Fibrinogen variants	Molecular weight (kDa)	Occurance (%)
HMW-FBG	340	70
LMW-FBG	305	26
LMW′-FBG	270	4
αE-FBG	420	1-2
γ′-FBG	340	8-15

Figure 1. Schematic representation of major fibrinogen variants in plasma
Bars indicate relative lengths of fibrinogen Aα, Bβ and γ chains, while the white bars represent elongated sequences of αE and γ′ fibrinogen variants. Adapted from de Maat et al., 2005.[46]

FIBRIN POLYMERIZATION

Fibrin formation is initiated by thrombin cleavage of a highly specific site Arg16 at the amino(N)-terminus of the Aα chains, resulting in release of fibrinopeptides A (FpA). Cleavage of FpA results in the exposure of a new polymerization site termed 'A'.[72] The complementary binding sites are called 'a', which are the adjacent molecules located between γ337 and γ379 in the D region, and are always exposed.[73-75] Fibrin monomers polymerize via these specific non-covalent A:a interactions and form half-staggered

oligomers that lengthen into double-stranded protofibrils (Figure 2). These intermolecular interactions have been described as knob-hole interactions.[76] During the lateral aggregation, branch points are created and which is essential to yield a complex three-dimensional fibrin network.[77-78]

Subsequently, thrombin cleavage after Arg14 at the N-terminal of the Bβ chains releases fibrinopeptides B (FpB) and exposes an independent N-terminal 'B' polymerization site. The 'B' polymerization site interacts with an exposed, complementary 'b' binding site in the Bβ chain of the D region of another molecule (Figure 2). This cleavage has been hypothesized to promote the lateral aggregation of existing fibrin protofibrils, resulting in an increase in the fiber thickness.[72] However, several snake venom enzymes (FpA release only) are possible to initiate fibrin formation, and also lateral aggregation can occur without FpB cleavage.[79-82]

The fibrin network is stabilized by plasma transglutaminase factor (F) XIII, which is activated by thrombin to form FXIIIa, and cross-links the fibrin clot covalently via glutamine–lysine bridging between two γ chains.[83-84] This intermolecular bridging also occurs more slowly between two α chains,[85-86] and even occurs between one γ and one α chain.[87] These cross-links further strengthen the fibrin clot and protect the clot against mechanical, chemical, and proteolytic degradation.[88]

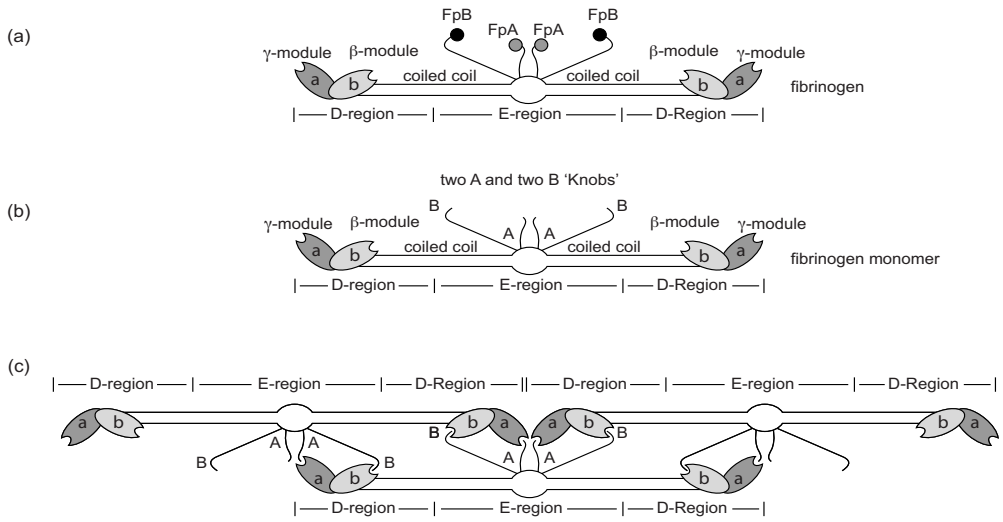


Figure 2. Schematic representation of fibrin monomer and their assembly into protofibrils
(a) Fibrin monomer. (b) Cleavage of FpA and FpB, results in the exposure of a new polymerization site termed 'A' and 'B'. (c) Fibrin monomers interact with adjacent molecules, assembly into protofibrils and promote the lateral aggregation. Adapted from Janmey et al., 2009.[89]

FIBRINOLYSIS

Fibrinolysis is a highly regulated system, which plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system. A dynamic balance between fibrin formation and fibrinolysis is essential, since excess activation of fibrinolytic system may lead to bleeding disorders whilst the opposite may lead to thrombosis.[90] In the fibrinolytic system (Figure 3), fibrin is degraded into soluble fibrin degradation products by the active serine protease plasmin, which is converted from its zymogen plasminogen by two types of plasminogen activators (PA).[91] In the circulatory system, the activation of plasminogen is enhanced by tissue-type plasminogen activator (t-PA) in the existence of fibrin or at the quiescent endothelial cell surface,[92] whereas urokinase-type plasminogen activator (u-PA) binds to a specific u-PA receptor (u-PAR) at the migrating capillary endothelial cells surface, inflammatory cells surface or endothelial microparticles,[93] thereby facilitates extracellular proteolysis during tissue remodeling and repair, regulates cell migration, adhesion and proliferation.[94]

The fibrinolytic system is controlled by several specific inhibitors, such as α_2-antiplasmin (α2-AP), plasminogen activator inhibitor 1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI). Most of them are belonged to the serine protease inhibitors (SERPINS) superfamily. The α2-AP inhibits plasmin rapidly by the formation of an irreversible complex via its free active site and free lysine binding sites.[95] Moreover, PAI-1 is the major physiological inhibitors of PA activity in the circulation, which inhibits the protease activity by formation of a reversible complex. Apart from PAI-1, the PA activity can be inhibited by plasminogen activator inhibitor-2, α2-macroglobulin, α2-AP, α1-antitrypsin, C1-inhibitor and plasminogen activator inhibitor-3.[96] TAFI is activated by thrombin and makes a connection between coagulation and fibrinolysis. Activated TAFIa degrades the C-terminal lysine and arginine residues from fibrin and provide a protection against plasmin.[96] In summary, fibrin plays an active role in fibrinolysis. It acts as substrate and co-activator for plasmin, and participates in its inhibition.

MODULATORS OF THE FIBRIN STRUCTURE

The fibrin clot structure can be described by the fiber thickness, length, porosity, permeability, degree of branching, and extent of cross-linking.[97] These variables determine properties of clot, such as stiffness and rate of fibrinolysis. Multiple factors regulate the fibrin clot formation, properties and function, including pH, ionic strength, calcium concentration,

fibrinogen concentration and thrombin concentration.[98-101] For example, an increased fibrinogen concentration and a decreased thrombin concentration lead to thicker fiber and more branch points, resulting in stiffer clots. Moreover, procoagulant activity affects the fibrin clot structure and stability, such as FXIIIa-induced fibrin cross-linking, interactions with platelet and vascular cells, which support the formation of dense fibrin networks that resist fibrinolysis.[102-103] In addition, fibrinogen polymorphisms (e.g. Bβ Arg448Lys[33] and Aα Thr312Ala polymorphisms[104]), fibrinogen variants (e.g. HMW-fibrinogen[52], LMW-fibrinogen[52] and γ′ fibrinogen) and pathophysiological condition (e.g. patients with type II diabetes mellitus,[105] premature coronary artery disease[106] or myocardial infarction[107]) also influence polymerization processes and may give an altered fibrin structure.

Taken together, the total effect of genetic and environmental influences determines the structure and function of the fibrin clot. The stability and characteristics of fibrin network play important roles in haemostasis and thrombosis. Alternations in fibrin structure could be a causative factor in the various hemorrhagic and thrombotic disorders, and understanding of the mechanism may lead to the development of therapeutic interventions to manage cardiovascular diseases.

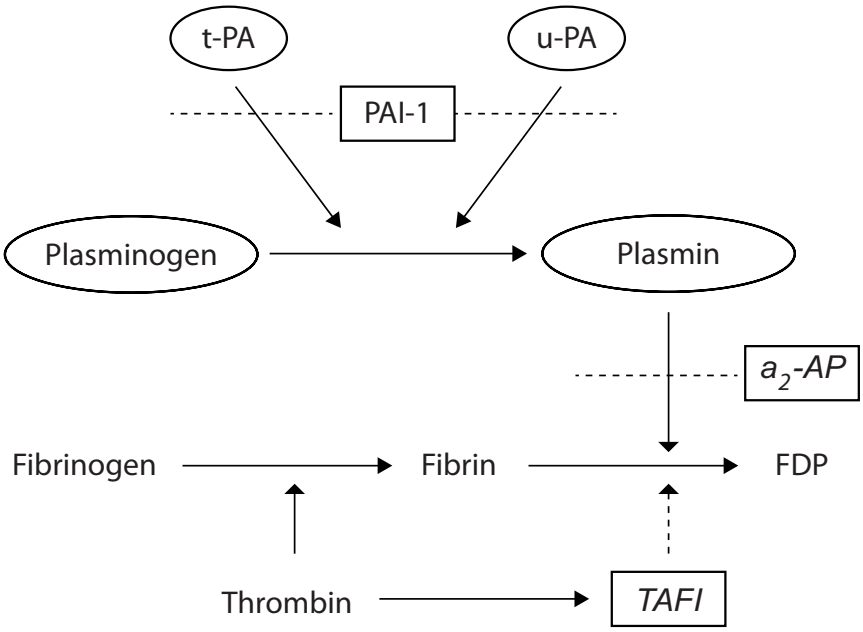


Figure 3. Fibrin degradation by the fibinolytic system

FIBRINOGEN AND WOUND HEALING

Wound healing is a dynamic, interactive and complex process, which consists of three sequential but overlapping phases: (1) inflammation (2) proliferation and (3) remodeling.[108] The wound healing process involves a series of cellular and biochemical events, such as cell adhesion, migration, proliferation, neovascularization, extracellular matrix (ECM) deposition and degradation. These events comprise the intricate interactions between many different cell types, extracellular matrix, soluble mediators (for instance growth factors and cytokines) and proteinases.[109-110]

Fibrinogen is a central protein in the coagulation cascade and it also plays a pivotal role in wound repair. After endothelial injury, a platelets plug is rapidly formed, followed by the formation of a fibrin network. The activated platelets are included in the fibrin network through the binding of fibrin to platelet integrin, the $\alpha_{IIb}\beta_3$ receptors. The resulting insoluble fibrin clot is the first protection against local hemorrhage in wounds. In addition to its function in haemostasis, the fibrin network provides an important provisional, biodegradable ECM that stabilizes the wound and facilitates cell invasion during wound healing process.

One of the important processes of wound healing is angiogenesis, which is the formation of new capillaries from pre-existing vessels in the ECM of the wounded tissue by migration and mitogenic stimulation of endothelial cells (EC).[110] This process consists of several steps, which including the stimulation of ECs by growth factors, degradation of the extracellular matrix by proteolytic enzymes, invasion of matrix by the migration and proliferation of EC, and finally the formation of capillary tube.[111] It is also highly depends on fibrin structure and the interactions between fibrin and proteins. The rate of fibrinolysis and angiogenesis are affected by the structure of fibrin matrix, for instance a faster fibrinolysis rate was observed in coarse matrix.[112] Furthermore, many haemostasis proteins (such as t-PA, plasminogen and FXIII) bind to fibrinogen and fibrin, which affect the fibrinolysis rate and hence influence the angiogenesis process.[113-114] The fibrin matrix is also physically associated with other matrix proteins, such as fibronectin and vitronectin, which provide a bridge between smooth muscle cells and fibrin.[16]

Besides being the temporary scaffold to support the wounded tissue, fibrin matrices actively recruit cells to modulate cell-cell and cell-matrix interactions. Previous studies have shown that many cell types have affinity to fibrin matrices, such as EC,[115] smooth muscle cells,[116] fibroblasts[117] and leukocytes.[118] These cells interact with fibrin matrices directly through cell surface integrin receptors (e.g. $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_{IIb}\beta_3$) and

non-integrin receptors, such as Vascular Endothelial(VE)-Cadherin, intercellular adhesion molecule (I-CAM), P-selectin and platelet glycoprotein Ib-α (GP1ba).[16,119] In addition, fibrin matrices serve as a reservoir for cytokines and growth factors during tissue repairing, in particular the high affinity binding with angiogenic growth factors vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).[120-121]

In summary, fibrin is an essential matrix to prevent local hemorrhage, to interact with proteins, proteases, protease inhibitors, growth factors and cells, to induce and modulate cell responses, and to facilitate the wound healing process. Since fibrin matrices are a great potential for wound repair and tissue regeneration, they have been utilized as fibrin sealants for multiple tissue engineering applications, including peripheral nerves repair, bone regeneration, skin grafting after burn, and induction of angiogenesis.[122-126]

AIM AND OUTLINE OF THE THESIS

The aim of this thesis is to investigate the role of fibrinogen variants and fibrinogen elastase degradation products in cardiovascular diseases (CVD), and to understand the contributions of fibrinogen γ′ variants in wound healing.

In **Chapters 2-4**, we investigate the relationship between common genetic variations of fibrinogen and CVD. In **Chapters 5-9,** one of the natural fibrinogen variants, the fibrinogen γ′ variant, is extensively studied. Finally, the relationship between fibrinogen elastase degradation products and stroke is evaluated in **Chapter 10**.

As previous described, an elevated plasma fibrinogen level is a well-known independent risk predictor for CVD. Genetic variations in the fibrinogen genes influence the plasma fibrinogen concentrations and the clot structure, thereby potentially contributing to the cardiovascular events. In **Chapter 2**, we investigate the association between plasma fibrinogen levels and the presence of cerebral small vessel disease (SVD) based on magnetic resonance imaging (MRI) of the brain. In addition, the relationship between common variations in *FGG*, *FGA* and *FGB* genes and SVD is examined. We perform the study as part of the Rotterdam Scan Study, a population based imaging study in individuals aged between 60 and 90 years.

Since vascular disease is associated with dementia and Alzheimer's disease, we hypothesized an association between altered fibrin clot structure and dementia. In **Chapter 3**, we investigate whether common variations in the *FGG* and *FGA* genes, which are involved in regulating clot structure, are associated with an increased risk of dementia and Alzheimer's disease. The study is performed in the Rotterdam Study, a population-based

prospective cohort study among persons of 55 years and over. In **Chapter 4**, we further investigate the involvement of genetic variations in the *FGG* and *FGA* genes in stroke in the Rotterdam Study.

Previous studies have reported associations between the plasma levels of fibrinogen γ′ variant, the fibrinogen γ′/total fibrinogen ratio and deep venous thrombosis, myocardial infarction and coronary artery disease. These results indicate that *FGG* gene variations, which are a main determinant of the plasma fibrinogen γ′ levels, may contribute to the pathology of thrombotic disease and influence the risk of CVD. In **Chapter 5**, we conduct a case-control study, the COntrolled study of genetically determined COagulation disorders in patients with transient ischemic attack or ischemic Storke (COCOS study), including 124 stroke patients and 125 population controls, to determine the role of fibrinogen γ′ levels and common *FGG* gene variations in ischemic stroke. In **Chapter 6**, we hypothesize that acute phase reaction alters the mRNA processing of fibrinogen γ′, thereby increasing the γ′/total fibrinogen ratio. In addition, an increased γ′ ratio during the acute phase of cardiovascular disease may modulate the secondary thrombotic risk. We test these hypotheses in patients with ischemic stroke (IS), pulmonary embolism (PE) and unstable angina pectoris (UAP).

Subsequently, we compare the differences in fibrinogen γ′ levels and γ′/total fibrinogen ratio between patients with ischemic stroke and control persons in **Chapter 7**. Furthermore, we study whether there is an association between these levels and short-term outcome in ischemic stroke. On the other hand, the role of fibrinogen γ′ in intracerebral hemorrhage (ICH) has not yet been elucidated, we therefore aim to determine the association between γ′ levels and the γ′/total fibrinogen ratio in patients with ICH compared with healthy controls in **Chapter 8**.

We suggest that the functional differences between γA and γ′ fibrin are associated not only with CVD, but also in combination with the altered matrix structure, influence angiogenesis and wound healing. In **Chapter 9,** we investigate the effects of the γA and γ′ fibrinogen variants on the *in vitro* endothelial cell characteristics and *in vivo* wound healing.

Previously, an association has been found between levels of leucocyte elastase and the risk of cardiovascular events and ischemic stroke. In **Chapter 10**, the effect of fibrinogen elastase degradation products (FgEDP) levels in patients with ischemic stroke is evaluated using a newly developed assay, which enables the assessment of elastase proteolytic activity by measuring FgEDP levels.

Finally, the results of our studies are discussed in **Chapter 11**. We attempt to increase the understanding and insight for the future prospects of fibrinogen variants in CVD and wound healing.

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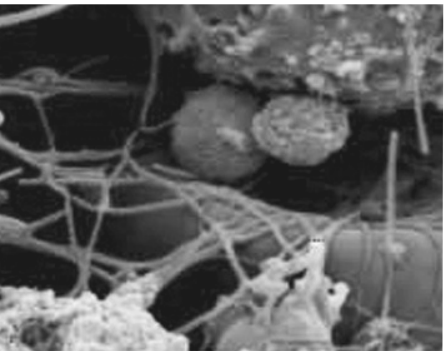

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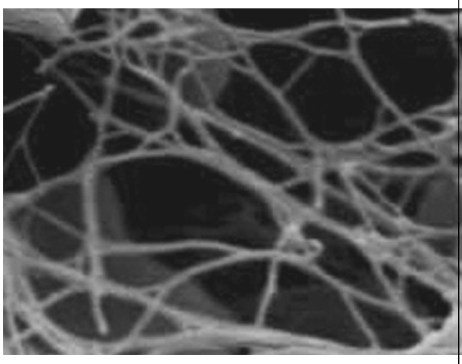

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Haplotypes of the fibrinogen gene and cerebral small vessel disease: the Rotterdam scan study

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ABSTRACT

Fibrinogen levels and fibrinogen clot structure have been implicated in the pathogenesis of vascular disease. We examined fibrinogen levels and variation in fibrinogen genes (fibrinogen γ (*FGG*), α (*FGA*) and β (*FGB*)), which have been associated with fibrin clot structure and fibrinogen levels, in relation to cerebral small vessel disease (SVD).

This study was performed as part of the Rotterdam Scan Study, a population based study in 1,077 elderly patients undergoing cerebral MRI. Plasma fibrinogen levels and haplotypes were determined. We examined the association between fibrinogen levels and haplotype with silent brain infarcts and white matter lesions using logistic regression models. We constructed seven haplotypes (frequency>0.01) that describe the total common variation in the *FGG* and *FGA* genes. Haplotype 2 (G**A**T**A**GT**G**) was associated with the presence of silent brain infarcts compared with the most frequent haplotype (GGTGGTA) (OR 1.41, 95% CI 1.03 to 1.94). Haplotype 3 (GG**C**G**A**TA) was associated with periventricular white matter lesions in the highest tertile of the distribution (OR 1.40, 95% CI 1.01 to 1.92). No association was found between plasma fibrinogen levels and SVD.

Our study provides evidence for an association of common variation in the *FGG* and *FGA* genes with cerebral SVD. It is possible that the structure of the fibrin clot rather than plasma fibrinogen levels plays a role in the pathogenesis of cerebral SVD.

INTRODUCTION

Silent brain infarcts and cerebral white matter lesions are commonly detected on brain imaging in the elderly. Both result from small vessel disease (SVD) and have been associated with an increased risk of stroke and dementia.[1] The pathogenesis of SVD is incompletely understood. Established risk factors are age and hypertension, although inflammatory, haemostatic and endothelial factors have also been implicated in the development of SVD.[2-3]

Fibrinogen has both inflammatory and haemostatic properties, and higher plasma levels have been associated with an increased risk of coronary artery disease, ischaemic stroke and dementia.[4-5] Also, there is increasing evidence that altered structure of the fibrin clot may be involved in the pathogenesis of atherosclerosis and thrombotic disease.[6] Genetic and environmental influences contribute to the variation in plasma fibrinogen concentration and fibrin clot structure. Fibrinogen is primarily synthesised by hepatocytes and consists of two symmetric sets of three chains (Aα, Bβ and γ), encoded by three separate genes, fibrinogen α (*FGA*), fibrinogen β (*FGB*) and fibrinogen γ (*FGG*), clustered on chromosome 4. The *FGB* gene is thought to be involved in determining fibrinogen plasma levels while the *FGA* and *FGG* genes are believed to play a role in regulating fibrin clot structure.[7]

We hypothesised a role for both fibrinogen plasma levels and clot structure in the pathogenesis of SVD. Therefore, we investigated the association between levels of fibrinogen in plasma and the presence of SVD, including silent brain infarcts and periventricular and subcortical white matter lesions, on MRI of the brain. In addition, we investigated the association between common variations in *FGG*, *FGA* and *FGB* genes, which are associated with fibrin clot structure and fibrinogen levels, and SVD. We performed the study as part of the Rotterdam Scan Study, a population based imaging study among those aged between 60 and 90 years.

METHODS

Study population

The Rotterdam Scan Study was designed to study causes and consequences of brain changes in the elderly.[8] In 1995–1996, participants aged 60–90 years were randomly selected in strata of age (5 years) and sex from two large ongoing population based studies, the Zoetermeer Study and the Rotterdam Study.[9] A total of 1077 non-demented elderly

subjects participated in the study (overall response 63%). The medical ethics committee of Erasmus Medical Centre approved the study and all participants gave informed consent.

Measurement of fibrinogen levels

Plasma concentrations of fibrinogen were determined in citrated plasma that was stored at –80°C. Prior to measurement, plasma samples were thawed at 37°C for 10 min. The method used was based on the Von Clauss[10] clotting rate assay (Fibrinogen-C kit; Instrumentation Laboratory, Breda, The Netherlands) and performed on an Automated Coagulation Laboratory (ACL 300; Instrumentation Laboratory).

Measurement covariates

We obtained information on the following variables by interview and physical examination in 1995–1996: systolic blood pressure, diastolic blood pressure, body mass index, smoking and carotid intima thickness as a measure of carotid atherosclerosis.[11-12]

Single nucleotide polymorphisms selection and genotyping

The Seattle Single Nucleotide Polymorphisms (SNPs) programme for Genomic Applications has identified various SNPs in the *FGG* and *FGA* genes and the common haplotypes can be identified by haplotype tagging SNPs. By genotyping seven haplotype tagging SNPs, we were able to infer haplotypes and describe the common variation across the *FGG* and *FGA* genes. The length (total number of SNPs known to date) of the *FGG* and *FGA* genes are 8.6 kb (64 SNPs) and 7.6 kb (44 SNPs), respectively. We genotyped in the *FGG* and *FGA* genes the *FGG* 5836 G>A (rs2066860, intron 7), *FGG* 7874 G>A (rs2066861, intron 8), *FGG* 9340 T>C (rs1049636, intron 9), *FGA* 2224 G>A (rs2070011, exon 1), *FGA* 3655 G>A (rs2070014, intron 2), *FGA* 3807 T>C (rs2070016, intron 2) and the *FGA* 6534 A>G (rs6050, exon 5) polymorphisms that tag haplotypes covering the total common variation in the *FGG* and *FGA* genes. In addition, we genotyped the functional –148 C>T polymorphism (rs1800787) in the *FGB* gene that has been shown to directly affect *FGB* promoter activity.[13] The *FGB* gene has a length of 8.1 kb and to date 75 SNPs have been identified. All polymorphisms have been described at http://www.ncbi.nlm.nih.gov/SNP.

DNA was isolated using standard procedures and genotyping was performed using baseline samples stored at –80°C. Genotypes were determined in 2 ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California,

USA). Primer and probe sequences were designed using the SNP assay by design service of Applied Biosystems. Reactions were performed with the Taqman Prism 7900HT 384 wells format in 2 μLof reaction volume.

Haplotype alleles present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/web/packages/haplo.stats/index.html), which computes maximum likelihood estimates of haplotype probabilities.[14-15] Information on all SNPs was available for 899 persons.

Outcome measures on MRI

All participants underwent MRI of the brain in 1995–1996. Axial T1 weighted, T2 weighted and proton density scans on 1.5T MRI scanners (for participants from Zoetermeer: MR Gyroscan, Philips, Best, the Netherlands and for participants from Rotterdam: MR VISION, Siemens, Erlangen, Germany) were made. Slice thickness was 5 or 6 mm, with an interslice gap of 20%.

Infarcts were defined as focal hyperintensities on T2 weighted images, 3 mm in size or larger. Proton density scans were used to distinguish infarcts from dilated perivascular spaces. Lesions in the white matter also had to have corresponding prominent hypointensities on T1 weighted images in order to distinguish them from cerebral white matter lesions. A history of stroke and transient ischaemic attack was obtained by self-report, and by checking medical records in all 1,077 participants. An experienced neurologist (PJK) subsequently reviewed the medical history and scans and categorized the infarcts as silent or symptomatic. Silent brain infarcts were defined as evidence of one or more infarcts on MRI, without a history of a (corresponding) stroke or transient ischaemic attack. Participants with both symptomatic and silent infarcts were categorised in the symptomatic infarct group.

White matter lesions were considered present if visible as hyperintense on proton density and T2 weighted images, without prominent hypointensity on T1 weighted scans. Two raters independently scored periventricular and subcortical located white matter lesions. Both intra-reader and inter-reader studies (n=100) showed good to excellent agreement (κ=0.79–0.90, r=0.88–0.95). A detailed description of the scoring method has been reported previously.[16] Briefly, severity of periventricular white matter lesions was rated semiquantitatively at three regions (grade 0–9). A total volume of subcortical white matter lesions was based on the approximate number and size of the lesions in the frontal, parietal, occipital and temporal lobes (volume range 0–29.5 ml).

Data analysis

Because of the skewed distribution of fibrinogen levels, we used log transformed fibrinogen in the analyses. We examined the association of levels of fibrinogen with the presence of silent brain infarcts and periventricular and subcortical white matter lesions (third tertile versus the lower tertile of the distribution) by means of logistic regression models, adjusted for age and sex. Only for the analyses on silent brain infarcts did we exclude persons with symptomatic brain infarcts. We examined the associations per standard deviation (SD) increase in log transformed fibrinogen and in quartiles using the lowest quartile as the reference category. In addition, we adjusted for cardiovascular factors, including systolic and diastolic blood pressure, body mass index, current smoking and carotid intima thickness.

Hardy–Weinberg equilibrium of fibrinogen polymorphisms was tested using χ^2 tests. We examined the association of the –148 C>T *FGB* promoter polymorphism with the presence of silent brain infarcts and periventricular and subcortical white matter lesions (third tertile versus the lower tertile of the distribution) by means of logistic regression models, using the CC genotype as the reference category. To test the associations of haplotypes of the *FGG* and *FGA* genes with levels of fibrinogen, silent brain infarcts and white matter lesions, we used the program Haplo Stats (http://cran.r-project.org/web/packages/haplo.stats/index.html).[14-15, 17] The probability for each haplotype pair in each individual was assigned and then an individual's phenotype was directly modelled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. The association between fibrinogen haplotypes and fibrinogen levels, silent brain infarction and white matter lesions was examined by means of the haplo.glm function of Haplo Stats.[14] This approach is based on generalised linear model, and computes the regression of a trait on haplotypes and other covariates. In these analyses the most frequent haplotype was used as the reference category. It is important to note that in this type of analysis, haplotypes rather than subjects are used as the exposure. We adjusted for age and sex, and additionally for cardiovascular factors. As plasma fibrinogen levels are an important determinant of clot structure we repeated the analyses adjusting for plasma fibrinogen. An interaction between fibrinogen levels and clot structure on disease risk has been suggested. Therefore, we repeated the analyses stratifying on high levels (above the median) and low levels (below the median) of fibrinogen.

RESULTS

We identified 213 persons (mean age 75.5 (SD 7.0) years, 58.0% female) with silent brain infarcts on brain MRI, most of which had lacunar infarcts (n=198), 358 persons (mean age 76.2 (SD 7.3), 54.0% female) with subcortical white matter lesions and 327 (mean age 76.7 (SD 7.1) years, 56.0% female) with periventricular white matter lesions. The baseline characteristics of the total study population are shown in Table 1. Table 2 shows the constructed haplotypes and their frequencies in our population.

Table 1. Baseline characteristics of the study population (n=1,077)

Characteristic	
Mean age (y)	72.2
Sex (female) (%)	52
Body mass index (kg/m^2)	26.7 (3.6)
Systolic blood pressure (mm Hg)	147.3 (21.6)
Diastolic blood pressure (mm Hg)	78.7 (11.7)
Intima media thickness (mm)	0.9 (0.2)
Current smokers (%)	17
Diabetes mellitus (%)	7
Fibrinogen (g/L)*	3.7 (3.2–4.4)
-148 C>T FGB (n (%))	
CC genotype	662 (65.4)
CT genotype	303 (29.9)
TT genotype	47 (4.6)

Values are mean (SD), unless otherwise stated.
*Because of skewed distribution, median (interquartile range) is given.

Levels of log transformed fibrinogen were not associated with silent brain infarcts or white matter lesions on MRI (Table 3). This did not change after additional adjustment for cardiovascular factors or when only lacunar infarcts (n=198) were analysed. All individual polymorphisms were in Hardy–Weinberg equilibrium.

No significant association was found between the –148 C>T *FGB* polymorphism and SVD. Compared with the CC genotype (reference), the age and sex adjusted odds ratio (OR) (95% confidence interval (CI)) for the presence of silent brain infarcts for the CT genotype was 1.36 (0.96 to 1.92) and 1.30 (0.61 to 2.78) for the TT genotype (p for trend 0.10).

Table 2. Construction of haplotypes and their frequencies

Haplotype	FGG gene			FGA gene				Frequency
	5836G>A rs2066860	7874G>A rs2066861	9340T>C rs1049636	2224G>A rs2070011	3655G>A rs2070014	3807T>C rs2070016	6534A>G rs6050	
1	G	G	T	G	G	T	A	0.26
2	G	**A**	T	**A**	G	T	**G**	0.25
3	G	G	**C**	G	**A**	T	A	0.18
4	G	G	T	G	G	**C**	A	0.13
5	G	G	**C**	**A**	G	T	A	0.11
6	**A**	G	T	G	G	T	A	0.03
7	G	G	T	**A**	G	T	**G**	0.01

FGA, fibrinogen α gene; FGG, fibrinogen γ gene.

Table 3.
Association of fibrinogen with silent brain infarcts, periventricular and subcortical white matter lesions.

	Silent brain infarcts OR (95% CI)	Periventricular white matter lesions OR (95% CI)	Subcortical white matter lesions OR (95% CI)
Log transformed fibrinogen per SD	1.03 (0.88-1.22)	0.98 (0.84-1.14)	0.94 (0.81-1.09)
1st quartile	1.00 (ref)	1.00 (ref)	1.00 (ref)
2nd quartile	1.47 (0.90-2.33)	0.85 (0.55-1.33)	1.13 (0.74-1.71)
3rd quartile	1.43 (0.89-2.31)	1.00 (0.65-1.54)	1.03 (0.68-1.57)
4th quartile	1.07 (0.65-1.74)	0.88 (0.57-1.37)	0.83 (0.54-1.27)

OR and 95% CI adjusted for age and sex.

Table 4. Association of haplotypes of the *FGG* and *FGA* gene with silent brain infarcts, periventricular and subcortical white matter lesions.

Haplotype	Silent brain infarcts OR (95% CI)	Periventricular white matter lesions OR (95% CI)	Subcortical white matter lesions OR (95% CI)
1	1.00 (ref)	1.00 (ref)	1.00 (ref)
2	**1.41 (1.03-1.94)**	1.29 (0.96-1.72)	0.90 (0.67-1.19)
3	1.33 (0.93-1.89)	**1.40 (1.01-1.92)**	0.87 (0.64-1.20)
4	1.39 (0.94-2.05)	1.25 (0.87-1.79)	0.95 (0.74-1.42)
5	1.30 (0.85-2.00)	1.08 (0.73-1.60)	0.76 (0.52-1.12)
6	0.43 (0.16-1.14)	0.99 (0.49-1.99)	0.66 (0.33-1.33)
7	1.03 (0.28-3.76)	2.08 (0.75-5.77)	0.98 (0.35-2.77)

OR and 95% CI adjusted for age and sex.

The age and sex adjusted OR (95% CI) for periventricular white matter lesions was 1.03 (0.75 to 1.42) for the CT genotype and 1.26 (0.64 to 2.48) for the TT genotype (p for trend 0.60). The age and sex adjusted OR (95% CI) for subcortical white matter lesions was 1.12 (0.82 to 1.53) for the CT genotype and 0.74 (0.36 to 1.50) for the TT genotype (p for trend 0.99). These estimates did not change after additional adjustments for cardiovascular factors. The –148 C>T *FGB* polymorphism was not associated with fibrinogen levels in our population. The age and sex adjusted mean of log transformed fibrinogen (95% CI) was 1.33 (1.31 to 1.35) for the CC genotype, 1.34 (1.31 to 1.37) for the CT genotype and 1.37 (1.30 to 1.44) for the TT genotype.

Haplotype reconstruction of the *FGG* and *FGA* genes resulted in 26 haplotypes, but only seven haplotypes had a frequency of >0.01 in our population. We disregarded 19 rare haplotypes with a frequency <1%, adding up to a total frequency of 3.8%. Haplotype alleles were coded as haplotype numbers 1 to 7 in order of decreasing frequency in the population (coding from 5836 G>A, 7874 G>A, 9340 T>C, 2224 G>A, 3655 G>A, 3807 T>C, 6534 A>G, haplotype 1=G-G-T-G-G-T-A, haplotype 2=G-**A**-T-**A**-G-T-**G**, haplotype 3=G-G-**C**-G-**A**-T-A, haplotype 4=G-G-T-G-G-**C**-A, haplotype 5=G-G-**C**-**A**-G-T-A, haplotype 6=**A**-G-T-G-G-T-A and haplotype 7=G-G-T-**A**-G-T-G). Haplotype analyses were based on 899 participants for whom information on all SNPs was available.

Compared with haplotype 1, haplotype 2 was associated with a higher prevalence of silent brain infarcts and haplotype 3 was associated with more periventricular white matter lesions in the highest tertile of the distribution (Table 4). Additional adjustment for cardiovascular factors did not markedly change the associations. If anything, they got stronger (OR (95% CI) for silent brain infarcts (haplotype 2) 1.48 (1.08 to 2.03) and for periventricular white matter lesions (haplotype 3) 1.42 (1.02 to 1.96)). Analysing lacunar infarcts only (n=198) gave similar results.

No association was found between haplotypes and plasma levels of fibrinogen, and adjustment for plasma fibrinogen did not change the estimates. Stratifying on high and low plasma fibrinogen also did not change the associations.

DISCUSSION

In this study, common variation in the *FGG* and *FGA* genes was associated with the presence of silent brain infarcts and periventricular white matter lesions on brain MRI. No associations were found between levels of fibrinogen or the –148 C>T *FGB* promoter polymorphism and silent brain infarcts or white matter lesions. Haplotypes of the *FGG*, *FGA* and *FGB* genes were not associated with fibrinogen levels.

Although an association between plasma fibrinogen levels and SVD has been suggested,[18] we were not able to confirm this finding. In addition, no association was found between the –148 C>T *FGB* promoter polymorphism and SVD. This is in contrast with a previous study by Martiskainen et al. that reported an association between the –455 G>A *FGB* promoter polymorphism, which is in perfect linkage disequilibrium (r^2=1) with the –148 C>T polymorphism, and lacunar infarction.[19] However, no fibrinogen levels were available in this study. Also, the study by Martiskainen et al. included only stroke patients while the Rotterdam Scan Study is population based, which may account for differences in results. Several other studies focused on the possible association between the –148 C>T polymorphism and ischaemic stroke, and their results were inconclusive.[20–22] It should be noted that in our population the –148 C>T *FGB* polymorphism was not significantly associated with fibrinogen levels. In an elderly population such as ours, the effects of the –148 C>T polymorphism on fibrinogen levels may be attenuated. Recently, such a decrease in raising effect on fibrinogen levels was shown for the –455 G>A polymorphism.[23]

The lack of an association between *FGG* and *FGA* haplotypes and plasma levels of fibrinogen is in line with previous findings.[24-25]

To date, no study has examined common genetic variation in *FGG* and *FGA* in relation to cerebrovascular disease. However, common variation in these genes has been associated with other manifestations of vascular disease, independent of fibrinogen levels. Recently, Uitte de Willige et al. studied haplotypes that describe the common variation in the *FGG*, *FGA* and *FGB* genes in relation to the risk of deep venous thrombosis.[25] The haplotype of *FGG* (*FGG*-H2), tagged by 7874 G>A (rs2066861) and comparable with haplotype 2 in our study, was associated with an increased risk of deep venous thrombosis. A study by Mannila et al. reported an association between haplotypes containing *FGG* SNP 9340 T>C (rs1049636; in their study named 1299+79 T>C), that tags haplotype 3 in our study, and *FGA* SNP 2224 (rs2070011; in their study named –58 G>A), that tags haplotype 2 in our study, and the risk of myocardial infarction.[24] Several studies have examined individual polymorphisms in the *FGG* and *FGA* genes and vascular disease risk.

The 6534 A>G (rs6050; also referred to as Thr312Ala) polymorphism in the *FGA* gene has been associated with venous thromboembolism, pulmonary embolism and post-stroke mortality among patients with atrial fibrillation.[26-27]

Although not associated with fibrinogen levels, there is evidence that variation in the *FGA* and *FGG* genes has functional implications. Uitte de Willige et al. reported that the *FGG*-H2 was associated with reduced levels of fibrinogen γ′ (and a reduced γ′/γ ratio), a product of alternative splicing of the *FGG* gene. It is not clear how this reduced ratio influences fibrin formation and degradation. Several studies have provided evidence for a role of plasma fibrinogen γ′ in disease risk.[28-29] Recently, Mannila et al. showed that the *FGA* 2224G>A SNP constitutes an independent determinant of fibrin clot porosity and is involved in epistatic interactions on plasma fibrinogen concentration.[30]

Also, evidence for a functional role of the *FGA* 6534 A>G SNP exists. This SNP occurs in a region important for FXIII dependent cross linking processes and may affect fibrin clot structure or stiffness. It is not clear how this increase in clot stiffness leads to a tendency to embolise. Perhaps stiffer clots are more brittle, leading to an increased tendency to fragmentize under conditions of blood flow.[7,31] Interestingly, the *FGA* SNP 2224 G>A in the study by Mannila et al. and the *FGG*-H2 haplotype in the study by Uitte de Willige et al. are in strong linkage disequilibrium with the 6534 A>G polymorphism.

In our study, common variation in the *FGG* and *FGA* genes was associated with silent brain infarcts and periventricular white matter lesions, but not with subcortical white matter lesions. It has previously been suggested that different pathophysiological mechanisms may underlie periventricular and subcortical white matter lesions.[32-33] For example, atrial fibrillation has been found to be predominantly related to periventricular white matter lesions.[34] A possible explanation for this may be that periventricular white matter is an arterial border zone and therefore more vulnerable while the subcortical white matter is better vascularised.[35]

In conclusion, in this population based imaging study, we found that common variation in the *FGG* and *FGA* genes of fibrinogen was associated with SVD on brain MRI. As this is the first report, our study needs to be replicated. Also, more functional studies need to be performed to elucidate the mechanism through which these genes influence the risk of vascular disease. Our findings suggest that mechanisms related to fibrin clot structure are involved in the pathogenesis of SVD rather than plasma concentration of fibrinogen.

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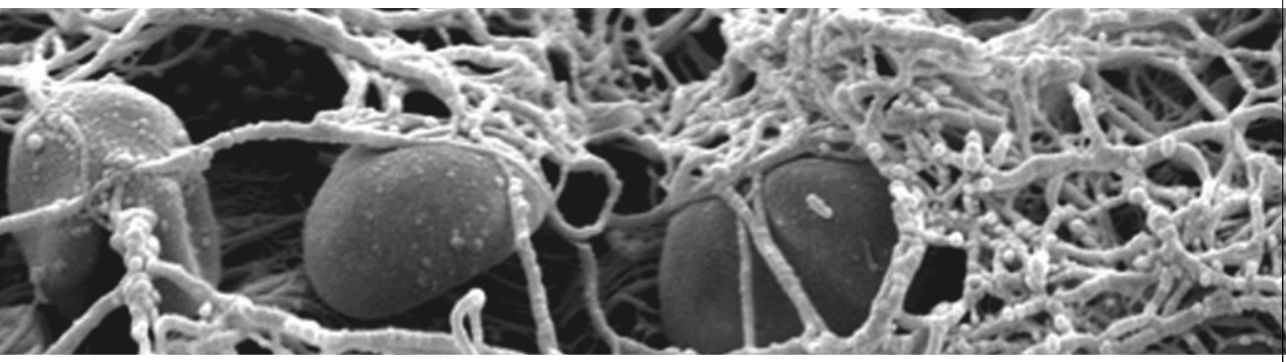

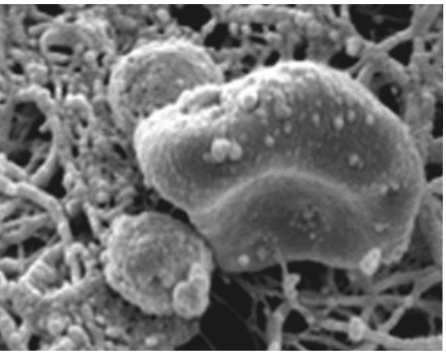

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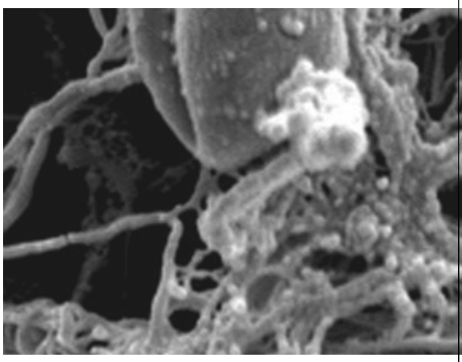

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Haplotypes in the fibrinogen gene and dementia

Submitted

ABSTRACT

Both inflammatory and hemostatic factors have been implicated in the pathogenesis of dementia. Previously we reported an association between higher plasma levels of fibrinogen and an increased risk of dementia. Whether fibrin clot structure plays a role in development of dementia is not known. We examined the association between variation in fibrinogen genes involved in fibrin clot structure (fibrinogen γ (*FGG*) and α (*FGA*)) and dementia.

The study was performed within the Rotterdam Study, a prospective population-based study among 7,983 persons of 55 years and over, ongoing since 1990. Seven tagging single nucleotide polymorphisms (SNPs) in the *FGG* and *FGA* genes were determined in blood samples drawn at the baseline examination (1990-1993). Until January 1st 2005, 743 dementia patients were identified. We examined the association between constructed haplotypes and dementia by means of logistic regression models.

Seven haplotypes (frequency >0.01) that describe the total common variation in *FGG* and *FGA* genes were constructed. Compared with the most frequent haplotype 1 (G-G-T-G-G-T-A) no association was found between haplotypes and dementia or Alzheimer's disease.

Our findings suggest that common variation in the *FGG* and *FGA* genes is not an important risk factor for dementia.

INTRODUCTION

Both inflammatory and hemostatic factors have been implicated in the pathogenesis of dementia.[1, 2] We recently reported an association between higher plasma levels of fibrinogen, which has inflammatory and hemostatic properties, and an increased risk of dementia, both Alzheimer's disease and vascular dementia.[3]

Fibrinogen is primarily synthesized by hepatocytes and consists of two symmetric sets of 3 chains (Aα, Bβ and γ), encoded by 3 separate genes, fibrinogen α (*FGA*), fibrinogen β (FGB) and fibrinogen γ (*FGG*) clustered on chromosome 4. The FGB gene is thought to be involved in determining fibrinogen plasma levels whereas *FGG* and *FGA* genes are believed to play a role in regulating fibrin clot structure.[4]

There is evidence that altered structure of the fibrin clot may be involved in the pathogenesis of vascular disease.[5] In a previous study performed in the Rotterdam Scan Study we found an association of haplotypes of the *FGG* and *FGA* gene with cerebral small vessel disease as assessed with brain imaging. Since vascular disease, including cerebral small vessel disease, is associated with dementia and Alzheimer's disease we hypothesized an association between altered fibrin clot structure and dementia.

In this study, we set out to investigate whether common variation in the *FGG* and *FGA* genes that are involved in regulating clot structure is associated with an increased risk of dementia and Alzheimer's disease. The study was performed in the Rotterdam Study, a population-based prospective cohort study among persons of 55 years and over.

METHODS

The Rotterdam Study is a population-based prospective cohort study among 7,983 persons of 55 years and over six baseline examinations (1990-1993) and follow-up examinations (1993-1994, 1997-1999 and 2002-2004) included physical examinations, screening and clinical work-up for dementia. The Medical Ethics Committee of the Erasmus Medical Center approved the study and written informed consent was obtained from all participants. Through linkage with records of general practitioners, the total cohort was continuously monitored for morbidity and mortality. This resulted in a virtually complete follow-up until January 1, 2005.

The Seattle SNPs program for Genomic Applications has identified various SNPs in the *FGG* and *FGA* genes and the common haplotypes can be identified by haplotype tagging SNPs. By genotyping seven haplotype tagging SNPs we were able to infer haplotypes

and describe the common variation across the *FGG* and *FGA* genes. In the *FGG* and *FGA* gene the *FGG* 5836 G>A (rs2066860), *FGG* 7874 G>A (rs2066861), *FGG* 9340 T>C (rs1049636), *FGA* 2224 G>A (rs2070011), *FGA* 3655 G>A (rs2070014), *FGA* 3807 T>C (rs2070016) and the *FGA* 6534 A>G (rs6050) polymorphisms that tag haplotypes describing the total common variation in the *FGG* and *FGA* gene were genotyped. These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/SNP.

DNA was isolated using standard procedures and genotyping was performed using baseline samples stored at -80º Celcius. Genotypes were determined in 2 ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California, USA). Primer and probe sequences were optimized by using the SNP-assay-by design service of Applied Biosystems. Reactions were performed with the Taqman Prism 7900HT 384 wells format in the 384-wells plate (Greiner Bio-One) in 2 μL of reaction volume.

Haplotype alleles present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html), which computes maximum likelihood estimates of haplotype probabilities.[7, 8] Information on all SNPs was available in 5,538 persons.

The diagnosis of dementia and subtypes of dementia was made following a three-step protocol as described previously.[9] We used internationally accepted criteria for dementia (DSM-IIIR),[10] Alzheimer's disease (NINCDS-ADRDA),[11] and vascular dementia (NINDS-AIREN).[12]

Data analysis

Hardy-Weinberg equilibrium of fibrinogen polymorphisms was tested by means of Chi square tests. To test the associations of fibrinogen haplotypes with dementia and Alzheimer's disease we used the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html).[7, 8, 13] The probability for each haplotype pair in each individual was assigned and then an individual's phenotype was directly modeled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. The association between fibrinogen haplotypes and dementia was examined by means of the haplo.glm function of Haplo Stats. This approach is based on generalized linear model, and computes the regression of a trait on haplotypes and other covariates. In these analyses the most frequent haplotype was used as the reference category. We adjusted for age and sex in the analyses. Because of low numbers, we were not able to assess the associations with vascular dementia.

RESULTS

In the study population at risk of dementia with complete data on haplotypes, we identified 563 patients with dementia, including 440 patients with Alzheimer's disease and 63 with vascular dementia. The baseline characteristics of the study population are shown in Table 1. All individual polymorphisms were in Hardy-Weinberg equilibrium. Haplotype reconstruction resulted in 24 haplotypes, but only seven had a frequency of >0.01 in our population. Haplotype alleles were coded as haplotype numbers 1 through 7 in order of decreasing frequency in the population (coding from 5836 G>A, 7874 G>A, 9340 T>C, 2224 G>A, 3655 G>A, 3807 T>C, 6534 A>G, haplotype 1=G-G-T-G-G-T-A, haplotype 2=G-**A**-T-**A**-G-T-**G**, haplotype 3=G-G-**C**-G-**A**-T-A, haplotype 4=G-G-T-G-G-**C**-A, haplotype 5=G-G-**C**-**A**-G-T-A, haplotype 6=**A**-G-T-G-G-T-A and haplotype 7=G-G-T-**A**-G-T-G). Table 2 shows the constructed haplotypes and their frequencies in our population.

We did not find associations of haplotypes with dementia or Alzheimer's disease (Table 3).

Table 1. Baseline characteristics of the study population (n=5,538)

Age, years	69.0 (8.7)
Sex, female, %	58.9
Current smokers, %	23.0
Presence of an APOE ε4 allele, %	24.8
Body mass index, kg/m^2	26.4 (4.0)
Systolic blood pressure, mmHg	139.4 (22.1)
Diastolic blood pressure, mmHg	73.9 (11.4)
Total cholesterol, mM	6.6 (1.2)
High density lipid (HDL) cholesterol, mM	1.3 (0.4)
Diabetes mellitus, %	10.0
Intima media thickness (IMT), mm	0.79 (0.16)
Fibrinogen, g/L*	2.7 (2.3-3.2)

Values are mean (SD), unless otherwise stated. *Because of skewed distribution, median (interquartile range) is given.

Table 2. Construction of haplotypes and their frequencies

Haplotype	FGG gene			FGA gene				Frequency
	5836G>A rs2066860	7874G>A rs2066861	9340T>C rs1049636	2224G>A rs2070011	3655G>A rs2070014	3807T>C rs2070016	6534A>G rs6050	
1	G	G	T	G	G	T	A	0.26
2	G	A	T	A	G	T	G	0.26
3	G	G	C	G	A	T	A	0.17
4	G	G	T	G	G	C	A	0.12
5	G	G	C	A	G	T	A	0.12
6	A	G	T	G	G	T	A	0.04
7	G	G	T	A	G	T	G	0.01

FGA, fibrinogen α gene; FGG, fibrinogen γ gene.

Table 3. The association of haplotypes in the *FGA* and *FGG* genes with dementia and subtypes of dementia.

Haplotype	OR (95% CI) for dementia	OR (95% CI) for Alzheimer's disease
1	1.00 (ref)	1.00 (ref)
2	0.91 (0.75-1.08)	0.86 (0.71-1.04)
3	0.89 (0.73-1.09)	0.83 (0.67-1.03)
4	0.89 (0.72-1.11)	0.93 (0.74-1.18)
5	0.89 (0.71-1.12)	0.84 (0.65-1.07)
6	1.02 (0.72-1.45)	1.11 (0.77-1.61)
7	1.13 (0.63-2.02)	0.89 (0.79-1.01)

OR and 95% CI adjusted for age and sex.

DISCUSSION

In this large population-based prospective study, we did not find an association between common variation in the *FGG* and *FGA* genes and dementia or Alzheimer's disease.

Though not associated with fibrinogen plasma levels, functional implications of variation in *FGG* and *FGA* on fibrin clot structure and stability that may explain an association with vascular disease have been described.[14, 15] Recently, several studies reported an association between common variation in these genes and vascular disease, including myocardial infarction[14] and venous thrombosis.[16] In the Rotterdam Scan Study, we found an association of haplotypes in the *FGG* and *FGA* gene with presence of silent brain infarcts and periventricular white matter lesions.

This is the first study to examine common genetic variation in *FGG* and *FGA* in relation to dementia. We cannot exclude that we have missed small haplotypes effects despite the large number of dementia cases in our sample. Also, the association with vascular dementia could not be studied because of low numbers.

To conclude, our findings suggest that common variation in the *FGG* and *FGA* genes is not an important risk factor for dementia.

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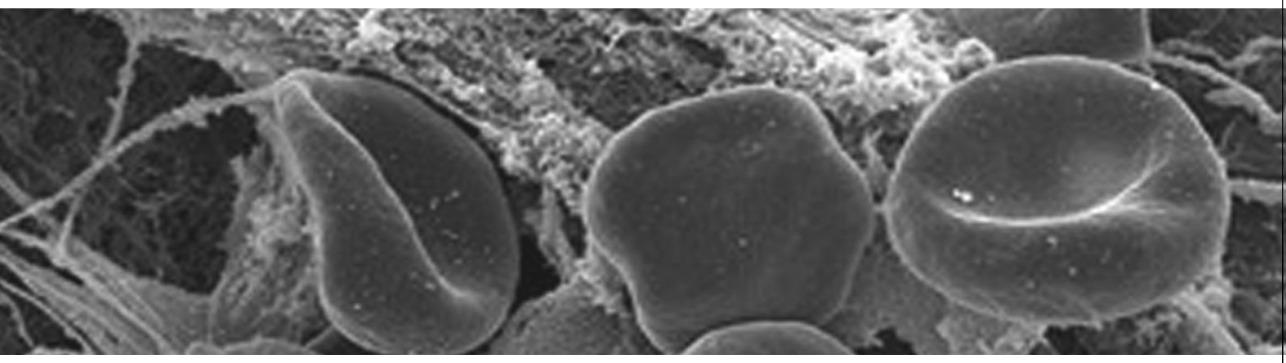

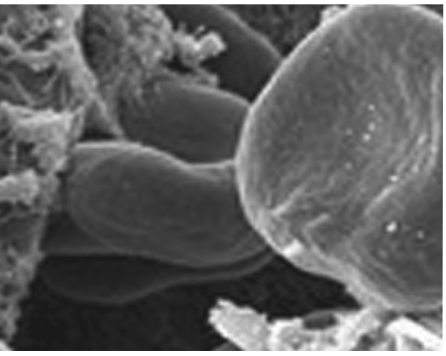

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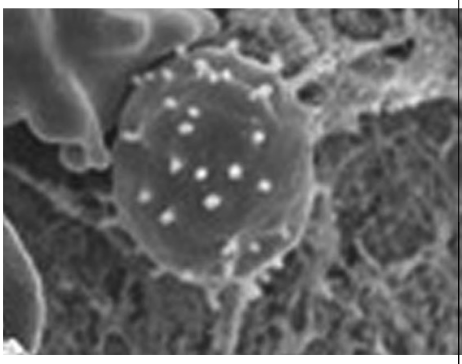

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Variation in fibrinogen *FGG* and *FGA* genes and risk of stroke. The Rotterdam Study

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ABSTRACT

Haplotypes of the fibrinogen gamma and alpha (FGG and FGA) genes are associated with the structure of the fibrin network and may therefore influence the risk of stroke. We investigated the relationship between common variation in these genes with ischemic and hemorrhagic stroke.

The study was based on 6275 participants of the prospective population-based Rotterdam Study who at baseline (1990-1993) were aged 55 years or over, free from stroke, and had successful assessment of at least one *FGG* or *FGA* single nucleotide polymorphisms (SNP). Common haplotypes were estimated using seven tagging SNPs across a 30 kb region containing the *FGG* and *FGA* genes. Follow-up for incident stroke was complete until January 1, 2005. Associations between constructed haplotypes and risk of stroke were estimated with an age and sex adjusted logistic regression model.

We observed 668 strokes, of which 393 were ischemic and 62 hemorrhagic, during a median follow-up time of 10.1 years. *FGG+FGA* haplotype 3 (H3) was associated with an increased risk of ischemic stroke (odds ratio (OR) 1.36, 95% confidence interval (CI) 1.09-1.69) and the risk estimate for hemorrhagic stroke was 0.71 (95% CI 0.46-1.09) compared to the most frequent H1. The *FGG* and *FGA* genes were not associated with stroke or its subtypes when analyzed separately.

In conclusion, risk of ischemic stroke was higher in *FGG+FGA* H3 than in H1. The results suggested that an opposite association may exist for hemorrhagic stroke.

INTRODUCTION

Fibrinogen is an important coagulation factor and its levels increase 2-4 times during acute phase reactions. The fibrinogen molecule comprises two identical disulphide-linked halves, each consisting of three polypeptide chains termed Aα, Bβ and γ,[1] which are encoded by the fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*) and fibrinogen gamma (*FGG*) genes, respectively, on chromosome 4.[2] At the final stage of the coagulation cascade, thrombin cleaves fibrinopeptide A and B from the amino termini of the fibrinogen Aα and Bβ chains, converting the soluble fibrinogen into fibrin monomers, which then polymerize to an insoluble fibrin clot.

Many prospective studies have confirmed that high total fibrinogen levels are associated with an increased risk of ischemic heart disease and stroke,[3-5] but it is still unclear whether fibrinogen is only an inflammatory marker of underlying atherosclerosis or whether it also directly contributes to the progression of cardiovascular disease (CVD) via effects on coagulation and/or atherogenesis.[6] One of the mechanisms by which fibrinogen can influence the pathogenesis of atherosclerosis is the structure of the fibrin clot.[7-8]

To determine whether fibrinogen is causally involved in stroke risk can be done using the approach of Mendelian randomization.[9] Briefly, when a variable is genetically determined and also associated with disease, an association between genetic variation and disease is expected. It has already been reported that the fibrin clot structure is partly determined by genetic factors.[10-11] that a number of single nucleotide polymorphisms (SNPs) in the *FGG* and *FGA* genes are associated with fibrinogen levels, functions and clot structure.[7,12-15] which are variables that have been associated with CVD.[16-18] Therefore, studies on genetic variation provide an opportunity to further investigate the involvement of fibrinogen in stroke. Since variations in both the *FGG* and the *FGA* gene are reported to be associated with fibrin clot structure, we focused this research project on the variation in the *FGG* and *FGA* genes. The aim of our study was to investigate the association between common genetic variations in the fibrinogen *FGG* and *FGA* genes and risk of stroke in the Rotterdam Study, a large, prospective, population-based cohort study.

STUDY DESIGN AND METHODS

Study population

The present study is part of the Rotterdam Study, a population-based cohort study on chronic and disabling diseases. The design of this study has been described in detail previously.[19]

All inhabitants of Ommoord, a district of the city of Rotterdam in the Netherlands, aged 55 years and over were invited to participate. Participation rate for the study was 78% and a total of 7,983 subjects participated in the study. The study was approved by the Medical Ethics Committee of Erasmus University Rotterdam and was performed in accordance with the Declaration of Helsinki. Written informed consent to retrieve information from treating physicians was obtained from all participants. Baseline measurements were obtained from 1990 through 1993 and consisted of a home interview and 2 visits to the research center for measurements and collection of blood.

Single nucleotide polymorphisms (SNPs) selection and genotyping

The SeattleSNPs program for Genomic Applications (http://pga.mbt.washington.edu/) has identified three haplotype-tagging SNPs in *FGG* and four SNPs in *FGA*, which tag the total common genetic variation in these genes in Caucasians. By genotyping these seven haplotype tagging SNPs, we were able to infer haplotypes and describe the common variation within the *FGG* and the *FGA* genes and also across the *FGG* and *FGA* genes. We performed haplotype analysis that covers both the *FGG* and *FGA* region because high linkage disequilibrium between the two genes complicates assigning the risk to one of the genes. We genotyped the *FGG* 4288G>A (rs2066860), *FGG* 6326G>A (rs2066861), *FGG* 7792T>C (rs1049636), *FGA* -58G>A (rs2070011), *FGA* 1374G>A (rs2070014), *FGA* 1526T>C (rs2070016) and the *FGA* 4253A>G (rs6050) polymorphisms that tag haplotypes covering the total common variation in the *FGG* and *FGA* genes. Annotation of the SNPs is in accordance with the nomenclature recommendations of the Human Genome Variation Society using GenBank Accession Number AF350254 for *FGG* and AF361104 for *FGA* as reference with nucleotide +1 being the A of the ATG translation initiation codon.[20] These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/SNP.

DNA was isolated using standard salting-out procedures and genotyping was performed using baseline samples stored at -80º Celsius. Genotypes were determined in 2 ng genomic DNA with the 5′ nuclease/Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences were designed using the SNP assay-by-design service of Applied Biosystems and are available upon request. Polymerase chain reactions were performed with fluorescent allele-specific oligonucleotide probes on GeneAmp PCR System 9700 thermal cyclers with 384 wells format in 2 μL of reaction volume (Applied Biosystems), and fluorescence clustered endpoint reading for allelic discrimination was read on an ABI Prism 7900HT sequence detection system (Applied Biosystems).

Assessment of stroke

History of stroke at baseline was positive if a stroke was reported during the baseline interview and confirmed by medical records. After enrollment into the Rotterdam Study, participants were continuously monitored for strokes through automated linkage of the study database with files from general practitioners and the municipality. Also nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized. For reported events, additional information (including brain imaging) was obtained from hospital records. Research physicians discussed information on all potential strokes with an experienced stroke neurologist to verify all diagnoses. A stroke was subclassified as ischemic if a computed tomography (CT) or magnetic resonance imaging (MRI) scan, made within 4 weeks after the stroke occurred, ruled out other diagnoses, or if indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours, atrial fibrillation in absence of anticoagulants treatment) pointed at an ischemic nature of the stroke. A stroke was subclassified as hemorrhagic if a relevant hemorrhage was shown on CT or MRI scan, or if the subject lost consciousness permanently or died within hours after onset of focal signs. If a stroke did not match any of these criteria, it was called unspecified. Follow-up was complete until January 1, 2005, for 98.7 % of potential person years.[21]

Population for analysis

Of all 7,983 Rotterdam Study participants, 261 had had a stroke before baseline and 176 refused informed consent for retrieval of stroke follow-up data. In addition, no blood was available for 1,217 participants and the SNP assays failed on all seven SNPs in 54 participants, leaving 6,275 participants eligible for the present study. Blood pressure was measured twice in the right arm using a random-zero sphygmomanometer, with the participant in a sitting position. We used the average of these 2 measurements. C-reactive protein, total cholesterol, high-density lipoprotein (HDL) cholesterol, and uric acid were measured in nonfasting baseline blood with automated enzymatic procedures. We considered diabetes mellitus to be present if a random or postload glucose level was 11.1 mM or higher, or if a person used antidiabetic medication. Atrial fibrillation at baseline was considered to be present if it was seen on electrocardiogram (ECG) during the center visit or if reported in medical records. The intima-media thickness was measured by longitudinal two-dimensional ultrasound of the carotid artery (mean of 4 locations). During the home interview, smoking status and medication use were assessed.

Measurement of fibrinogen

Fibrinogen levels were derived from the clotting curve of the prothrombin time assay using Thromborel S as a reagent on an automated coagulation laboratory (ACL 300, Instrumentation Laboratory).

Haplotypes construction and data analysis

We assessed the associations between individual SNPs and risk of stroke, ischemic stroke, and hemorrhagic stroke with Cox proportional hazards models, adjusting for age and sex. Hardy-Weinberg equilibrium of each fibrinogen polymorphism was tested using Chi square analysis. When a minor homozygotes group contains <10 subjects (e.g. *FGG4288),* we combined the minor homozygotes with the heterozygotes. Haplotype alleles present in the population were inferred by means of the *haplo.em* function of the *Haplo Stats* suite implemented in the *R* package (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html),[22-24] which computes maximum likelihood estimates of haplotype probabilities, accounting for missing genotype data by including in the analysis the likelihood for all possible genotypes for the incomplete loci. Only haplotypes with frequencies at least 5% were used in the analyses. The haplotypes were numbered in order of decreasing frequency in the population.

We calculated odds ratios (OR) for stroke in fibrinogen haplotypes relative to the risk of stroke in the most common fibrinogen haplotype using the *Haplo Stats* suite. The probability for each haplotype pair in each individual was assigned and then an individual's phenotype was directly modeled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. An estimate of the haplotype frequencies was based on the expectation-maximization (EM) algorithm. The association between fibrinogen haplotypes and risk of stroke was examined by means of the *haplo.glm* function of *Haplo Stats*.[23] This approach is based on the binary family of generalized linear models, and computes the regression of a trait on haplotypes and other covariates. In these analyses the most frequent haplotype was used as the reference category. We adjusted for age and sex.

RESULTS

During on average 10.1 years of follow-up, 668 strokes occurred, of which 393 were classified as ischemic, 62 as hemorrhagic, and in 213 patients the stroke class was not specified. The median age at start of follow-up was 68 years and 59% of participants were female (Table 1). The total risk of stroke was highest in subjects with plasma

fibrinogen levels in the highest quartile (OR 1.3, 95%CI 1.0-1.8). A similar relationship was observed for ischemic stroke (OR 1.3, 95%CI 0.9-1.9)(Table 2). No analyses could be performed for hemorrhagic stroke, since fibrinogen levels were measured in only 29 cases.

Table 1. Baseline characteristics of study population.

Number of participants	6275
Age, *yrs*	68 (62-75)
Female sex, *%*	59
Both parents Caucasian, *%*	98.5
Antihypertensive drug use, *%*	13
Systolic blood pressure, *mmHg*	137 (123-153)
C-reactive protein, *mg/L*	1.85 (0.89-3.60)
Total cholesterol, *mM*	6.6 (5.8-7.4)
HDL cholesterol, *mM*	1.3 (1.1-1.5)
Uric acid, *µmol/L*	310 (267-370)
Intima-media thickness, *mm*	0.77 (0.68-0.88)
Body mass index, *kg/m²*	26.0 (23.8-28.4)
Waist to hip ratio	0.90 (0.84-0.97)
Diabetes mellitus, *%*	15
Ever smokers, *%*	65
Current smokers, *%*	23
Atrial fibrillation, *%*	5
Previous myocardial infarction, *%*	12

Values are percentage (%) or median (25th and 75th percentile) when appropriate.

Table 2. Relationship between plasma fibrinogen levels and risk of stroke

Fibrinogen	Stroke (n=340)	Ischemic stroke (n=192)
Quartile 1	1	1
Quartile 2	1.08 (0.78-1.52)	0.79 (0.50-1.25)
Quartile 3	1.16 (0.85-1.59)	1.25 (0.85-1.84)
Quartile 4	1.34 (0.98-1.82)	1.29 (0.86-1.92)
Per SD	1.10 (0.98-1.22)	1.13 (0.98-1.31)

Analysis in 2,583 participants, with adjustment for age and sex

The genotypic distributions for all 7 haplotype-tagging SNPs were in Hardy-Weinberg equilibrium. A total of 6.7% of subjects had 1 or 2 missing FGG or FGA SNPs and were included in the haplotype analysis, 1.8% of subjects was excluded because they had 3-6 missing SNPs. Analyses of the single SNPs in the FGG and FGA showed a slightly increased risk for ischemic stroke in carriers of the A-allele of the FGG4288, but no association was seen between the other variants and risk of stroke (Supplementary Table 1).

Supplementary Table 1. Association between individual SNPs in *FGG* and *FGA* and risk of stroke.

SNP	Genotype	Frequency (strokes/N)	Stroke OR (95%CI)	Ischemic stroke OR (95%CI)	Hemorrhagic stroke OR (95%CI)
FGG4288	GG	594/5677	1 (ref)	1 (ref)	1 (ref)
	GA&AA	55/456	1.14 (0.87-1.50)	1.41 (1.01-1.96)	1.37 (0.59-3.18)
FGG6326	GG	338/3330	1 (ref)	1 (ref)	1 (ref)
	GA	266/2365	1.08 (0.92-1.27)	1.10 (0.89-1.36)	0.94 (0.57-1.56)
	AA	46/405	1.14 (0.84-1.55)	0.87 (0.55-1.36)	
FGG7792	TT	337/3045	1 (ref)	1 (ref)	1 (ref)
	TC	268/2595	0.95 (0.81-1.11)	1.19 (0.96-1.46)	0.60 (0.69-1.01)
	CC	49/506	0.88 (0.65-1.19)	1.00 (0.68-1.48)	
FGA-58	GG	228/2261	1 (ref)	1 (ref)	1 (ref)
	GA	327/2966	1.08 (0.91-1.28)	1.09 (0.88-1.36)	1.14 (0.68-1.94)
	AA	100/909	1.12 (0.88-1.41)	1.03 (0.76-1.41)	
FGA1374	GG	441/4184	1 (ref)	1 (ref)	1 (ref)
	GA	187/1734	1.00 (0.84-1.19)	1.20 (0.96-1.48)	0.71 (0.39-1.26)
	AA	15/184	0.84 (0.50-1.40)	0.98 (0.52-1.85)	
FGA1526	TT	503/4649	1 (ref)	1 (ref)	1 (ref)
	TC&CC	152/1469	0.96 (0.81-1.15)	0.95 (0.75-1.20)	1.39 (0.81-2.39)
FGA4253	ThrThr	312/3156	1 (ref)	1 (ref)	1 (ref)
	ThrAla	275/2422	1.14 (0.97-1.34)	1.20 (0.97-1.48)	0.75 (0.51-1.44)
	AlaAla	53/455	1.21 (0.90-1.62)	0.92 (0.60-1.41)	

Presented are odds ratio and 95% confidence intervals (OR, 95%CI), adjusted for age and sex.

Three common haplotypes were inferred in *FGG*. The most common *FGG* haplotype (H1), which was estimated to be present in 41% of participants, was G-G-T (Supplementary Table 2). Two other *FGG* haplotypes (H2: G-G-**C** and H3: G-**A**-T) were present in more than 5% of the participants. Inferring haplotypes resulted in five haplotypes in *FGA*, of which the most common *FGA* haplotype (H1: G-G-T-A) was estimated to be present in 31% of participants. Four other *FGA* haplotypes (H2-H5) had a prevalence of at least 5%.

The risk of stroke was similar for the various *FGG* haplotypes: the OR for stroke for *FGG*-H2 and *FGG*-H3 were 0.97 (95% CI 0.84-1.12) and 1.10 (95% CI 0.95-1.27), respectively, compared with *FGG*-H1 (Table 3). Likewise, risk of stroke was similar for the *FGA* haplotypes. When we studied only ischemic stroke, the risks in all haplotypes were slightly increased when compared with *FGG*-H1 or *FGA*-H1 (Table 3). Since the linkage disequilibrium between the *FGG* SNPs and the *FGA* SNPs was very high (D′ of 0.94), we also constructed extended haplotypes covering both the *FGG* and *FGA* genes. Haplotype reconstruction resulted in five *FGG+FGA* haplotypes with a frequency >5%, and together these five haplotypes covered 93% of the total genetic variation of this region (Supplementary Table 2). The total risk of stroke was similar in all *FGG+FGA* haplotypes. However, when we studied only ischemic stroke we observed for *FGG+FGA*-H3 an association with an OR of 1.36 (95% CI 1.09-1.69) compared with the reference haplotype *FGG+FGA*-H1 (Table 3). When we studied only hemorrhagic stroke, we observed an opposite risk estimate (OR 0.71 [95% CI 0.46-1.09]), and there was only minimal overlap between the 95% CI for ischemic and hemorrhagic stroke in H3. Furthermore, a slightly increased risk estimate for ischemic stroke was seen for *FGG+FGA*-H2 (OR 1.20, 95% CI 0.98-1.46). To account for multiple testing, permutation p-values (1000 permutations) were computed. The p-value for the extended *FGG+FGA* haplotypes was 0.07 for the ischemic stroke group and larger for the other analyses. The results were similar in men and women (results not shown). In the subgroup of subjects where fibrinogen levels were available (n=2,583) we also analyzed the relationship between the fibrinogen haplotypes and plasma fibrinogen levels and no clear relationship was observed. Only H4 showed a 3% (p=0.05) higher plasma fibrinogen level (Table 4).

Supplementary Table 2. Frequency of fibrinogen *FGG* and *FGA* haplotypes separate and combined. Only haplotypes with frequency > 5% were studied.

Fibrinogen chain	Haplotype	4288G>A rs2066860	6326G>A rs2066861	7792T>C rs1049636	-58G>A rs2070011	1374G>A rs2070014	1526T>C rs2070016	4253A>G rs6050	Frequency in study	Name used in reference (16)	Name used in reference (26)(32)	Name used in reference (33)
FGG	H1	G	G	T					40.8%		FGG-H1	
	H2	G	G	**C**					29.4%	FGG*2	FGG-H3	
	H3	G	**A**	T					26.1%		FGG-H2	
FGA	H1				G	G	T	A	30.5%	FGA*1	FGA-H1 or FGA-H5	
	H2				**A**	G	T	**G**	27.1%	FGA*2	FGA-H2	
	H3				G	**A**	T	A	17.3%		FGA-H7	
	H4				G	G	**C**	A	13.0%		FGA-H4	
	H5				**A**	G	T	A	11.8%	FGA*3	FGA-H3	
FGG + FGA	H1	G	G	T	G	G	T	A	26.4%	FGG-FGA*1		A1
	H2	G	**A**	T	**A**	G	T	**G**	25.6%			A2
	H3	G	G	**C**	G	**A**	T	A	16.8%			A3
	H4	G	G	T	G	G	**C**	A	12.2%			A1
	H5	G	G	**C**	**A**	G	T	A	11.5%	FGG-FGA*4		A4

Table 3. Association between fibrinogen haplotypes and risk of any, ischemic and hemorrhagic stroke

FGG Haplotype	Stroke OR (95%CI)	Ischemic stroke OR (95%CI)	Hemorrhagic stroke OR (95%CI)
H1 (GGT)	1 (ref)	1 (ref)	1 (ref)
H2 (GG**C**)	0.97 (0.84-1.12)	1.17 (0.98-1.40)	0.72 (0.46-1.14)
H3 (G**A**T)	1.10 (0.95-1.27)	1.12 (0.92-1.35)	0.88 (0.56-1.37)
FGA Haplotype			
H1 (GGTA)	1 (ref)	1 (ref)	1 (ref)
H2 (**A**GT**G**)	1.10 (0.94-1.28)	1.11 (0.92-1.35)	0.92 (0.59-1.43)
H3 (G**A**TA)	0.99 (0.83-1.18)	1.23 (0.99-1.52)	0.66 (0.37-1.18)
H4 (GG**C**A)	0.99 (0.81-1.20)	1.07 (0.83-1.37)	1.12 (0.66-1.91)
H5 (**A**GTA)	0.94 (0.77-1.16)	1.07 (0.83-1.39)	1.00 (0.56-1.79)
FGG + *FGA* Haplotype			
H1 (GGT-GGTA)	1 (ref)	1 (ref)	1 (ref)
H2 (G**A**T-**A**GT**G**)	1.12 (0.96-1.32)	1.20 (0.98-1.46)	0.98 (0.68-1.41)
H3 (GG**C**-G**A**TA)	1.02 (0.85-1.22)	1.36 (1.09-1.69)	0.71 (0.46-1.09)
H4 (GGT-GG**C**A)	1.02 (0.83-1.25)	1.18 (0.92-1.52)	1.25 (0.81-1.91)
H5 (GG**C**-**A**GTA)	0.94 (0.76-1.16)	1.14 (0.88-1.47)	0.90 (0.60-1.33)

Analysis performed in 6,275 participants; 668 strokes, 393 ischemic and 62 hemorrhagic strokes were observed. Presented are odds ratio and 95% confidence intervals (OR, 95%CI), with adjustment for age, sex, systolic blood pressure, intima-media thickness, serum C-reactive protein level, waist-hip ratio, ever smoking, antihypertensive drug use, history of myocardial infarction and diabetes mellitus at baseline.

Table 4. Fibrinogen levels for *FGG+FGA* haplotype carriers.

	Fibrinogen level*		
FGG + *FGA* Haplotype	g/L	Standard error	P for difference with H1*
H1 (GGT-GGTA)	2.83	0.04	
H2 (G**A**T-**A**GT**G**)	2.83	0.05	0.87
H3 (GG**C**-G**A**TA)	2.85	0.05	0.59
H4 (GGT-GG**C**A)	2.91	0.05	0.05
H5 (GG**C**-**A**GTA)	2.86	0.06	0.46

* Estimated with generalized linear model. Analyses performed in 2,583 participants.

DISCUSSION

In this population-based cohort study, we observed that the risk of ischemic stroke was associated with *FGG+FGA*-Haplotype 3 and that this relationship was opposite for ischemic and hemorrhagic stroke. This finding strengthens the hypothesis that fibrin structure may be a causal factor in the development of stroke.

Strengths of our study are the prospective inclusion of the stroke cases and the meticulous and nearly complete follow-up (loss of potential person-years 1.3 %). To our knowledge, this is the first study that assessed the association between *FGG* and *FGA* haplotypes and risk of stroke in the general population. Our stringent stroke monitoring procedures allowed us to include also stroke patients who had not been referred to a neurologist. In the Netherlands, the general practitioners do not refer all stroke patients to the neurologist, and therefore in our study population, 31% of all stroke cases had not been referred. As neuroimaging had not been performed in these non-referred cases, we could subclassify only 16% of them into ischemic or hemorrhagic. In contrast, 92% of strokes that had been diagnosed by a neurologist could be subclassified. In total, 32% of all stroke cases could not be subclassfied. The study would have had a greater power for the subgroup analysis when all strokes would have been subclassified.

In this study, we focused on the *FGG and FGA* genes, since these genes determine the fibrin clot structure, which is suggested to directly affect atherosclerosis and thrombotic disease.[7,25] Although the *FGG* and *FGA* gene encode for different peptides, we decided to also perform a combined haplotype analysis because both the α and γ chains are part of one single protein, fibrinogen, and their effects on fibrin structure cannot be separated. In addition, variants in both genes are reported to be associated with fibrin clot structure and functionality has been proposed for variants in both genes.[17-18,26] Because there is high linkage disequilibrium between the two genes, the effects cannot be assigned to one of the genes. In our study the strongest association with stroke risk was seen when we combined variants in both genes.

The *FBG* promoter SNPs have been much studied previously, since increased plasma fibrinogen levels have been identified as a consistent risk indicator for cardiovascular disease and stroke[3-5] and the fibrinogen β chain synthesis is rate limiting step in the production of mature fibrinogen *in vitro*.[27] Indeed, several studies reported that *FGB* promoter SNPs contributed to the regulation of plasma fibrinogen concentration,[28] but no clear and consistent association was found between these SNPs and stroke.[29-31] We did not include the *FGB* gene in this study because it has not been shown to be associated with

fibrin structure and because it is generally considered not to be associated with thrombosis risk.[29] In the Rotterdam study, the *FGB* promoter SNP rs1800787 (-148C/T) was also not associated with risk of stroke (unpublished data).

We did not observe associations between individual SNPs in the *FGG* and *FGA* genes with ischemic or hemorrhagic stroke, although participants with variant SNPs were at slightly higher risk of ischemic stroke than participants with wild type SNPs. In studies with other vascular endpoints no significant association was seen between *FGG*-H3 and myocardial infarction[16,32] (Supplementary Table 2) but *FGG*-H3 (named FGG-H2 in that study) was associated with increased risk of venous thrombosis.[26] Moreover, *FGG*-H2 was associated with the risk of myocardial infarction in one study but not in another[16,32] and not associated with risk of deep vein thrombosis (*FGG*-H2 was named *FGG**2 or *FGG*-H3 in these studies).[26] Recently, a case-control study on ischemic stroke reported that haplotype H5 (named A4 in that study), was associated with an increased risk and also an increased risk in that study was seen for H2 (named A2).[33] All studies used the haplotype with common alleles for all SNPs as reference group. Also reported were associations of single SNPs in the *FGA* gene (-58G>A (rs2070011) and 4253A>G (rs6050)) or *FGG* gene (7792T>C (rs1049636)) that are associated with myocardial infarction, plasma fibrinogen concentration or fibrin clot characteristics,[7,15-18] but these SNPs were not associated with stroke in our study. The differences of the observed associations between genetic variation in *FGG* and *FGA* with stroke, myocardial infarction and deep vein thrombosis may be explained by differences in etiology of these endpoints, medication and methods for statistical analysis.

With the extended haplotypes composed of SNPs in the fibrinogen *FGG* and *FGA* genes, we observed an association between *FGG+FGA*-H3 and ischemic stroke. In only one previous study, Mannila and colleagues combined SNPs in the *FGG* and *FGA* genes and they observed a decreased risk of myocardial infarction for a haplotype combining *FGG* 7792T>C (rs1049636) and *FGA* -58G>A (rs2070011).[16] This haplotype would cover our *FGG+FGA*-H5, and contains the rare alleles of these two SNPs, but we did not see an association of this haplotype with stroke.

We were able to separate ischemic and hemorrhagic stroke in the majority of our stroke cases. Although the group of patients with a hemorrhagic stroke was small with only 62 cases, it was very interesting to observe an association between *FGG+FGA*-H3 and ischemic stroke and that our data suggest that an opposite association may exist for hemorrhagic stroke. However, these results need to be considered with great caution and this observation needs to be replicated in independent populations. Interestingly, opposite

associations are what one would expect if thrombosis and bleeding were important mechanisms in the ischemic and hemorrhagic stroke, respectively.

In our study, the individual SNPs were not associated with risk of ischemic stroke, but we observed associations with ischemic stroke when we combined the SNPs and constructed haplotypes. This illustrates that haplotype analysis is a more powerful analysis when it is uncertain if functional variants are being studied or in the presence of multiple susceptibility alleles, in the pattern or magnitude of their allelic associations.[34]

It has recently been reported that the 8486C>T SNP, tagged in haplotype *FGG+FGA*-H3, affects a cleavage stimulatory factor binding site near the γA specific polyadenylation site and would therefore result in a relative decrease of the formation of fibrinogen γ′ specific mRNA, thus altering the proportion of the γ′ chains.[35] This would ultimately result in a changed fibrin clot formation since γ′ fibrinogen has an antithrombin effects because it binds thrombin, misses the platelet binding site and can bind factor XIII.[36] It has also been shown that this SNP is associated with elevated risk of deep venous thrombosis by reducing plasma fibrinogen γ′ levels.[26] However, we did not see an association between this SNP and risk of stroke in this study, and also not in another case-control study in ischemic stroke.[37]

In conclusion, we found a higher risk of ischemic stroke in subjects with H3 in the extended *FGG+FGA* haplotype but not in individual SNP analysis in this population-based cohort study and that an opposite association may exist for hemorrhagic stroke. Our data illustrate that haplotype analysis may reveal associations with risk of stroke that may be missed by single SNP analysis.

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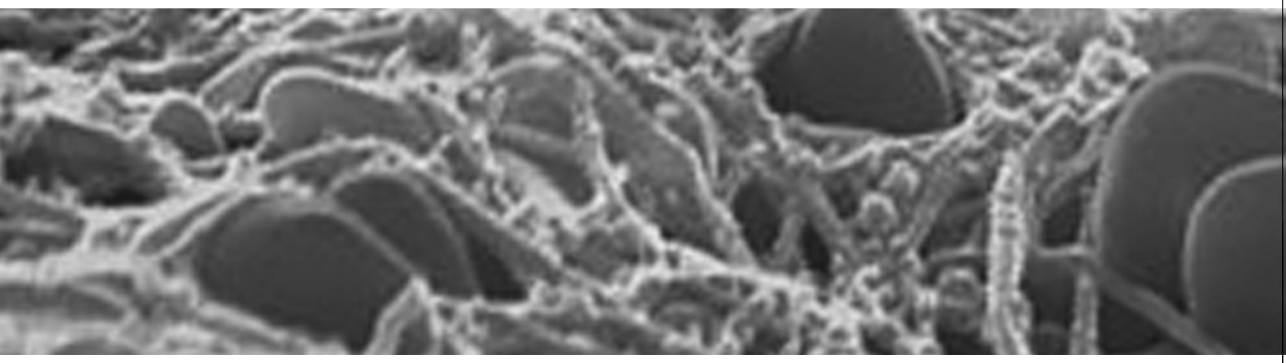

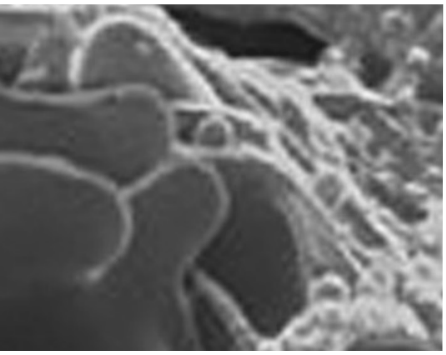

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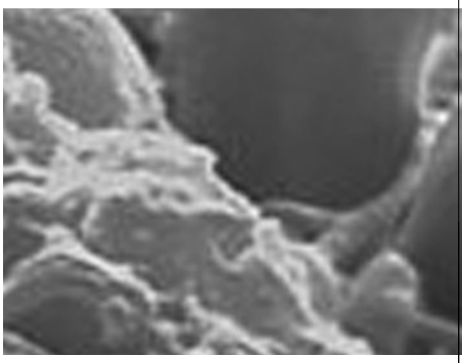

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Fibrinogen γ' in ischemic stroke
A case-control study

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ABSTRACT

We determined the contribution of fibrinogen γ′ levels and *FGG* haplotypes to ischemic stroke. Associations between fibrinogen γ′ levels, fibrinogen γ′/total fibrinogen ratio, and *FGG* haplotypes with the risk of ischemic stroke were determined in 124 cases and 125 controls. Fibrinogen γ′/total fibrinogen ratio was higher in patients than in controls during the acute phase of the stroke and lower in the convalescent phase 3 months after the stroke. *FGG* haplotype 3 (H3) was associated with a reduced risk of ischemic stroke (odds ratio 0.60; 95% CI, 0.38 to 0.94), but not with the fibrinogen γ′/total fibrinogen ratio. In contrast, FGG-H2 was associated with a decreased fibrinogen γ′/total fibrinogen ratio, but not with risk of stroke. Fibrinogen γ′/total fibrinogen ratio is associated with ischemic stroke, especially in the acute phase of the disease. In addition, FGG-H3 haplotype appears to be protective against ischemic stroke.

Table 1. Baseline characteristics of study participants

	Patients (n = 124)	Controls (n = 125)	P
Demographics			
Age, years (SD)	56 (±12)	56 (±12)	NS
Sex (female)	58 (47%)	59 (47%)	NS
Index event			
Stroke: TIA	115:9	NA	NA
Risk factors			
Smoking	61 (49%)	37 (30%)	0.004
Hypertension	60 (48%)	24 (19%)	<0.001
Diabetes mellitus	18 (14%)	5 (4%)	0.004
Hypercholesterolemia	78 (63%)	84 (67%)	0.700
Positive family history for cardiovascular disease	75 (61%)	56 (45%)	0.013

Data for age are presented as mean±SD. Other data are counts and (percentages). SD= Standard Deviation; TIA= Transient Ischemic Attack; NA= Not Applicable; NS= Not Significant

INTRODUCTION

Fibrinogen, a central protein in the hemostatic system, has a high degree of heterogeneity in healthy individuals. One variant, fibrinogen γ′, carries an extended γ chain formed by alternative processing of the fibrinogen γ pre-mRNA and comprises 7% to 15% of the fibrinogen molecules. Fibrinogen γ′ has both antithrombotic (binding sites for thrombin and disruption of platelet binding to fibrinogen) and prothrombotic properties (binding site for the factor XIII B subunit).[1] Associations with deep venous thrombosis (DVT)[2] and coronary artery disease[3] indicate that plasma fibrinogen γ′ levels may contribute to the pathology of thrombotic disease. Fibrinogen γ gene (*FGG*) variation is associated with fibrinogen γ′ levels, fibrinogen γ′/total fibrinogen ratio, risk of DVT[2] and myocardial infarction (MI).[4,5] The aim of this study was to determine the role of fibrinogen γ′ levels and common *FGG* gene variations in ischemic stroke.

PATIENTS AND METHODS

We performed a case-control study with 124 first-ever ischemic stroke or transient ischemic attack (TIA) patients, and 125 population controls, aged 18 to 75 years old. The study design has been described previously. [6] Baseline characteristics are given in Table 1. Blood was collected from an unselected subgroup of 47 patients 7 to 14 days (acute phase) and 3 months after the stroke in tubes containing 1/10 volume of 0.129 mol/L sodium citrate.

Fibrinogen γ′ antigen levels were measured by ELISA as described previously.[2] Pooled normal plasma calibrated against purified human γ′ fibrinogen (a gift from Dr M. Mosesson, Blood Center of Wisconsin, Milwaukee, WI) was used as calibrator.

Total fibrinogen levels were measured according to von Clauss, and C-reactive protein (CRP) was measured using an in-house ELISA with polyclonal rabbit antihuman CRP antibodies (DAKO).

Genetic Analyses

We genotyped 3 haplotype-tagging single nucleotide polymorphisms (SNPs) that tag the total common genetic variation in *FGG* in whites (http://pga.gs.washington.edu). SNP 8486C>T (rs2066865, 10034C>T by SeattleSNPs) tagged *FGG* haplotype 2 (H2), SNP 7792T>C (rs1049636, 9340T>C by SeattleSNPs) tagged FGG-H3 and SNP 4288G>A (rs2066860, 5836G>A by SeattleSNPs) tagged FGG-H4. FGG-H1 was assigned to subjects who possessed the common alleles of the 3 SNPs. Annotation of the SNPs uses AF350254

as reference sequence with nucleotide +1 being the translation initiation nucleotide. We genotyped the 3 SNPs using 5′ nuclease/TaqMan assays (primer sequences available on request).

Statistical Analyses

Differences between groups were examined by analysis of variance (ANOVA) or by paired-samples *t* test for acute phase versus convalescent phase in patients. We adjusted for risk factors for vascular diseases (smoking, hypertension, diabetes mellitus, and hyperlipidemia). The association between haplotypes, taking the haplotype ambiguity into account, and risk of stroke, fibrinogen γ′ level and fibrinogen γ′/total fibrinogen ratio were determined using Haplo.Stats version 1.2.2 (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html).

RESULTS

In the acute phase of stroke, the mean plasma fibrinogen γ′ level was higher in patients (0.42±0.17 g/L) than in controls (0.34±0.10 g/L; $P<0.001$;Table 2).The fibrinogen γ′/total fibrinogen ratio was calculated to adjust for the acute phase increase of fibrinogen, and this ratio was also significantly higher in patients (0.113±0.034) than in controls (0.100±0.029; $P=0.002$; Table 2). Adjustment for CRP and common cardiovascular risk factors did not influence these relationships.

In the samples collected 3 months after the event, the mean fibrinogen γ′ level and fibrinogen γ′/total fibrinogen ratio (0.29±0.09 g/L and 0.088±0.02 respectively) were significantly lower than during the acute phase of stroke, and also significantly lower than in controls (Table 2).

Carriers of the FGG-H3 allele had a significantly reduced risk of ischemic stroke (OR 0.60; 95% CI, 0.38 to 0.94). No clear association with stroke was observed for the other haplotypes (FGG-H1: reference; FGG-H2: OR 0.91; 95% CI, 0.58 to 1.43; FGG-H4: OR 0.75; 95% CI, 0.30 to 1.88).

Only FGG-H2 was associated with a significantly decreased fibrinogen γ′/total fibrinogen ratio in patients during the acute phase (decrease of 0.093±0.013 and 0.085±0.020, respectively, both $P<0.001$), in the convalescent phase (decrease of 0.060±0.015, $P<0.001$) and in controls (Figure) No association between *FGG* haplotypes and total fibrinogen levels was present. Adjustment for cardiovascular risk factors and CRP levels did not affect these relationships.

Table 2. Fibrinogen γ′ levels, γ′/total fibrinogen ratio and fibrinogen levels in patients and controls

	Patients		Controls			
	Acute phase	Convalescent phase				
	N=114	N=47	N=119	*P**	*P*†	*P*‡
Fibrinogen γ′ (g/L)	0.42±0.17	0.29±0.09	0.34±0.10	<0.001	0.01	<0.001
Fibrinogen γ′/total fibrinogen ratio	0.113±0.034	0.088±0.020	0.100±0.029	0.002	0.01	<0.001
Fibrinogen (g/L)	3.69±1.06	3.34±0.76	3.41±0.61	0.006	0.54	0.003
CRP§ (mg/L)	2.33 (0.49-8.18)	1.15 (0.62-5.17)	1.30 (0.61-3.18)	0.007	0.11	0.85

Presented are means±SD, § median and inter quartile range (IQR); *P**: *P*-value between controls and patients in acute phase, *P*†: *P*-value between controls and patients in convalescent phase, *P*‡: *P*-value between patients in acute phase and in convalescent phase.

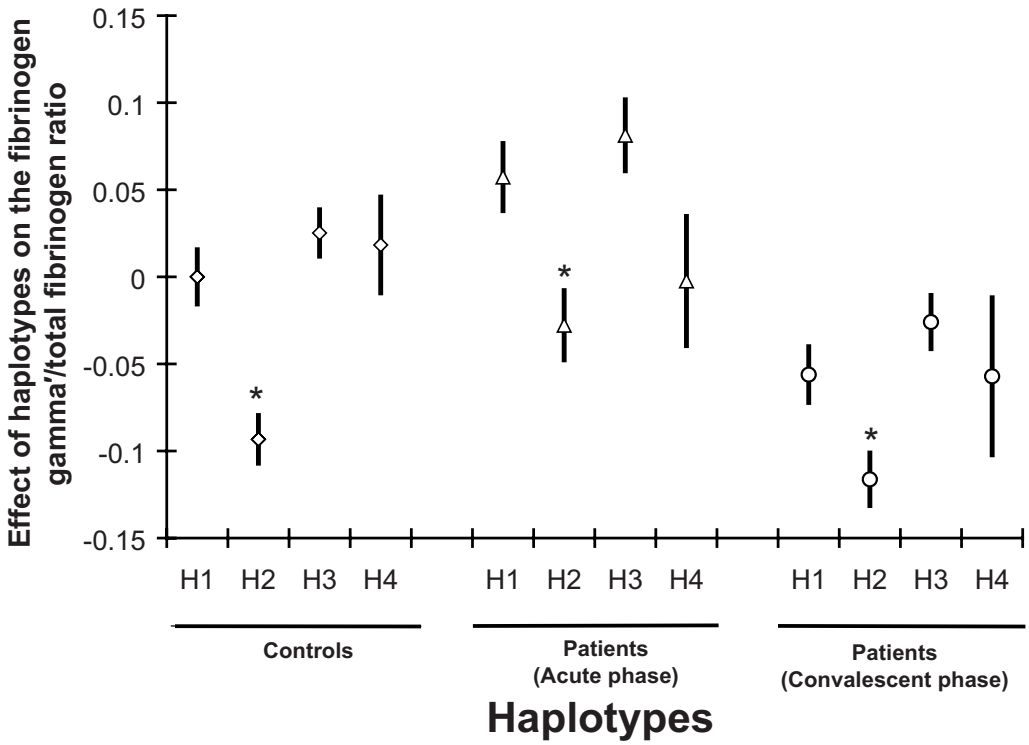

Figure. Effect of *FGG* haplotypes on the fibrinogen γ′/ total fibrinogen ratio
H1 in controls was used as reference group. * indicates a significant different from H1 of each group.

DISCUSSION

Fibrinogen γ′ levels were elevated in the acute phase of ischemic stroke and decreased in the convalescent phase. The increase in the acute phase is probably a result of the event. The decrease in the convalescent phase may more reflect the prestroke levels, which would suggest that the antithrombotic properties of fibrinogen γ′ are more important than its prothrombotic properties. This seems to be in contrast with previous studies where increased fibrinogen γ′ levels or fibrinogen γ′/total fibrinogen ratios were associated with increased risk of arterial thrombotic disease,[3,7] but these latter results may have been influenced by the acute phase. It is a limitation of our study that it has a relatively small sample size, especially for the unselected group of patients that has been followed-up in the convalescent phase.

Fibrinogen γ′/total fibrinogen ratio is only elevated in the acute phase of stroke and not in the convalescent phase suggesting that the acute phase affects alternative splicing as already suggested for other genes.

We observed that carriers of FGG-H3 have a reduced risk of ischemic stroke, but this is not consistent with previous studies.[2,4,5,8] It is unclear what the underlying mechanism might be because FGG-H3 gave only a slight, nonsignificant increase of fibrinogen γ′ levels or fibrinogen γ′/total fibrinogen ratios. As expected,[2] the fibrinogen γ′/total fibrinogen ratio was strongly reduced in FGG-H2, both in cases (acute phase and convalescent phase) and in controls. This observation may be explained by the improved cleavage stimulatory factor binding site near the γA specific polyadenylation site in the FGG-H2 allele, which may inhibit the formation of fibrinogen γ′ specific mRNA.[8] No relationship between FGG-H2 and risk of stroke was observed, which is consistent with previous studies.[4,8]

In conclusion, this study shows that the fibrinogen γ′/total fibrinogen ratio is increased in the acute phase of stroke, which may reflect an antithrombotic defense mechanism of the human body. Additionally, carriers of the FGG-H3 haplotype appear to be protected against ischemic stroke.

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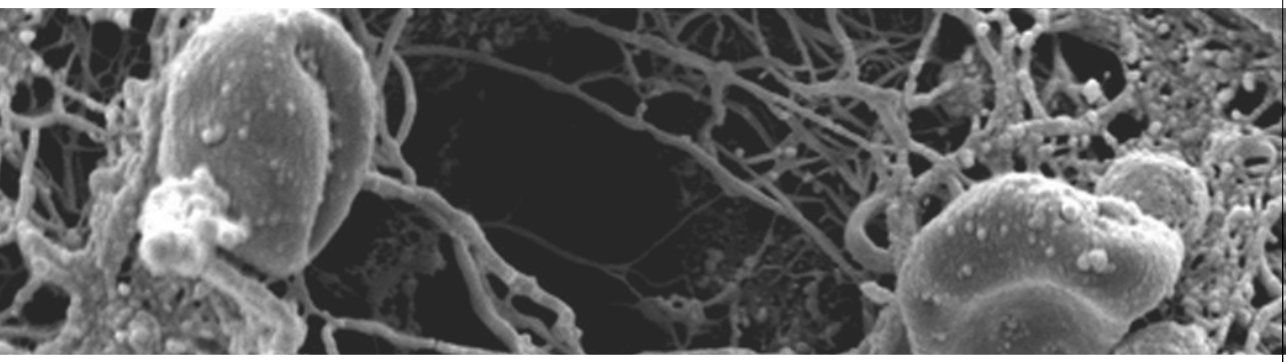

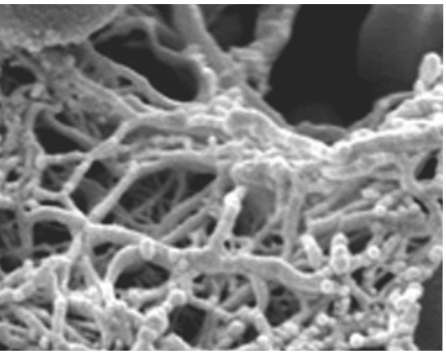

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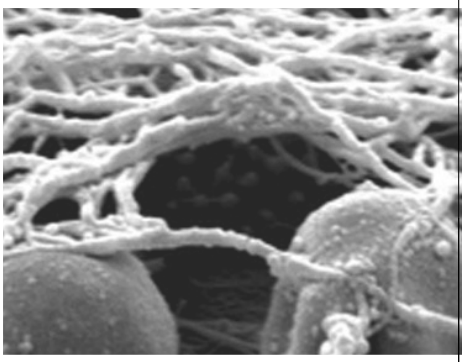

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Elevated fibrinogen γ' ratio is associated with cardiovascular diseases and acute phase reaction but not with clinical outcome

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TO THE EDITOR

Recently, Nowak-Göttl et al. reported on the association between the fibrinogen γ′/total fibrinogen ratio (γ′ ratio) and thromboembolism in children.[1] We and others previously reported on this association in adults.[2-6] However, the underlying mechanism remains unknown. Recently, we reported that the γ′ ratio is higher in the acute phase of ischemic stroke (IS), but not in the convalescent phase.[2] This suggests that the acute phase reaction alters the mRNA processing of fibrinogen γ, thereby increasing the γ′ ratio. Furthermore, the anti- and prothrombotic properties of γ′ fibrinogen suggest that an increased γ′ ratio during the acute phase of cardiovascular disease may influence the secondary thrombotic risk.

Fibrinogen γ′ antigen levels were measured by enzyme-linked immunosorbent assay as described previously.[4] Total fibrinogen levels were measured according to von Clauss, and C reactive protein (CRP) levels was measured using an in-house high-sensitivity enzyme-linked immunosorbent assay. Statistical analysis was performed using SPSS 16.0 for Windows. All investigations were approved by the Medical Ethics Committee of Erasmus University Medical Center and were performed in accordance with the recommendations of the Declaration of Helsinki.

We confirmed our previous finding of an elevated γ′ ratio in IS and observed significantly higher γ′ ratios in the acute phase of IS (independent cohort of 53 patients), pulmonary embolism (PE, n=202)[7] and refractory unstable angina pectoris (UAP, n=72)[8] compared with the γ′ ratios in healthy controls (P <0.001; Table 1). These observations are in contrast to those of Nowak-Göttl et al., who reported a decreased γ′ ratio in children with thromboembolism (U. Nowak-Göttl, H. Weiler, personal communication, July 2009),[1] and also with results from adult patients with deep venous thrombosis.[4] One explanation for the discrepancy may be the timing of blood collection. In our studies, blood was collected in the acute phase of the diseases. We observed a significant correlation between γ′ ratios and CRP levels in the acute phase (Rs=0.23, P<0.001) in the patients with IS.[2] Moreover, γ′ ratios were slightly higher (0.24±0.19, n=16) in PE patients with CRP levels >100 mg/L, than in patients who did not have an acute phase response (CRP levels µ10 mg/L;0.20±0.06, n=13, P=0.54). These results together support our hypothesis that the mRNA processing of the fibrinogen γ alters during an acute phase reaction.

The characteristics of γ′ fibrinogen suggest that a high ratio during the acute phase of disease prevents thrombotic events in patients with UAP. In our patient group, there was no difference in ratios between stabilized (n=130) and refractory (n=72) patients (both

0.14±0.04, *P*=0.59). Neither were the ratios associated with clinical outcome during 18 months of follow-up. These results suggest that elevated ratio does not predict the risk of acute coronary syndromes in patients with UAP.

In conclusion, the fibrinogen γ′/total fibrinogen ratios are elevated in patients with various cardiovascular diseases (IS, PE or UAP) compared with the γ′ ratios in healthy controls. An altered mRNA processing of fibrinogen γ during the acute phase may contribute to this observation. However, the fibrinogen γ′/total fibrinogen ratio does not predict short or long term outcome in patients with UAP.

Table 1. Fibrinogen levels, fibrinogen γ′ levels and fibrinogen γ′/fibrinogen ratios in various cardiovascular diseases.

Groups	n	Fibrinogen, g/L	Fibrinogen γ′, g/L	Fibrinogen γ′/total fibrinogen ratio	*P*-value*	*P*-value†
Population Controls	173	3.30 (0.59)	0.33 (0.10)	0.10 (0.03)		
Patients						
Ischemic stroke (IS)	53	3.87 (1.19)	0.49 (0.18)	0.13 (0.02)	<0.001	
Pulmonary embolism (PE)						
Non acute phase	13	2.69 (1.42)	0.53 (0.27)	0.20 (0.06)	<0.001	0.54
Acute phase	16	4.39 (2.30)	0.79 (0.33)	0.24 (0.19)	<0.001	
Unstable angina pectoris (UAP)						
Stabilized	130	3.36 (0.76)	0.46 (0.15)	0.14 (0.04)	<0.001	0.59
Refractory	72	3.82 (1.00)	0.53 (0.23)	0.14 (0.04)	<0.001	

Data presented are means (SD).
**P* value of fibrinogen γ′/total fibrinogen ratio between patients and healthy controls after adjustment for age and sex.
†*P* value of fibrinogen γ′/total fibrinogen ratio between patient groups after adjustment for age and sex.

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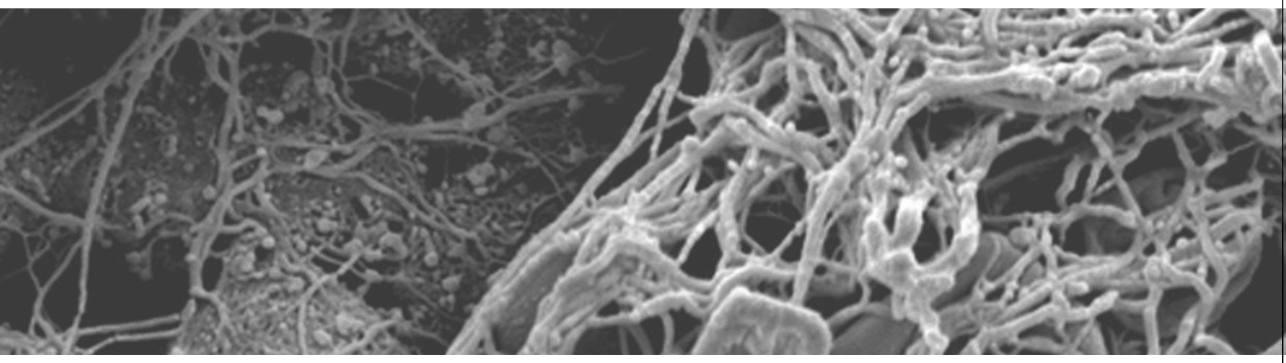

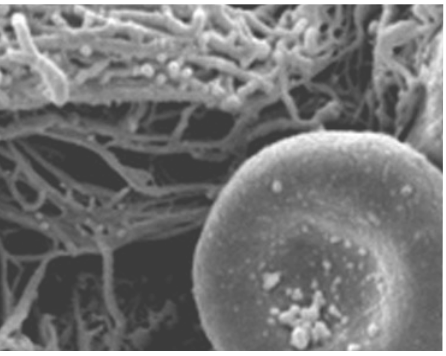

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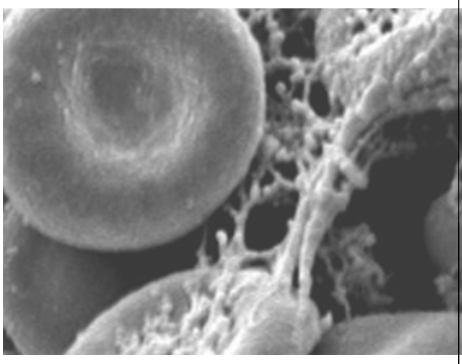

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γ'/total fibrinogen ratio is associated with short-term outcome in ischemic stroke

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ABSTRACT

Fibrinogen γ′ (γ′) is a natural isoform of fibrinogen, and alters the rate of formation and the properties of clots. It could therefore affect outcome after ischemic stroke. The prognostic significance of γ′ fibrinogen levels is, however, still unclear. We assessed levels of γ′ in ischemic stroke, and its association with short-term outcome.

We included 200 ischemic stroke patients and 156 control persons. Total fibrinogen and γ′ levels were measured; outcome at discharge was assessed by means of the modified Rankin Scale score (defined as unfavorable when >2). We compared levels between patients and controls using multiple linear regression analysis, and logistic regression analysis was used to assess the relationship between levels and outcome. All analyses were adjusted for age and sex.

Mean γ′ levels were significantly higher in patients with ischemic stroke than in controls (0.37 vs 0.32 g/L, $p<0.001$), and patients also had a higher γ′/total fibrinogen ratio (0.102 vs 0.096, $p=0.19$). The γ′/total fibrinogen ratio is associated with unfavorable outcome in patients with ischemic stroke (odds ratio per unit increase of γ′/total fibrinogen ratio 1.27, 95%CI 1.09-1.47).

Our study shows that patients with ischemic stroke have increased levels of fibrinogen γ′ and suggests a trend towards an increased γ′/total fibrinogen ratio in ischemic stroke. Increased fibrinogen γ′ relative to total fibrinogen levels are associated with unfavorable outcome in the early phase after stroke.

INTRODUCTION

Human plasma fibrinogen is an important protein in the hemostatic system, in which soluble fibrinogen is converted to insoluble fibrin, leading to clot formation. Fibrinogen is a heterogeneous protein, existing as several natural isoforms. The fibrinogen variant containing γ′ is one such isoform, comprising 10 ± 3% of the circulating fibrinogen molecules.[1] In the systemic circulation, γ′ is mostly present as a heterodimeric protein with the common γA form (γA/γ′), while <1% is present in the γ′/γ′ form.[2] Fibrinogen γ′ is a result of alternative mRNA processing, in which 20 amino acids replace the 4 amino acids at the C-terminal of γA.[3,4] The extension of fibrinogen γ′ contains additional binding sites for thrombin (hence the alternative name antithrombin I for γ′-containing fibrinogen molecules)[5] and for the factor XIII B-subunit,[6] enhancing binding of factor XIII. The platelet binding site for integrin $\alpha_{IIB}\beta_3$ is disrupted, which deducts platelet binding capacity.[7]

Recently, we and others reported that an elevated γ′ level or γ′/total fibrinogen ratio is associated with cardiovascular disease,[1,8-12] especially in the acute phase of ischemic stroke. Results of our previous studies suggest that fibrinogen γ′ levels as well as the γ′/total fibrinogen ratio alter during an acute phase reaction.[1,8] This may lead to a different clot formation rate and to different characteristics of the fibrin network, and may therefore contribute to the pathology of thrombotic diseases and stroke.

The role of fibrinogen γ′ and the γ′/total fibrinogen ratio in the precipitation and course of ischemic stroke is as yet unclear. To our knowledge, no studies have been published on fibrinogen γ′ in relation to outcome of cardiovascular disease, ischemic stroke in particular.

We aimed to investigate the differences in fibrinogen γ′ levels and γ′/total fibrinogen ratio, between patients with ischemic stroke and control persons. Furthermore, we studied whether there is an association between these levels and short-term outcome in ischemic stroke.

MATERIALS AND METHODS

Study population

All patients and controls participated in the Erasmus Stroke Study (ESS), which is an ongoing prospective registry of all patients with transient ischemic attack (TIA) or stroke treated at Erasmus MC University Medical Center Rotterdam since December 2005. From all patients, detailed clinical and radiological data, blood samples and DNA are collected.

The ESS also includes population-based control persons, mostly friends and spouses of patients, but no family members. All participants in the ESS provided informed consent. The study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

For this case-control study we included 200 patients with ischemic stroke, and 156 age- and sex matched stroke-free control persons. In all patients, citrated blood was collected and centrifuged at 4000 rpm for 15 minutes. Citrated plasma was stored at -80°C within 2 hours from collection. Blood was drawn 8 days (interquartile range 3-22 days) from the date of onset, either in the stroke unit or the outpatient clinic.

Total fibrinogen and fibrinogen γ′ antigen measurements

We determined total fibrinogen levels according to Von Clauss[13] on a fully-automated blood coagulation analyzer (Sysmex CA-1500 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Normal Reference Plasma was used as reference plasma in this assay (Precision BioLogic, Dartmouth, ON, Canada), the intra-assay coefficient of variation was 3.5%.

Fibrinogen γ′ antigen levels were measured with an enzyme-linked immunosorbent assay as described previously,[9] with minor modifications. Briefly, plastic 96-well microtiter plates (Nunc maxisorp, Roskilde, Denmark) were coated with mouse anti-human γ′ fibrinogen (2.G2.H9; Millipore, Billerica, MA, USA) and then incubated overnight at 4°C. Wells were blocked with bovine serum albumin for 1 hour at room temperature. Plasma samples were added to each well in duplicate. After 1 hour incubation at room temperature, horseradish peroxidase-conjugated rabbit anti-human fibrinogen (DAKO A/S, Glostrup, Denmark) was added to tag the immobilized patient fibrinogen γ′, and incubated for 1 hour at room temperature. Color was developed with Tetramethylbenzidine substrate solution (BioMérieux, Marcy l′Etiole, France) and was terminated with H_2SO_4 after 15 minutes. The plate was read at 450 nm spectrophotometrically. Wells were washed 3 times between all incubation steps. Pooled normal plasma calibrated against purified human γ′ fibrinogen was used as calibrator.[1] The intra-assay variation was less than 10%.

Definitions

Ischemic stroke was defined as focal neurological deficit of presumed vascular origin, lasting >24 hours or leading to death within 24 hours, with brain imaging showing no abnormalities or typical signs of brain infarction. Hypertension was defined as the use of antihypertensive drugs before the event, hypercholesterolemia as the use of cholesterol

lowering drugs before the event. Diabetes mellitus was defined as the use of oral antidiabetic drugs and/or insulin before the event. All strokes were classified according to TOAST criteria[14] based on all available information.

Functional short-term outcome was assessed by means of the modified Rankin Scale (mRS)[15] score at time of discharge from the stroke unit for hospitalized patients, and from the outpatient clinic for ambulant patients. The mRS is a score for handicap and level of dependency. The scale ranges from 0 (no symptoms) to 6 (dead), with each increase denoting more severe disability. At a score of 2, patients have neurological symptoms affecting their daily life but are still able to live completely independent; at a score of 3 patients cannot live independently due to symptoms. The mRS was dichotomized between scores of 2 and 3, as favorable (≤2, independent living) or unfavorable (>2, dependent or institutionalized living).

Statistical analysis

Differences between baseline characteristics of patients and controls were assessed with Student′s t-test. Differences in fibrinogen levels between patient groups and controls were assessed using Student′s t-test; adjustment for confounders was performed using analysis of variance (ANOVA). Associations between fibrinogen levels and outcome were assessed using multiple logistic regression analysis and expressed as odds ratio (OR) with 95% confidence intervals (CI). Analyses were also performed in 2 subgroups of equal size, based on time from event to blood drawing. This allowed us to study levels of γ′ in the acute and subacute stage after ischemic stroke. All analyses were adjusted for age and sex. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois). A probability value <0.05 was considered significant.

RESULTS

Baseline characteristics of patients and controls are shown in Table 1. As expected, prevalence of cardiovascular risk factors (smoking, hypertension and hypercholesterolemia) was different between patients and controls.

Levels of total fibrinogen and fibrinogen γ′ were significantly higher in patients compared with controls, after adjustment for age and sex (Table 2A). Levels of the γ′/total fibrinogen ratio were similar in patients and controls.

The median time from onset of symptoms to blood drawing was 7.5 days. Two subgroups of patients were formed, by dividing patients in 2 equal groups based on

time from onset of symptoms to blood drawing, the acute phase and the subacute phase. Differences in γ′/total fibrinogen ratio between patients and controls were most pronounced in the group with blood drawing in the acute phase of stroke (Table 2B). In this group γ′/total fibrinogen ratio was significantly increased, in line with our previous results.[8] In the group of patients in the subacute phase of ischemic stroke, the γ′ ratio was not different from controls.

Outcome was unfavorable (modified Rankin Scale score >2) in 13.5% of patients with ischemic stroke. These patients could no longer live independently due to neurological symptoms, had severe disabilities, or died.

Table 1. Characteristics of the study population.

		Patients	Controls	p-value
N		200	156	
Age (years), mean (SD)		62 (13)	59 (12)	0.04
Female, %		45	51	0.29
Current smoking, %		36	24	0.02
Hypertension, %		55	29	<0.001
Hypercholesterolemia, %		34	18	0.001
Diabetes Mellitus, %		17	13	0.37
Body Mass Index, mean (SD)		26.0 (4.2)	26.7 (4.5)	0.22
TOAST	Large artery atherosclerosis	39 (20)		
classification	Cardioembolism	21 (11)		
N(%)	Small vessel occlusion	57 (29)		
	Other etiology	12 (6)		
	Undetermined etiology	71 (36)		
Time from onset of symptoms to blood drawing (days), median (IQR)		7.5 (3-22)		
Modified Rankin Scale at discharge, median (IQR)		1 (1-2)		

Levels of fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio with respect to favorable or unfavorable outcome are shown in Table 3. After adjustment for confounders, γ′ and total fibrinogen levels and the γ′/total fibrinogen ratio were significantly higher in patients with unfavorable outcome, compared with patients with a favorable outcome. Increased fibrinogen γ′ levels and γ′/total fibrinogen ratio were significantly associated with unfavorable outcome (OR per unit increase γ′ 1.48, 95%CI 1.15-1.91 and OR per

unit increase γ′ ratio 1.27, 95%CI 1.09-1.47; both adjusted for age, sex and time to blood drawing; Table 4). The γ′/total fibrinogen ratio adjusts fibrinogen γ′ levels for total fibrinogen levels, the prognostic effect of fibrinogen γ′ on outcome was thus independent of total fibrinogen level. Effects were similar in subgroups based on time to blood drawing, showing that the γ′/total fibrinogen ratio was associated with unfavorable outcome in the first week as well as later in the course of ischemic stroke (OR 1.25, 95%CI 1.05-1.49 and OR 1.20, 95%CI 0.84-1.70, respectively).

Table 2A. Levels of total fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio in patients and controls.

	Controls	Patients	Model 1, p-value*	Model 2, p-value†	Model 3, p-value‡
Total fibrinogen (g/L)	3.37 (0.72)	3.69 (0.96)	<0.001	0.001	<0.001
Fibrinogen γ′ (g/L)	0.32 (0.13)	0.37 (0.15)	<0.001	<0.001	0.002
γ′/total fibrinogen ratio	0.096 (0.028)	0.102 (0.036)	0.19	0.30	0.495

Presented are means (SD). *Model 1: adjustment for age and sex †Model 2: adjustment for age, sex and time to blood drawing ‡Model 3: adjustment for age, sex, time to blood drawing, current smoking, hypertension and hypercholesterolemia

Table 2B. Levels of total fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio in subgroups of patients and controls. Subgroups: patients were divided in 2 equal groups of 100 patients based on time from event to blood drawing.

	0-7 days	≥ 8 days	0-7 days vs controls, p-value*	>= 8 days vs controls, p-value*
Total fibrinogen (g/L)	3.76 (1.11)	3.62 (0.78)	<0.001	0.033
Fibrinogen γ′ (g/L)	0.40 (0.17)	0.34 (0.12)	<0.001	0.194
γ′/total fibrinogen ratio	0.110 (0.040)	0.095 (0.029)	0.015	0.64

Presented are means (SD). *p value after adjustment for age and sex

Table 3. Levels of total fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio in patients with favorable or unfavorable outcome

	Favorable outcome (modified Rankin Scale ≤ 2)	Unfavorable outcome (modified Rankin Scale >2)	Model 1*, p-value	Model 2†, p-value
N	173	27		
Total fibrinogen, g/L (SD)	3.64 (0.89)	3.97 (1.34)	0.112	0.013
fibrinogen γ′, g/L (SD)	0.36 (0.14)	0.47 (0.18)	<0.001	<0.001
γ′/total fibrinogen ratio, SD	0.098 (0.029)	0.129 (0.059)	<0.001	0.006

*Model 1: adjusted for age and sex †Model 2: adjusted for age, sex, current smoking, hypertension and days to blood drawing

Table 4. Associations between total fibrinogen levels, fibrinogen γ′ levels and γ′/total fibrinogen ratio and outcome of ischemic stroke.
OR (95% CI) for unfavorable outcome (modified Rankin Scale >2) per unit increase in levels

Unfavorable outcome, N (%)	27 (13,5)	
	Model 1*	Model 2†
Total fibrinogen (g/L)	1.35 (0.90-2.01)	1.31 (0.87-1.96)
Fibrinogen γ′ (dg/L)	1.52 (1.18-1.96)	1.48 (1.15-1.91)
γ′/total fibrinogen ratio (%)	1.28 (1.10-1.48)	1.27 (1.09-1.47)

* Model 1: adjusted for age and sex † Model 2: adjusted for age, sex and time to blood drawing

DISCUSSION

Our study shows that levels of total fibrinogen, fibrinogen γ′ and the γ′/total fibrinogen ratio are increased in the acute phase of ischemic stroke. Furthermore, this is the first study to show that increased relative levels of fibrinogen γ′ are significantly associated with unfavorable outcome. The γ′/total fibrinogen ratio was associated with unfavorable outcome both in the acute and subacute phase after ischemic stroke.

Some methodological issues of our study have to be discussed. Strengths of our study are the extent and quality of clinical data and availability of short-term follow-up, which enabled us to study fibrinogen γ′ in relation to outcome of stroke. Limitations of our study are the relatively small number of patients, and the low rate of unfavorable outcome among our patients. However, this study provided sufficient precision to allow meaningful conclusions about the relationship between fibrinogen γ′ and outcome.

We observed that the γ′ levels were increased most in patients who were presented at the hospital soon after the onset of stroke. In general, patients with severe stroke were admitted to the hospital sooner after the onset of complaints than patients with milder stroke. Therefore, we cannot distinguish between the effects of the acute phase reaction that was initiated by the stroke, and the severity of stroke. Still, adjustment in the analysis for the time since onset of complaints has only a minor effect on the associations of γ′ levels with outcome of stroke. After adjustment for cardiovascular risk factors, levels of fibrinogen γ′ were still significantly increased in patients, but the γ′ ratio was similar in patients and controls. Also, when looking at associations with outcome, the absolute γ′ level had a stronger association with outcome than the γ′/total fibrinogen ratio. This may indicate that the absolute level of fibrinogen γ′ is perhaps more important than the γ′/total fibrinogen ratio.

Our results confirm those of our previous studies that showed increased total fibrinogen and fibrinogen γ′ levels in the acute phase of ischemic stroke.[8] Also, earlier studies have found a relation between higher post-stroke total fibrinogen levels and poor outcome measured on the modified Rankin Scale.[16] We found that higher total fibrinogen levels are associated with unfavorable outcome as well, but in addition we found that the association between increased fibrinogen γ′ levels and unfavorable outcome after ischemic stroke is independent of total fibrinogen levels.

Fibrinogen γ′ has antithrombotic properties; one of these is a high affinity binding site for thrombin, which may result in lower levels of circulating thrombin available for clot formation.[17] Also, γ′ fibrinogen has a disrupted binding site for platelet integrin $\alpha_{IIb}\beta_3$, which diminishes its capacity to facilitate platelet aggregation.[7] On the other hand, fibrin formed from γ′-containing fibrinogen has a finer, more tightly branched network and smaller pores than fibrin formed from γA fibrinogen.[18,19] *In vitro* studies have reported that fibrinogen γ′-rich clots show a lower clot lysis rate than clots formed from γA fibrinogen.[20,21]

Increased total fibrinogen levels have been shown to increase risk of cardiovascular disease.[22-24] Several studies in the convalescent phase of thrombotic diseases show a decreased fibrinogen γ′ level, which is thought to resemble the level before occurrence of an event. When considering fibrinogen γ′ as a protein with mostly antithrombotic properties, it is possible that increased baseline fibrinogen levels, combined with a low fibrinogen γ′/total fibrinogen ratio, could predispose to atherothrombotic disease. In the acute phase of thrombotic diseases, the fibrinogen γ′ level then increases, as was seen in previous studies[1] as well as our study. Based on findings from previous studies and our study, it is still too early to use fibrinogen γ′ assays for diagnostic or prognostic evaluation in acute stroke, for which further studies are needed.

In conclusion, we have found that levels of fibrinogen γ′ are increased in the acute phase of ischemic stroke. Furthermore, increased levels of fibrinogen γ′ are associated with unfavorable outcome. Future research should be aimed at unraveling the role of fibrinogen γ′ in the pathogenesis and prognosis of cardiovascular diseases such as ischemic stroke, in which the genetic background of fibrinogen γ′ levels may be of particular interest.

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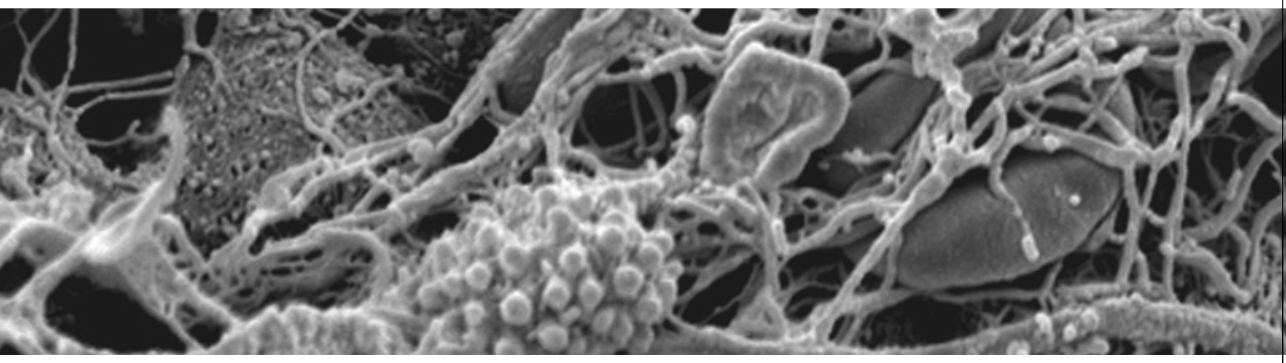

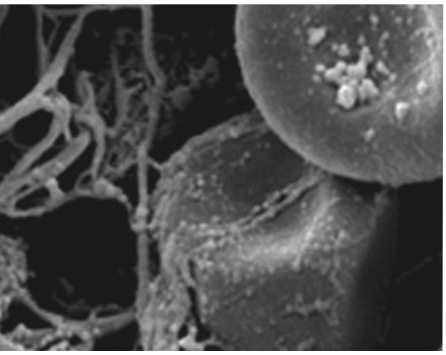

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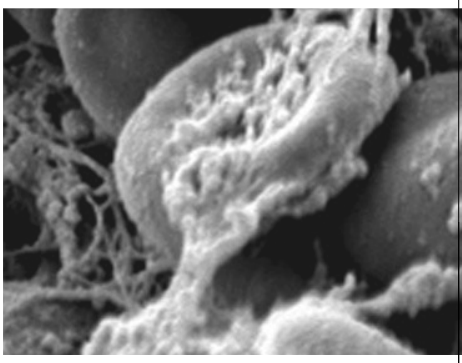

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Fibrinogen γ' levels in patients with intracerebral hemorrhage

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ABSTRACT

The fibrinogen γ′ variant (γ′) has both antithrombotic and prothrombotic properties when compared to normal fibrinogen. It may therefore be of relevance in intracerebral hemorrhage and intraventricular extension of the bleeding. We studied the role of γ′ in intracerebral hemorrhage, and in intraventricular extension of the hemorrhage.

We performed a case-control study in 156 controls and 55 patients with intracerebral hemorrhage, with and without intraventricular extension. Levels of fibrinogen γ′ and the γ′/total fibrinogen ratio were measured in all participants.

Levels of γ′ were increased in patients with intracerebral hemorrhage when compared with controls (0.40 vs 0.32 g/L, $p<0.001$). The γ′/total fibrinogen ratio was similar in patients and controls (0.092 vs 0.096, $p=0.42$). There was evidence for an unfavorable outcome in patients with fibrinogen levels in the highest tertile compared with the lowest tertile (OR 4.0, 95%CI 1.1-15.2), and a nonsignificant trend toward unfavorable outcome with higher levels of γ′ (p-value for trend=0.06).

Our study shows that absolute levels of fibrinogen γ′ are increased in patients with intracerebral hemorrhage, but relative levels are similar in patients and controls, suggesting that the absolute rise in γ′ is an acute phase response.

INTRODUCTION

Fibrinogen is one of the central proteins in hemostasis, and several isoforms are present in the blood. One of these isoforms is fibrinogen γ′ (γ′), which contains an altered γ chain that is present in 8 to 15% of fibrinogen molecules.[1] Fibrinogen γ′ is produced after alternative mRNA processing of the γ chain gene product.[2,3] It has antithrombotic properties such as a binding site for thrombin[4] and a disrupted binding site for platelet integrin $\alpha_{IIB}\beta_3$,[5] as well as a high-affinity binding site for factor XIII:B, which is considered prothrombotic.

Intracerebral hemorrhage (ICH), resulting from rupture of blood vessels in the brain, is a severe neurologic disorder leading to a high rate of mortality and disability.[6] Hemostasis plays an important role in the pathophysiology of hemorrhagic disorders, such as ICH, and plays a role in hematoma growth.[6] Larger hematomas are associated with extension of the bleeding into the brain ventricles (intraventricular extension, IVE).[7] IVE is associated with an unfavorable prognosis, resulting in a higher mortality.[8]

In several cardiovascular disorders, such as myocardial infarction and ischemic stroke, a pathogenic role of γ′ has been suggested but not yet proven.[9,10] The role of γ′ in hemorrhagic disorders such as ICH has, however, not yet been studied. We therefore aimed to determine the association between γ′ levels and the γ′/total fibrinogen ratio and occurrence of ICH, and with intraventricular extension.

METHODS

We performed a case-control study in 55 consecutive patients admitted to our hospital with non-traumatic intracerebral hemorrhage between July 2006 and July 2009, and 156 age- and sex matched stroke-free controls. Participants had no history of coagulation disorders. All individuals participated in the Erasmus Stroke Study (ESS), a hospital-based registry of patients with cerebrovascular disorders treated at our hospital. The ESS also includes population-based controls, mostly friends and spouses of patients, but no family. All participants provided written informed consent. The institutional Medical Ethics Committee approved the study. Upon admission, all patients underwent a plain brain CT scan to confirm the diagnosis and to determine the presence or absence of intraventricular extension of the hemorrhage. Functional outcome was evaluated at discharge by means of the modified Rankin Scale (mRS),[11] which is a score for handicap and disability ranging from 0 (no symptoms) to 6 (death). At a score of 2 patients have disabilities interfering with activities but are still able to live completely independent; at a score of 3 patients are

dependent for activities of daily living. Unfavorable functional outcome was defined as mRS>2, signifying loss of independency.

Citrated blood was drawn 3-6 days after admission and centrifuged at 1700*g* for 15 minutes. Citrated plasma was frozen at -80°C within 2 hours from collection and stored until analysis.

Laboratory analyses

Total fibrinogen levels were measured according to von Clauss on a fully-automated coagulation analyzer (Sysmex CA-1500 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Fibrinogen γ′ antigen levels were measured with an enzyme-linked immunosorbent assay as described previously[12] using anti-γ′ fibrinogen antibodies for catching and HRP-labeled rabbit anti-fibrinogen antibodies for tagging.

Statistical analysis

Apart from fibrinogen γ′ levels we also determined the γ′/total fibrinogen ratio, to adjust for total fibrinogen levels, which are known to increase as results of an acute phase reactions. Differences in γ′ levels and γ′/total fibrinogen ratio between patients and controls and within patient subgroups were assessed by means of multiple linear regression, adjusted for age and sex. The relation between γ′ levels and the γ′/total fibrinogen ratio and functional outcome was assessed using logistic regression and expressed as odds ratio (OR) per tertile increase in fibrinogen level, the lowest tertile being the reference category. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois). A probability value <0.05 was considered significant.

RESULTS

Median age was 63 years in patients and 60 years in controls (difference not significant), 55% of patients and 49% of controls were men (not significant). Of all patients, 8 (15%) had intraventricular extension of the hemorrhage.

Total fibrinogen and γ′ were significantly higher in patients than in controls (Table 1). Table 1 also shows levels in patients with and without intraventricular extension of the hemorrhage. We found no difference in the γ′/total fibrinogen ratio between patients and controls. However, the subgroup of patients with IVE had a significantly higher γ′/total fibrinogen ratio than controls and patients without IVE.

The median modified Rankin Scale score at discharge was 2 (interquartile range 2-4).

Patients with fibrinogen levels in the highest tertile had a significantly higher probability of unfavorable outcome, compared with patients in the lowest tertile. There were also signs of a linear relationship (p-value for trend over tertiles 0.12, Table 2). Levels of fibrinogen γ′ seemed to be higher in patients with unfavorable outcome, but not statistically significant. These risks were not attenuated when patients with IVE were left out of the analysis.

Table 1. Levels of total fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio in patients and controls

	Patients			Controls				
	All N = 55	No IVE N = 47	IVE present N = 8	N = 156	P value*	P value†	P value‡	P value§
Total fibrinogen (g/L)	4.41 (1.21)	4.41 (1.23)	4.40 (1.13)	3.37 (0.72)	<0.001	0.95	<0.001	<0.001
Fibrinogen γ′ (g/L)	0.40 (0.14)	0.38 (0.14)	0.51 (0.13)	0.32 (0.13)	<0.001	0.02	0.01	<0.001
γ′/total fibrinogen ratio	0.092 (0.031)	0.087 (0.032)	0.119 (0.032)	0.096 (0.028)	0.42	0.01	0.08	0.03

Presented are means (SD). All analyses are adjusted for age and sex. *P value between all patients and controls; †P value between patients with and without IVE; ‡P value between patients without IVE and controls; §P value between patients with IVE and controls

Table 2. Risk of unfavorable functional outcome (mRS >2) and levels of fibrinogen, fibrinogen γ′ and the γ′/total fibrinogen ratio, in tertiles of the distribution.

	Tertiles			p-trend
	1	2	3	
Fibrinogen				
Distribution	<3.8 g/L	3.8 – 4.4 g/L	>4.4 g/L	
All patients	1.00 (ref)	1.56 (0.41-5.95)	4.00 (1.05-15.21)	0.12
Only patients without IVE	1.00 (ref)	1.35 (0.32-5.71)	2.62 (0.66-10.48)	0.39
Fibrinogen γ′				
Distribution	<0.33 g/L	0.33 – 0.44 g/L	>0.44 g/L	
All patients	1.00 (ref)	5.60 (1.36-23.06)	2.80 (0.71-11.10)	0.06
Only patients without IVE	1.00 (ref)	6.24 (1.44-27.06)	2.60 (0.56-12.02)	0.05
γ′/total fibrinogen ratio				
Distribution	<0.078	0.078 – 0.101	>0.101	
All patients	1.00 (ref)	0.80 (0.21-3.00)	1.72 (0.47-6.32)	0.50
Only patients without IVE	1.00 (ref)	0.83 (0.21-3.35)	2.25 (0.54-9.45)	0.38

Shown are OR (95%CI)

DISCUSSION

In this case-control study, we found that levels of fibrinogen γ′ were strongly elevated in patients with intracerebral hemorrhage as compared to control persons. In the total patient group, the fibrinogen γ′/total fibrinogen ratio was similar to controls, but subgroup analyses revealed an increased γ′/total fibrinogen ratio in patients with intraventricular extension of the hemorrhage, while in patients without IVE, this γ′/total fibrinogen ratio was somewhat lower, although not statistically significant, than in controls.

Strengths of our study are the novelty of studying fibrinogen γ′ in intracerebral hemorrhage, and the availability of a functional outcome measure. A methodological concern of our study is the small number of patients. Intracerebral hemorrhage, a rare disorder when compared to ischemic stroke, has a high mortality and many patients die before arriving in the hospital or within hours from admission.[6,14] This makes any study of patients with intracerebral hemorrhage, including our own, vulnerable to bias as many patients are not included. A second point is the case control design of the study, which makes it difficult to study causal relationships between fibrinogen γ′ and ICH.

Although the anti- and prothrombotic properties of γ′ have been studied quite extensively, most clinical research in this field has been done in thromboembolic disorders such as venous thrombosis, myocardial infarction and ischemic stroke.[10,12,15-17] No studies have yet been performed focusing on the antithrombotic properties of γ′ in relation to hemorrhage.

The γ′ chain has a binding site for thrombin, with both antithrombotic or prothrombotic consequences, which have been subject of discussion.[4,18,19] Despite this, thrombin binding to the γ′ chain overall seems to be antithrombotic, reducing the free thrombin content and inhibiting thrombin-mediated platelet activation.[20,21] Also, the binding of thrombin leads to a selective inhibition of the intrinsic coagulation pathway.[19] An additional antithrombotic property of γ′ is the decreased binding to platelet integrin $\alpha_{IIB}\beta_3$ by the γ′ chain, leading to a diminished platelet aggregation activity.[22] Given the antithrombotic properties of γ′, one might hypothesize that γ′ might also affect the risk and outcome of (intracerebral) hemorrhage. Thus far, no studies have been done on this particular subject.

In our study we found that γ′ levels were indeed increased in patients with intracerebral hemorrhage. However, due to the case control design we can′t rule out that this finding is the result of an acute phase response in ICH patients.[9]

Previous studies have shown a stronger acute phase response in patients with blood in the intraventricular or subarachnoid space.[23-25] The relevance of intraventricular blood may

play a role in our finding that relative fibrinogen γ′ levels seem to increase significantly only in patients with extension of bleeding into the ventricles. However, in our study the presence or absence of IVE did not affect total fibrinogen levels. The reason for this discrepancy remains unknown, but it has been hypothesized that the direct contact between blood and cerebrospinal fluid might play a role.[23]

Our finding that patients with high levels of total fibrinogen had a significantly higher chance of unfavorable outcome is in line with findings from previous studies.[26,27] The prognostic value of fibrinogen γ′ in patients with ICH is still unclear. The risk estimates we found for unfavorable outcome in higher tertiles of fibrinogen γ′, was present in all patients, also after removing patients with IVE from the analysis. Larger studies are required to explore the prognostic value of fibrinogen γ′ in patients with ICH.

Any hemorrhage leads to consumption of coagulation factors; for intracerebral hemorrhage, literature is very limited with very little knowledge on consumption in this category of patients. The volume of the hemorrhage is usually small, varying depending on the method used, between 30-50 ml.[28,29] Considering the small size of the hemorrhage, systemic consumption of coagulation factors will be minimal and not affect the results that we measured in our study.

In conclusion, the level of fibrinogen γ′ is increased in patients with intracerebral hemorrhage compared with controls; however, the ratio of γ′/total fibrinogen ratio is similar, suggesting that the rise in fibrinogen γ′ may reflect an acute phase response.

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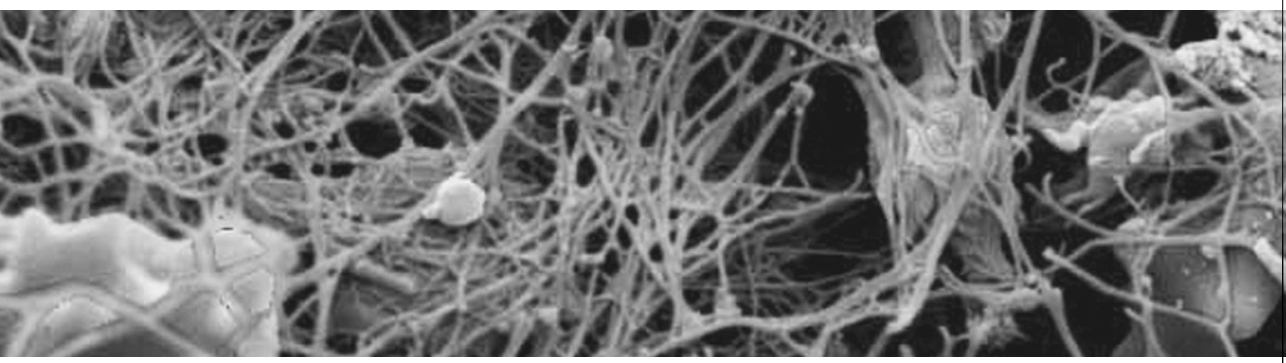

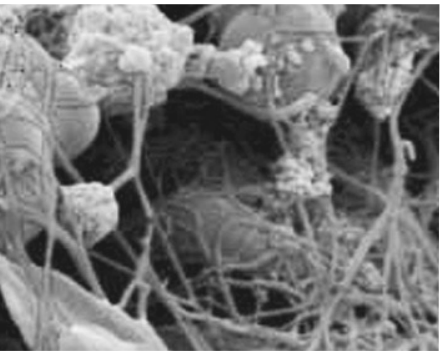

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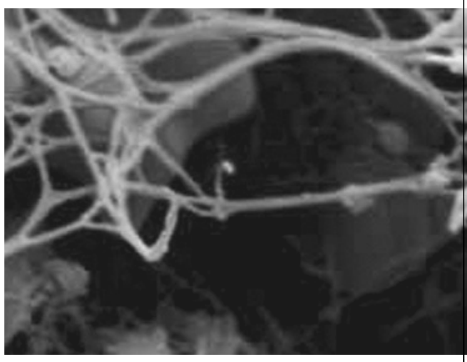

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The effect of fibrinogen γ variants on wound healing

Submitted

ABSTRACT

Fibrin plays an important role in hemostasis, but it also provides a temporary, degradable matrix for tissue repair. In the final step of the coagulation cascade fibrinogen is converted to fibrin. Plasma fibrinogen is very heterogeneous and one form is the alternative messenger RNA processing variant γ′. This fibrinogen form shows increased thrombin and factor XIII binding and decreased platelet binding, as compared to the common γA form. In this study the effects of γ variants on angiogenesis and wound healing were investigated.

The endothelial cell characteristics on γA and γ′ fibrin matrices were investigated *in vitro* using adhesion, proliferation, migration and tube-formation assays. Moreover, the progress of wound healing was studied *in vivo* with a rat full-thickness wound healing model (wound closure rate, new vessel formation, perfusion and wound breaking strength). Purified γ′ fibrinogen contained more factor XIII (FXIII) and formed a fibrin network with thinner fibers than γA fibrinogen, this affect was abolished by using equal FXIII concentrations. The *in vitro* tube formation was slightly more pronounced in γA than in γ′ fibrin matrices and was accompanied with an increased fibrinolysis. Endothelial cell adhesion, migration and proliferation were similar on γA and γ′ fibrin. The *in vivo* wound healing model suggested that γA was promoting wound healing slightly, but not significantly, better than γ′ fibrinogen.

In conclusion, matrices of γA and γ′ fibrin govern different structural and functional characteristics. Small, non-significantly increased *in vitro* tube formation and *in vivo* wound healing were shown in γA fibrin, compared to γ′ fibrin. However, more data are necessary before conclusions can be drawn.

INTRODUCTION

Wound healing is a dynamic, interactive and complex process, which involves interactions between many different cells and the extracellular microenvironment. Fibrinogen is a central protein in the coagulation cascade and following endothelial injury, a fibrin clot is formed, which is the first protection against hemorrhage. In addition to its role in hemostasis, fibrin provides a temporary, biodegradable matrix for cell invasion during wound healing.[1-3] The formation of new capillaries (angiogenesis) depends on the extracellular matrix in the wounded area, and on the migration and mitogenic stimulation of endothelial cells.[1] Fibrin facilitates cell adhesion, migration, proliferation and stimulates angiogenesis.[2] Moreover, fibrin binds growth factors that influence these processes, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).[1-3]

The fibrinogen molecule comprises two identical disulphide-linked halves, each consisting of three polypeptide chains termed Aα, Bβ and γ.[4] Numerous fibrinogen forms are present in healthy individuals, which are the result of alternative processing of fibrinogen messenger RNA, posttranslational modifications and degradation of the protein.[5] One naturally occurring isoform is the γ′ fibrinogen variant, which comprises 10% ± 3% of the total circulating fibrinogen in healthy individuals.[6] γ′ fibrinogen is the result of alternative messenger RNA processing at intron 9 and exon 10, resulting in the replacement of the last 4 amino acids (408-411) at the C-terminus of the γ-chain by 20 amino acids (408-427).[7]

The extension of γ′ fibrinogen contains a high affinity binding site for thrombin, which results in antithrombin I activity.[7] In addition, γ′ fibrinogen contains an extra binding site for the factor XIII (FXIII) B subunit[8, 9] and lost its platelet integrin $\alpha_{IIb}\beta_3$ binding site, which results in reduced platelet-fibrin(ogen) interactions.[10] In the circulation, γA/γA fibrinogen (abbreviated as γA) and fibrinogen molecules containing one or two γ′ chain(s) (γA/γ′ or γ′/γ′, both abbreviated as γ′) are present. Several studies reported structural and functional differences between γA and γ′ fibrin matrices, such as slower fibrinopeptide B release, slower fibrin polymerization for γ′ fibrinogen, thinner fibers and more branch points with scanning electron microscopy on γ′ fibrin matrices.[11, 12]

We hypothesized that the functional differences between γA and γ′ fibrin, in combination with the altered matrix structure, influences angiogenesis and wound healing. The denser structure of γ′ fibrin is likely to be associated with impaired fibrinolysis[13, 14] and local degradation of the extracellular matrix is an important determinant for angiogenesis and wound healing.[15, 16] The interaction between endothelial cells and γA or γ′ fibrin matrices might be modified, and hence affect the inflammatory, proliferative and remodeling phases in wound repair. In this study, we investigated the effects of the γA and γ′ fibrinogen variants on the *in vitro* endothelial cell characteristics and *in vivo* wound healing.

MATERIAL AND METHODS

γA and γ′ fibrinogen characterization

Purification and characterization of fibrinogen γ variants

γA and γ′ fibrinogen variants were purified by anion-exchange chromatography[8] from plasma-purified fibrinogen (*in vitro* experiments)[17] or from commercial fibrinogen (*in vivo* experiments) (Enzyme Research Laboratories, South Bend, IN). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4–15%

gradient gel (Criterion Tris-HCL precast, Biorad, Hercules, CA) and total protein was stained with colloidal blue (Invitrogen, Carlsbad, CA). Western blotting was performed using polyclonal rabbit anti-fibrinogen (Dako, Glostrup, Denmark) and monoclonal anti-human γ′ fibrinogen antibodies (2.G2.H9; Millipore, Billerica, MA) followed by detection with fluorescent labeled secondary antibodies: goat anti-rabbit 680 (Invitrogen, Paisley, UK) and goat anti-mouse 800 (Invitrogen, Paisley, UK) for fibrinogen and γ′ fibrinogen, respectively. Factor XIII activity was determined photometrically according to manufacturer's instructions (Berichrom Factor XIII, Siemens healthcare diagnostics**,** Deerfield, IL). Plasminogen activity was measured with chromogenic substrate S-2251 following manufacturer's instructions (Instrumentation laboratory, Lexington, MA). The clottability was determined by measuring the protein content (optical density at 280nm) before and after 2 hours polymerization of purified fibrinogen (1 mg/mL) at ambient temperature with 0.5 U/mL thrombin (Organon Teknika, Boxtel, the Netherlands) and 2.5 mM $CaCl_2$.[18]

Confocal microscopy of fibrin matrices

Fibrinogen variants were labeled with fluorescein isothiocyanate isomer I (FITC) (Sigma-Aldrich, Saint Louis, MI) and fibrin matrices (2 mg/mL) were formed by adding 0.5 U/mL thrombin and 2.5 mM $CaCl_2$ in a total volume of 100 μL in microchambers. The FXIII concentration in the γA *f*ibrinogen matrix was supplemented with human plasma coagulation FXIII (Fibrogammin P, CSL Behring, Marburg, Germany) to compensate for the higher FXIII activity in γ′ fibrinogen. After 2 hours polymerization, the matrices were extensively washed with Tris-HCl buffer. Confocal images were visualized on a Zeiss LSM 510 Meta inverted confocal microscope (Carl Zeiss MicroImaging, Jena, Germany*)* with 63x oil immerse objective lens in combination with a 5-W argon laser and 488 nm band-pass laser filter for the excitation. Fibrin fiber diameters (n=50) were measured using LSM Image Browser software, version 4.2 (Carl Zeiss MicroImaging, Jena, Germany*)*.

In vitro **endothelial cell behavior**

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords, kindly provided by the Department of Obstetrics of the Amstelland Hospital, Amstelveen. HUVEC were isolated, cultured and characterized as previously described.[19] Human microvascular endothelial cells (HMVEC) were isolated from foreskin, kindly provided by the Department of Dermatology of the VUMC, Amsterdam. HMVEC were isolated, cultured and characterized as previously described.[20]

Fibrinogen coatings

Fibrinogen coatings were prepared on polystyrene culture plates (Costar, Corning, Amsterdam, The Netherlands) as previously described.[21] Plates were coated with fibrinogen (0.2 μM; 0.068 mg/mL) in phosphate buffered salt solution (PBS) for 1 hour at 37°C, and then washed with PBS. Endothelial cells were seeded on fibrinogen coatings to determine their adhesion, migration and proliferation.

In vitro adhesion, proliferation and migration

Adhesion was performed by seeding HUVEC on fibrinogen coatings in a density of 2 x 10^4 cells/cm^2 in medium (M199) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (p/s) and 1% human serum albumin (HSA, Sanquin, Amsterdam, The Netherlands). After 0.5, 1, 2 and 4 hour(s) the wells were carefully washed and cells were fixated. Pictures were taken using a Qimaging camera on a Zeiss microscope connected to a computer with Optimas image analysis software (Media Cybernetics, Bethesda, USA). Cell counting was performed using Image J 1.42 software (National institutes of health, Bethesda, USA).

Proliferation of HUVEC was determined using ^{3}H-thymidine incorporation. HUVEC were seeded in a density of 6 x 10^3 cells/cm^2 in M199 supplemented with p/s and 1% HSA. Cells adhered to the fibrinogen coatings in 4 hours, followed by 72 hours stimulation of endothelial cell proliferation with M199 supplemented with p/s, 10 ng/mL vascular endothelial growth factor (VEGF) and 10% heat-inactivated newborn calf serum (NBCSi). To quantify the proliferation 1 μCi ^{3}H-thymidine was added during the last 16 hours of growth. The beta-emission was measured with Ultima Gold scintillation fluid on 1900 TR Liquid Scintillation Analyzer (Packard Bioscience, Massachusetts, USA).

The migration of HUVEC was studied by determining their migration into a cell free area. Endothelial cells were seeded in a density of 6 x 10^4 cells/cm^2 in M199 supplemented with p/s and 1% HSA. Prior to cell seeding, a device of 1.5 x 20 mm was placed into the well to prevent cell adhesion. Cells adhered to the fibrinogen coating, the device was removed and migration was stimulated for 16 hours by addition of M199 supplemented with p/s, 10 ng/mL VEGF and 10% NBCSi. Five measurements of cell-free area per picture were performed using Photoshop 5.0 software (Adobe, San Jose, USA).

In vitro tube formation

The *in vitro* tube formation was determined using three-dimensional (3D) fibrin matrices and HMVEC, as described by Koolwijk et al.[22] Prior to polymerization, all fibrinogen

fractions were dialyzed to M199 supplemented with p/s, and the γA fibrin matrix was supplemented with human plasma coagulation FXIII (final concentration 0.31 U/mL). Fibrin matrices were prepared by addition of thrombin (0.01 U/mL) to 2 mg/mL fibrinogen solution. After 4 hours polymerization, thrombin was inactivated by 16 hours incubation with M199 supplemented with 10% heat-inactivated human serum (HSi) and 10% NBCSi. HMVEC were seeded in confluent density and after 24 hours, and subsequently at 48 hours intervals, the HMVEC were stimulated with M199, 10% HSi, 10% NBCSi, 10 ng/mL tumor necrosis factor-α (TNFα, Sigma, St Louis, USA) and 25 ng/mL VEGF or 10 ng/mL fibroblast growth factor-2 (FGF-2, Preprotech, London, UK). The tubular structure formation of HMVEC into the fibrin matrices was analyzed after staining the F-actin filaments with rhodamine-phalloidine (Molecular Probes, Eugene, USA) and nuclei with Hoechst (Molecular Probes). The Zeiss Axiovert 200 Marianas™ inverted microscope with 2.5 and 10x magnification was used and controlled with Slidebook software (Intelligent Imaging Innovations, Denver, USA). The amount of fibrin degradation products in conditioned media of the tube formation assay were determined with the enzyme-linked immunosorbent assay FDP14-DD13, as described previously.[23]

In vivo wound healing

Full-thickness excisional wound healing model

The wound healing model was performed as previously described by Tong et al.[24] Briefly, 24 male 9-week-old WAG/RijHsd rats (Harlan, Zeist, the Netherlands) were anesthetized with *5*% isoflurane for induction and 2% isoflurane for maintenance (Isoflurane, Rhodia Organique Fine Limited, Bristol, UK) and two full-thickness wounds (15 mm diameter circles, the centers were 3 cm apart and 4 cm caudal to the scapulae) were excised. 100 μL fibrinogen (2 mg/mL), mixed with 1U/mL thrombin, was topically applied or an equal amount of Tris-HCl buffer was applied for control wounds. Experiments were conducted with approval of the Animal Experiments Committee of the Erasmus University Medical Center.

Wound closure rates were measured with standardized digital photographs of the wounds taken on day 0, 3, 7, 10, 14 and 21 after surgery with an Olympus camera (Olympus, Tokyo, Japan). The wound area was determined with Image J 1.42, and the wound closure rate was calculated.

The skin perfusion was determined using Laser Doppler perfusion measurements (LDPM, Perimed, Periflux System 5000, Perimed AB, Stockholm, Sweden) of the wounded and normal skin on day 14 and 21 after wounding, as described previously.[24]

A stable measurement at 33°C with the thermostatic Laser Doppler probe (PROBE 457, Perimed AB, Stockholm, Sweden) was set as baseline. Subsequently, the skin was heated to 44°C and the microcirculatory response was measured for 10 minutes using continuous coherent laser light at a wavelength of 780 nm. The reactive hyperemia rate was expressed as percentage change in baseline.

The breaking strength of the wounded skin was measured as previously described by Tong et al.[24] Briefly, after sacrificing the rat on day 21, the skin containing the wounded areas and the normal skin between the wounds were excised and uniformly cut into standardized dumbbell-shaped strips (central width 4 mm and length 45 mm). Subsequently, the strips were fixed perpendicularly between the two clips of the tensiometer (Testometrics AX, M250-2.5KN, Testometric Company, Lancashire, UK) and a constant strain rate of 60 mm/minute using a 1.0 kg force transducer was applied. Before the skin collapsed, the maximum load was recorded in Newton (N). The ratio of the wound breaking strength was expressed relative to the breaking strength of normal skin.

Paraffin sections of the wounds were immunohistochemically stained for CD34 on endothelial cells (goat-anti-rat CD34 IgG, R&D system, Minneapolis, MN).[25] The positive-signal density of CD34 was quantified using the Cell^D Imaging Solution software (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by univariate ANOVA, and differences between different time points were analyzed by ANOVA with pairwise comparisons (Bonferroni correction). Statistical analyses were performed with Statistical Package for the Social Sciences for windows (SPSS), Version 15.0 (Chicago, IL). Results were considered statistically significant with two-sided P-values <0.05.

RESULTS

Characterization of γA and γ¢ fibrinogen and fibrin

SDS-PAGE analysis showed the presence of the γ′-chain at approximately 50 kDa in the purified fraction of γ′ fibrinogen, and the absence hereof in the γA fibrinogen fraction (Figure 1A). Western blotting with fibrinogen and γ′ fibrinogen specific antibodies confirmed this result (Figure 1B). In the γ′ fibrinogen fraction an extra band of approximately 77 kDa was visible, mass spectrometry confirmed that this was the FXIII B unit (data not shown). In the γ′ fibrinogen fraction the FXIII activity was significantly higher than those measured in the γA fraction (34 U/mg and 1 U/mg, respectively). The plasminogen activity in the γA and γ′ fibrinogen fractions was similar (0.012 U/mg and 0.011 U/mg, respectively). Purified γA and γ′ fibrinogen were shown to be >95% clottable. Confocal microscopy on the fibrin matrices showed thicker fibers in γA fibrin without FXIII (0.37 ± 0.08 μm) than in γ′ fibrin (0.29 ± 0.04 μm). However, the thickness of the γA fibers (0.29 ± 0.04 μM) was similar to that of γ′ fibrin, when equal amounts of FXIII were supplemented as in γ′fibrin (Figure 2). In addition to the fiber thickness, we also observed that the distribution of the fibers and the number of branch points differed for γ′ and γA. This is not yet studied in more detail, but we included it in the discussion and others have also seen this.

In vitro endothelial cell behavior

The adhesion of HUVEC to γA and γ′ fibrinogen coatings revealed no significant differences in the number of adhering endothelial cells. However, the endothelial cells adhered slightly faster to γA fibrinogen coatings ($P<0.05$) after 0.5 and 1 hour. The adhesion of endothelial cells to gelatin coatings was 2–fold better than to γA and γ′ fibrinogen coatings, whereas BSA coatings showed hardly any cell adhesion (Figure 3A, n=3). The proliferation of HUVEC cultured on γA and γ′ fibrinogen coatings did not show significant differences in cell growth after adjustment for the cell adhesion (84 ±31% and 85 ±35% respectively) (Figure 3B, n=3). Also the migration of HUVEC cultured on γA and γ′ fibrinogen coatings was similar (1.89 ± 0.57 A.U and 1.89 ± 0.50 A.U. respectively) (Figure 3C, n=6), whereas on gelatin coatings the migration of endothelial cells was slightly better (2.83 ± 0.30 A.U). The *in vitro* tube formation of HMVEC in 3D γA fibrin matrices showed massive and uncontrolled tube formation, with many sprouting cells and a high fibrinolytic activity explaining the bright structures (Figure 4A). In the γ′ fibrin matrix the endothelial cells formed stable tube-like structures with a normal morphology. Quantification of the *in vitro* tube formation revealed 2.2x more ingrowth of endothelial cells into γA fibrin (Figure 4B).

The fibrin degradation products in conditioned media of the *in vitro* tube formation assay was significantly higher in γA fibrin when compared to those measured in γ′ fibrin (Figure 4C, $p<0.05$).

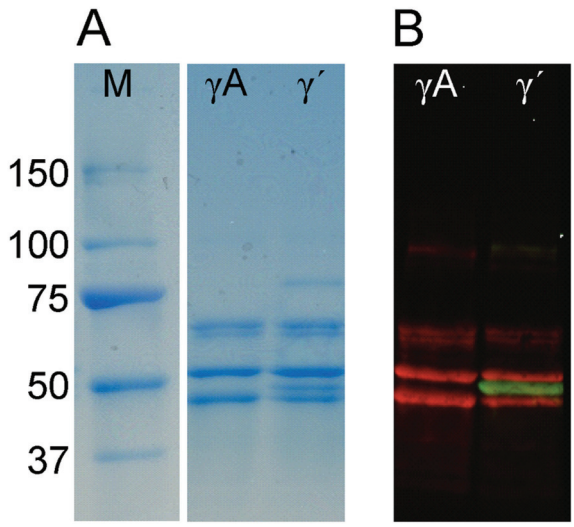

Figure 1. Characterization of the γA and γ′ fibrinogen fractions.
(A) SDS–PAGE with total protein staining and (B) Western blotting of fibrinogen with polyclonal human fibrinogen antibody (red) and monoclonal human γ′ fibrinogen antibody (green). M: protein electrophoresis marker.

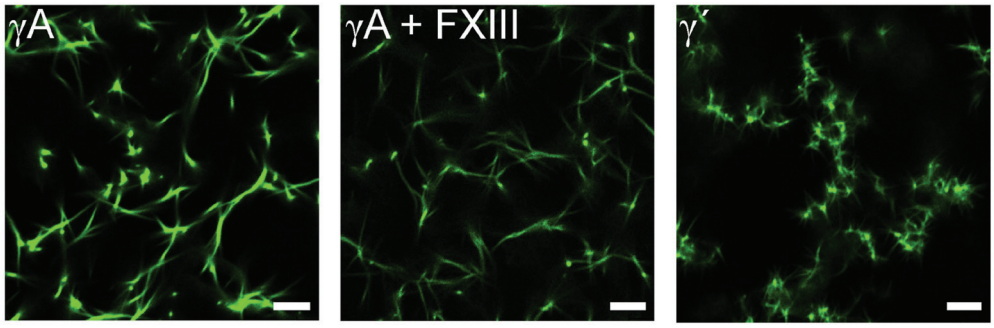

Figure 2. Confocal microscopy of γA and γ′ fibrin matrices.
Fibrin matrices prepared from γA fibrinogen without additional FXIII (γA), γA supplemented with FXIII (γA+FXIII) and γ′ fibrin (γ′) as described in Materials and methods. The scale bars indicate 5 μm.

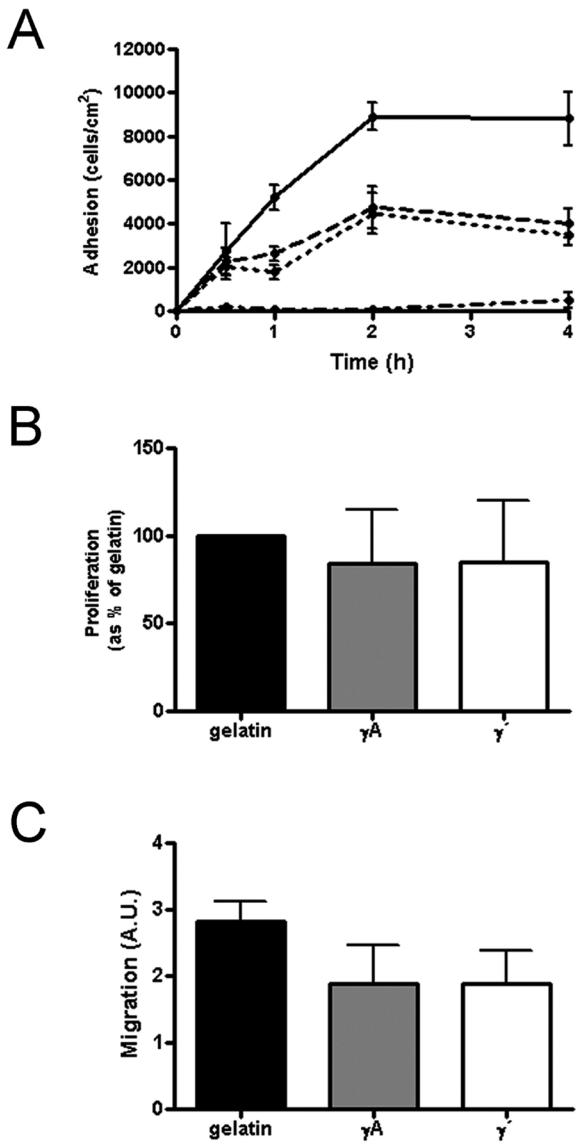

Figure 3. *In vitro* functional characteristics of HUVEC on various fibrinogen coatings. (A) Adhesion of HUVEC to gelatin (solid line), γA fibrinogen (dashed line), γ′ fibrinogen (dotted line) and 1% BSA (dashed-dotted line) coatings (n=3). (B) The proliferation of HUVEC on gelatin, γA and γ′ fibrinogen coatings, measured by ^{3}H-thymidine incorporation (n=3). The values were corrected for cell adhesion and expressed as percentage of HUVEC proliferation on gelatin coatings. (C) The migration of HUVEC on gelatin, γA and γ′ fibrinogen coatings, after 16 hours of migration the area of migration was determined (n=6).

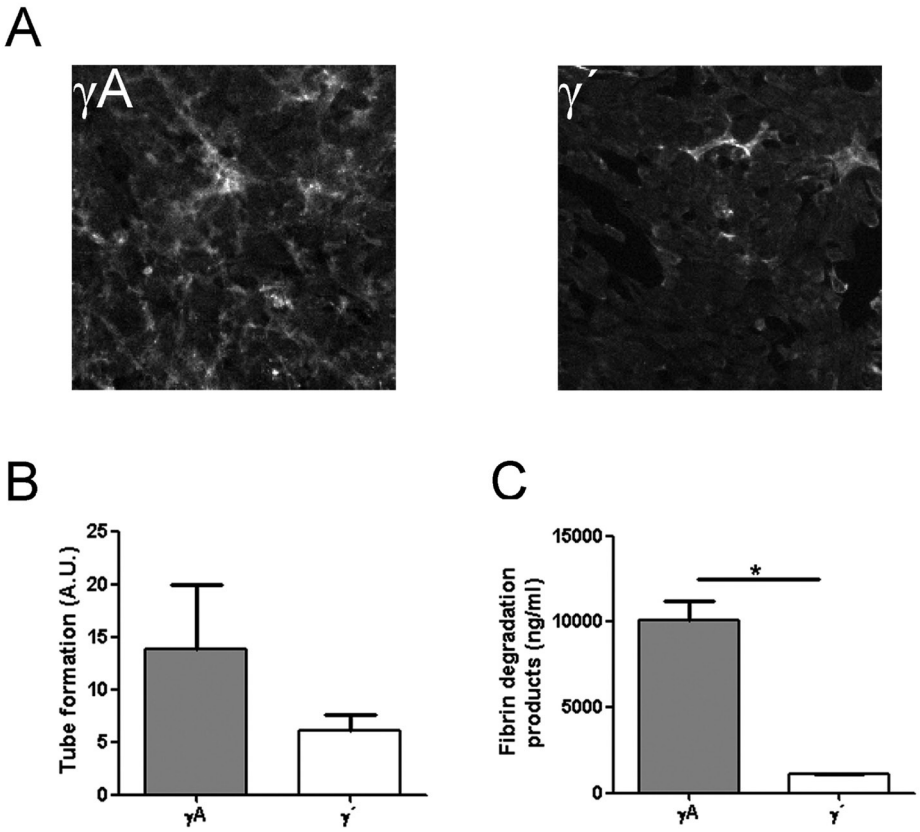

Figure 4. Fluorescent microscopy of *in vitro* tube formation by HMVEC.
HMVEC were seeded on three dimensional γA and γ′ fibrin matrices and tubular structure formation was stimulated. (A) Representative pictures of the *in vitro* tube formation assay. The staining represents the F-actin filaments of endothelial cells, the bright structures represent the tubular structures in the fibrin matrix. (B) Quantification of the amount of tube formation in the various fibrin matrices (n=3). (C) Fibrin degradation products that were present in conditioned medium of the *in vitro* tube formation assay, as determined by ELISA (n=3).

In vivo skin wound healing

The closure rate of the dorsal full-thickness wounds of the rats was similar for controls and unfractionated, γA and γ′ fibrin treated wounds (Table 1 and Figure 5). All wounds were closed 21 days after surgery. The wound closure rate on day 7 and day 10 tended to be slightly higher in the γA fibrin treated group, when compared to the γ′ fibrin treated group (Table 1). In addition, the Laser Doppler perfusion measurements showed that the γA fibrin treated wounds had a slightly, but not significantly, higher vasodilator response in the skin at day 21 than γ′ fibrin treated wounds (53.0 ± 10.0% versus 41.3 ± 6.5%). Also

the breaking strength ratio was slightly, but not significantly, higher in the γA fibrin treated group, when compared to the γ′ fibrin treated group (0.15 ± 0.03 versus 0.11 ± 0.02).

The vessel density and vessel size in the wounds were determined from paraffin embedded sections after CD34 staining of endothelial cells. No significant differences in vessel density and vessel size were observed in control and fibrin treated animals. The vessel density showed a higher vessel number in the γA fibrin treated group than the γ′ fibrin treated group (127 ± 47 versus 67 ± 13 vessels/mm^2). Moreover, the vessel size was slightly, but not significantly, smaller in the γA fibrin treated group, when compared to the γ′ fibrin treated group (196 ± 25 versus 232 ± 16 μm^2/vessel).

Table 1. *In vivo* wound healing in the rat model.

Parameters	Control	Unfractionated fibrinogen	γA fibrinogen	γ′ fibrinogen
Wound closure rate (%)				
day 3	0.3 ± 2.6	-0.1 ± 4.1	0.9 ± 2.6	-2.1 ± 3.7
day 7	43.7 ± 3.4	37.7 ± 4.9	48.5 ± 3.5	45.9 ± 4.6
day 10	82.0 ± 1.8	81.6 ± 2.1	85.0 ± 2.8	81.2 ± 2.8
day 14	97.1 ± 0.5	95.4 ± 0.7	96.2 ± 0.7	97.1 ± 0.7
day 21	100 ± <0.1	100 ± <0.1	99.8 ± 0.2	100 ± <0.1
Laser Doppler perfusion (%)				
day 14	27.3 ± 6.7	34.5 ± 6.0	20.4 ± 5.5	28.6 ± 6.4
day 21	26.2 ± 3.6	37.4 ± 4.2	53.0 ± 10.0 *	41.3 ± 6.5
Breaking strength ratio				
day 21	0.112 ± 0.021	0.104 ± 0.013	0.151 ± 0.028	0.107 ± 0.017
Immunohistochemistry				
Vessels density (n/mm2)	59 ± 7	44 ± 8	127 ± 47	67 ± 13
Vessel size (μm^2)	245 ± 14	302 ± 60	196 ± 25	232 ± 16
Vessel area (%)	1.43 ± 0.13	1.31 ± 0.29	2.04 ± 0.44	1.49 ± 0.23

The data are given as mean ± SEM. Laser Doppler perfusion is expressed relative to baseline measurements at 33°C (% increase). The breaking strength is expressed as ratio relative to the breaking strength of normal skin. * P<0.01 γA fibrin versus control treated wounds.

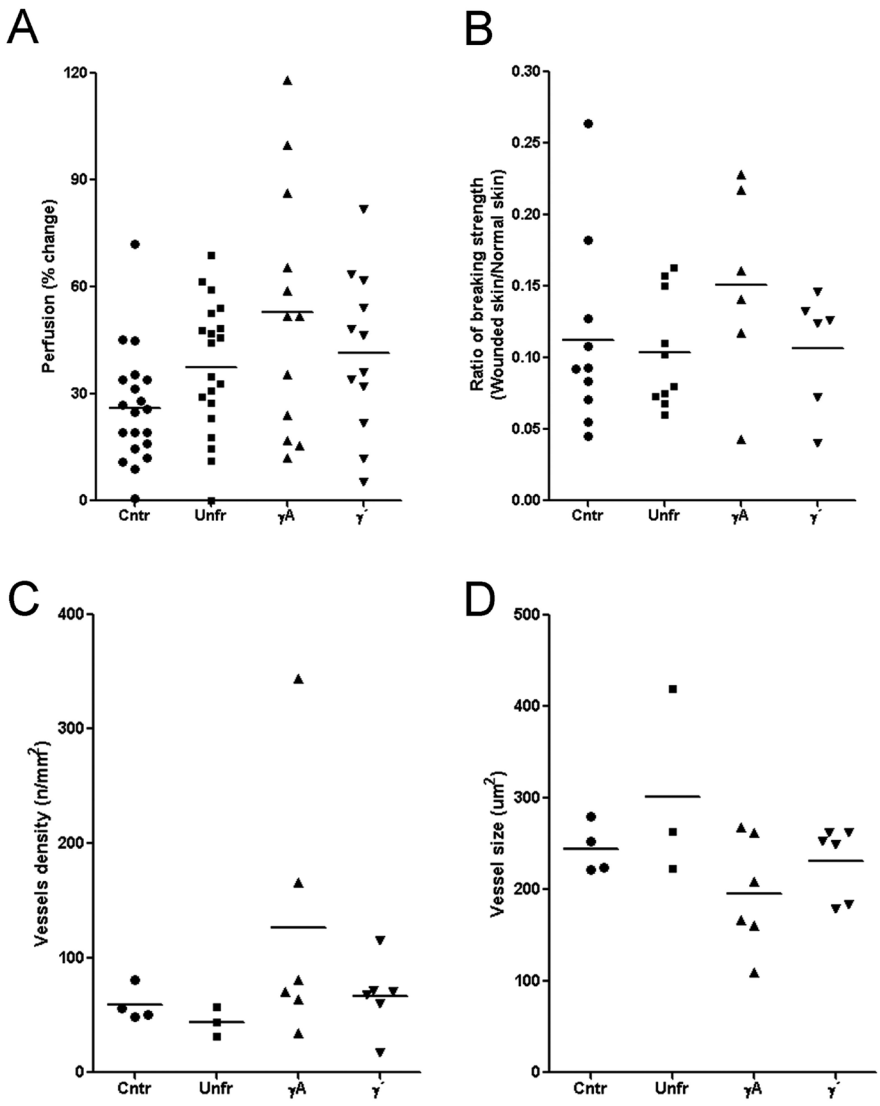

Figure 5. Overview of the perfusion, breaking strength and vessel properties of the *in vivo* wound healing assay. Cntr, Unfr, γA and γ′ represent control (Tris-HCl), unfractionated fibrinogen, γA fibrinogen and γ′ fibrinogen treated wounds. (A) The perfusion was measured with Laser Doppler and results are expressed as percentage change from baseline measurement in the same wound. (B) The breaking strength ratio of wounded and normal skin is given. The vessel density (C) and the vessel size (D) were determined with immunohistochemistry after staining CD34 on endothelial cells. Means are indicated in the graphs.

DISCUSSION

In this study we showed that γA and γ′ fibrin matrices have different effects on the *in vitro* tube formation, wherein the γA fibrin matrix shows the highest angiogenic potential. No clear differences in functional endothelial cell characteristics on γA and γ′ fibrinogen coatings were seen in the *in vitro* endothelial cell adhesion, proliferation and migration assays. In the full-thickness wound healing model with these fibrinogen forms, the data suggest that γA fibrin has a slightly better wound-healing promoting property. Although only minor effects were found on the wound closure rate, the vascularization at day 21 and restoration of biomechanical strength. Furthermore, we observed different structures for γA and γ′ fibrin matrices, which could be partly explained by FXIII that was co-purified in the γ′ fraction, since supplementing FXIII in the γA fraction abolished the difference in fibrin diameter.

The *in vitro* endothelial cell adhesion, proliferation and migration showed no significant differences between γA and γ′ fibrinogen coatings. Coating of fibrinogen to culture plastic has previously been shown by Filova et al.[26] Furthermore, we showed an altered migration and proliferation on HMW and LMW-fibrinogen coatings,[21] which was consistent with the *in vitro* endothelial cell tube formation.[27] Unlike the tube formation in γA and γ′ fibrin, no significant differences in adhesion, migration and proliferation responses of HUVEC on γA or γ′ fibrinogen coatings were found. Endothelial cells are known to interact with various sites on the C-terminus of the α-chain (such as α95-97 and α572-574),[28] but the interactions with the C-terminus of γA or γ′ fibrinogen were not shown to play a significant role in endothelial cell adhesion.[29]

The *in vitro* tube formation showed more tube-like structures and higher fibrinolysis of HMVEC in γA fibrin, than that of γ′ fibrin. γ′ fibrin facilitated controlled tubular structure formation, whereas γA fibrin showed massive fibrinolysis and sprout formation. The increased fibrinolysis rate was confirmed with higher levels of fibrin degradation products in the conditioned media of γA fibrin matrices and is in concordance with previous studies.[30, 31] Differences in fiber thickness did not explain the effects on angiogenesis and fibrinolysis, since the results of confocal microscopy showed that the fiber thickness was similar in γA and γ′ fibrin matrices with equal FXIII levels. We did observe that γA fibrin matrices were more opaque than γ′ fibrin matrices, and since this can not be explained by the different fiber thickness, this may be the result of different clot structure that we observed for γ′ fibrin. A similar observation on clot structure was reported by Gersh et al., who also reported a higher number of branch points and free fiber ends in γA fibrin matrices.[32]

We observed thinner fibrin fibers in γ′ than in γA fibrin matrices, this has also been previously reported.[11, 12] Our data suggest that the difference in fiber thickness may be the result of differences in FXIII concentration. We showed that γA fibrinogen contains very little FXIII, while γ′ fibrinogen contains a considerable amount of FXIII. Therefore, we supplemented γA fibrinogen with FXIII to obtain similar FXIII concentrations during our *in vitro* experiments. We then observed that γA fibrin matrices have similar fiber thickness as γ′ fibrin matrices. In line with Collet et al. no significant differences were observed when FXIII was supplemented to γA fibrin.[31]

The *in vivo* experiments using the full-thickness wound healing model showed no clear differences between γA and γ′ fibrin matrices on day 21 after surgery. Several methodological reasons may be underlying this discrepancy; The vascularization parameters were determined on day 14 (LDPM) or day 21 (LDPM, immunohistochemistry), this may not be the optimal time point. Much of the vascularization may already taken place and earlier time points may show better distinctions. On the other hand, the breaking strength increases during the process and continues for months, hereof later time points should be used. Furthermore, we used healthy rats that showed a very rapid wound healing. Fibrinogen did not show benefits over controls in these wounds, an explanation might be that the wound healing process is already optimal in healthy rats and improvement cannot be made with fibrin matrices. Choosing a model in which wound healing is disturbed, such as the diabetic db/db mouse,[33] may show an improvement for application of fibrinogen and its specific forms.

In conclusion, this study shows that matrices of γA and γ′ fibrin have different structural and functional characteristics, and alter the *in vitro* tube formation and fibrinolysis of endothelial cells. In addition, the *in vivo* experiments suggest that γA fibrin matrices may be beneficial, but more data are needed before any firm conclusion can be drawn.

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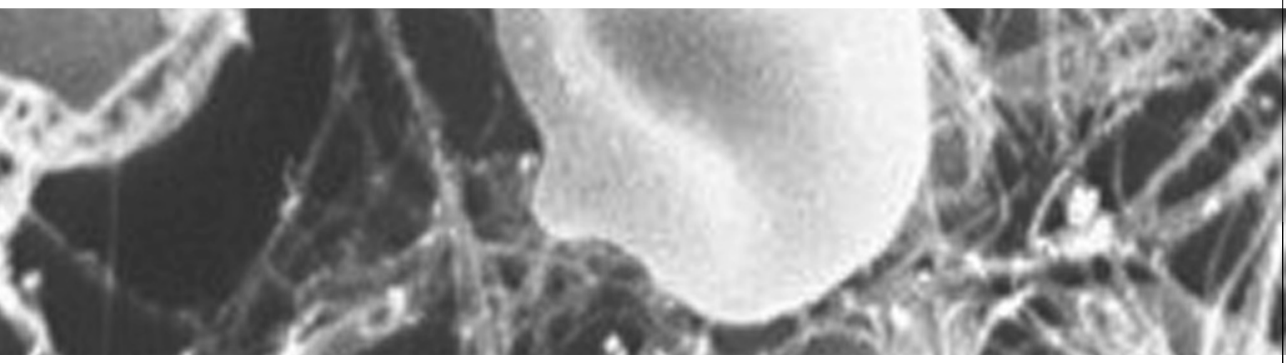

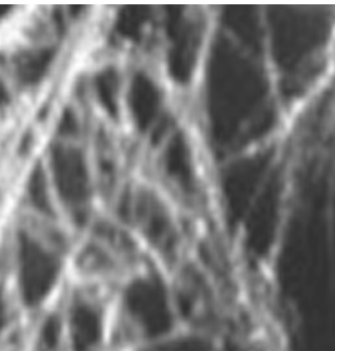

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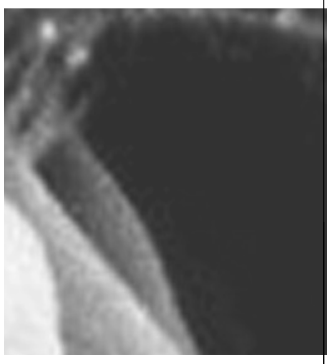

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Strongly increased levels of fibrinogen elastase degradation products in patients with ischemic stroke

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ABSTRACT

Ischemic stroke is associated with leucocyte activation. Activated leucocytes release elastase, an enzyme that can degrade fibrinogen. Fibrinogen elastase degradation products (FgEDP) may serve as a specific marker of elastase proteolytic activity. In a case-control study of 111 ischemic stroke patients and 119 controls, significantly higher FgEDP levels were observed in cases than in controls, both in the acute phase and in the convalescent phase. Results were only slightly affected by adjustment for cardiovascular risk factors, C-reactive protein and fibrinogen. Our findings suggest that FgEDP might be involved in the pathogenesis of stroke.

INTRODUCTION

The accumulation and activation of leucocytes have been demonstrated in ischemic stroke.[1] Activated leucocytes, particularly polymorphonuclear leucocytes (PMNs), release several proteolytic enzymes including elastase, which can degrade components of the extracellular matrix and plasma proteins.[2-3] Previously, an association has been found between levels of leucocyte elastase and the risk of cardiovascular events and ischemic stroke.[4-5] However, elastase levels may not accurately reflect the proteolytic activity of elastase *in vivo*, as proteolysis is dependent on protease levels as well as the concentration and activity of protease inhibitors.[6-7] Until recently, specific biochemical markers for the proteolytic activity of elastase were lacking. One of the proteins that can be degraded by elastase is fibrinogen, resulting in fibrinogen elastase degradation products (FgEDP). An assay has recently been developed, based on a monoclonal antibody with a high affinity and specificity for elastase-degraded fibrinogen, which enables the assessment of elastase proteolytic activity by measuring FgEDP levels.[7]

PATIENTS AND METHODS

We performed a case-control study that consecutively recruited patients (n = 124) with a first-ever ischemic stroke who were admitted to the department of Neurology of the Erasmus Medical Centre, Rotterdam.[8] Age- and sex-matched population controls (n = 125) were partners, friends or neighbours of the patients, without a history of stroke. Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. A brain computerized tomography (CT) scan was made in all patients within 3 days after symptom onset to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke. All patients underwent a standard twelve lead electrocardiogram and further cardiological work-up if indicated, and duplex ultrasound or angiography of the carotid or vertebral arteries. Stroke severity was assessed with the National Institutes of Health Stroke Scale (NIHSS). Aetiological stroke type was classified according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria. Patients with a definite non-atherosclerotic cause for stroke, such as a mechanical heart valve, endocarditis, or dissection, as well as persons >75 years or using oral anticoagulants were excluded. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam approved the study and written informed consent was obtained from all participants.

In both patients and controls, we collected detailed information about medical history and cardiovascular risk factors and fasting venous blood samples were drawn under strictly standardized conditions.[8] Venepuncture in patients was performed between 7 and 14 days after the stroke. In a subgroup of 64 patients, a second blood sample was obtained under the same conditions when they visited the outpatient department in the convalescence phase, 3 months after the stroke. Blood was collected into citrate (0.105 mol/l) with use of the Vacutainer (Becton-Dickinson, Plymouth, UK) system. Blood was centrifuged (2000 ***g*** for 30 min at 4°C) and plasma was stored in aliquots at −80°C until use. C-reactive protein (CRP) and fibrinogen levels were measured according to a previously described protocol.[8]

Plasma levels of FgEDP were measured by enzyme-linked immunosorbent assay using a monoclonal antibody against FgEDP (1-1/B3) as catching antibody and a monoclonal antibody directed to the D-region of fibrinogen as tagging antibody. The test had a within-run coefficient of variation of <5% and between-run coefficient of variation of <20%.[7] FgEDP levels were expressed as nanograms of fibrinogen equivalents (FE) per ml, identifying the proportion of fibrinogen that is degraded by elastase directly. Plasma for measuring FgEDP levels was available for 111 patients in the acute phase and 48 patients in the convalescence phase and for 119 control persons.

Student's *t*-test was used to compare levels of FgEDP between cases and controls and between patients with mild ischemic stroke (NIHSS score <3) and those with more severe stroke (NIHSS score ≥3, with 3 being the median NIHSS score in our patient group). The association between FgEDP levels and the occurrence of ischemic stroke was analysed through multivariate logistic regression, with FgEDP concentration categorized into quartiles of the distribution in the control group. The lowest quartile was set as the reference category. Analyses were adjusted for smoking, hypertension, diabetes mellitus, hypercholesterolaemia, a positive family history of cardiovascular diseases and levels of CRP and fibrinogen. All analyses were performed using spss software, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Smoking, hypertension, and diabetes were more frequent among cases, and ischemic stroke patients had higher levels of CRP and fibrinogen than healthy controls (Table 1). FgEDP levels in the acute phase were significantly higher in cases [mean ± standard deviation (SD), 251 ± 121 ng FE/ml] compared with control persons (169 ± 41 ng FE/ml; $P < 0.001$). Levels of FgEDP seemed to be slightly higher in patients with more severe stroke (259 ± 109 ng FE/ml) as compared with those with mild stroke (235 ± 141 ng FE/ml), although the difference was not statistically significant ($P = 0.33$).

Individuals in the highest quartile of FgEDP had a significantly higher risk of ischemic stroke compared with those in the lowest quartile (odds ratio [OR], 9.6; 95% confidence interval [CI], 3.9–23.2, Table 1). The association was only slightly affected by adjustment for cardiovascular risk factors and levels of CRP and fibrinogen (OR, 7.5; 95% CI, 2.2–25.0). In the samples collected 3 months after the event, FgEDP levels were lower than in the acute phase (mean ± SD, 203 ± 58 ng FE/ml; $P = 0.004$), but still significantly higher than in the control group ($P < 0.001$). Patients for whom second FgEDP measurements were available were not significantly different from those without a second measurement with respect to baseline characteristics, including NIHSS score.

Table 1. Baseline characteristics of the study population

Characteristic	Cases	Controls	p-value
n	111	119	
Women	52 (47%)	56 (47%)	NS
Age (yr), mean (SD)	55 (±12)	56 (±12)	NS
Smoking	54 (49%)	34 (29%)	0.002
Hypertension	53 (48%)	22 (19%)	<0.001
Diabetes	18 (16%)	5 (4%)	0.002
Hypercholesterolemia	70 (63%)	80 (67%)	0.700
Family history of cardiovascular disease	66 (60%)	60 (50%)	0.120
CRP (mg/l), median (interquartile range)	2.06 (0.41-7.89)	1.42 (0.58-3.34)	0.008
Fibrinogen, (g/l) mean (SD)	3.7 (±1.1)	3.4 (±0.6)	0.012
NIHSS score, median (interquartile range)	3.0 (2.0-5.0)		

Values are numbers (percentages), unless stated otherwise. NS, not significant; SD, standard deviation; CRP, C-reactive protein; NIHSS, National Institutes of Health Stroke Scale.

Table 2. Association between FgEDP levels and risk of first ever ischemic stroke. Odds ratio (OR) with 95% confidence interval (CI) for quartiles of the distribution.

Quartiles	Q1	Q2	Q3	Q4
FgEDP (ng FE/ml)	< 138.7	138.7 - 163.2	163.2 - 189.8	>189.8
OR (95% CI)				
Model 1	1.0 (ref.)	1.3 (0.5 - 3.8)	1.57 (0.6 - 4.4)	9.6 (3.9 -23.2)
Model 2	1.0 (ref.)	1.1 (0.3 - 3.8)	0.7 (0.2 - 2.3)	7.5 (2.2 -25.0)

Model 1: unadjusted model, Model 2: adjusted for smoking, hypertension, diabetes, hypercholesterolaemia, family history of cardiovascular diseases, C-reactive protein and fibrinogen.
OR, odds ratio; CI, confidence interval; FgEDP, fibrinogen elastase degradation products.

DISCUSSION

To our knowledge, this is the first study to evaluate the relationship of FgEDP with ischemic stroke. Among the strengths of this study are the consecutive inclusion of cases, brain CT-imaging in all patients, the use of population-based controls and the detailed collection of data on potential confounders. Limitations are the relatively small number of participants and the fact that we cannot reach a conclusion regarding the causality of FgEDP in stroke pathogenesis because of the case-control design of the study. It is possible that the elevated levels of FgEDP in stroke patients were due to an acute phase reaction. Yet, adjustment for the acute phase proteins CRP and fibrinogen did not affect the risk estimates. In addition, FgEDP levels in patients 3 months after the event, although lower than in the acute phase, were still significantly higher than those in controls. Furthermore, data on leucocyte counts might have provided more insight in the mechanism to explain the association between FgEDP levels and ischemic stroke, but unfortunately these were not available in our study.

We used an assay that has been proven to be very specific for FgEDP. The monoclonal antibody (1-1/B3) has a high affinity for elastase-degraded fibrinogen, while no reactivity was found with plasma fibrinogen or with fibrinogen degradation products generated by plasmin or other active proteases, such as metalloproteases or cathepsins (unpublished results). FgEDP levels may thus be considered a good marker of PMN-derived elastase activity.

The exact significance of the finding that elastase activity is increased in patients with ischemic stroke is, as yet, unclear. Previous studies have shown a relationship between leucocyte elastase levels and the risk of cardiovascular events and stroke.[4-5] Elastase levels are generally regarded as a marker of leucocyte activation.[2, 4] Thus far, it is not

known whether the association between elastase levels and stroke indicates a causal role of elastase in stroke pathogenesis or just reflects the involvement of leucocyte activation. Accumulation and activation of leucocytes, particularly PMNs, were shown to occur in cerebral ischemia.[1] Activated PMNs accumulate on platelet thrombi and incorporate into the fibrin clot and may thus play a role in activation of the coagulation cascade.[2-3, 9] Elastase released by activated PMNs might also directly contribute to stroke pathogenesis because of its pro-atherogenic properties[5] or through activation of coagulation pathways.[3-4] Alternatively, leucocyte activation and increased elastase activity might only reflect an inflammatory reaction, and not be causally related to stroke risk. An association between inflammatory markers and the risk of stroke has repeatedly been observed, although it remains controversial whether inflammation actually contributes to the aetiology of ischemic stroke or rather occurs in response to atherosclerosis or ischemia.[10] A final explanation for our findings could be a direct effect of FgEDP on the occurrence of ischemic stroke. Evidence suggests that split products of fibrinogen resulting from degradation by elastase might be able to influence coagulation activity by prolonging the thrombin-induced generation of fibrin.[11-12] Whether elastase activity or increased FgEDP levels in itself play a causal role in stroke pathogenesis or are only markers of PMN activation or inflammation needs clarification in further studies.

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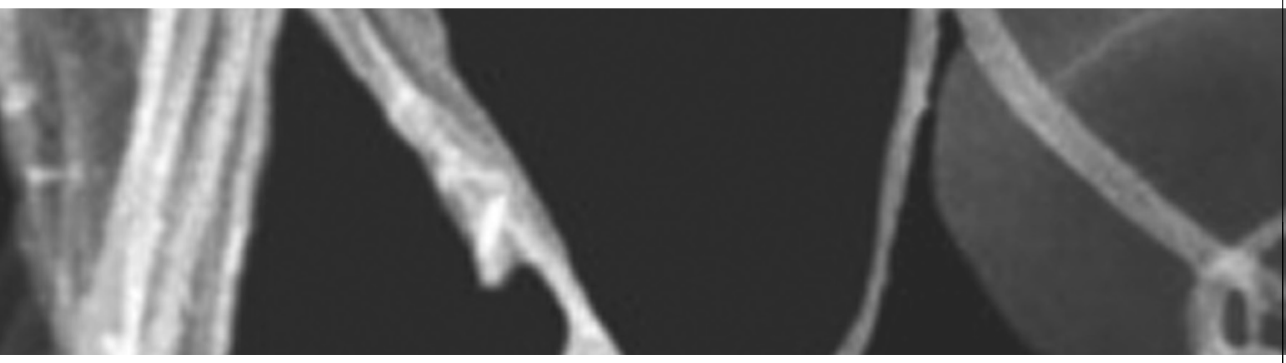

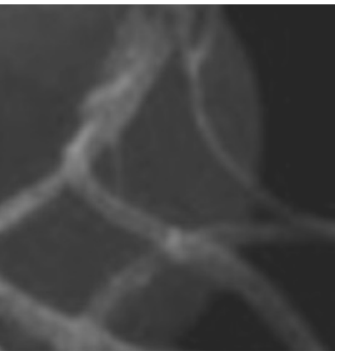

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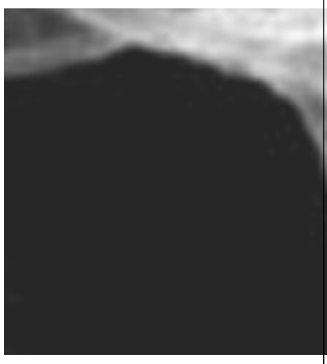

General discussion

In this thesis, the main aim was to study the role of genetic variants and protein variants of fibrinogen in cardiovascular diseases (CVD) and in wound healing. In addition, the relationship between fibrinogen elastase degradation products (FgEDP) and cardiovascular disease was studied. In this chapter, the overall interpretations and implications of the results will be discussed. Furthermore, we explore the perspectives for future research.

FIBRINOGEN GENE VARIATIONS IN CARDIOVASCULAR DISEASES

The investigations that were performed in the Rotterdam study, a population-based cohort study, provide evidence for an association of common variations in the FGG and FGA genes with cerebral small vessel disease (Chapter 2) and ischemic stroke (Chapter 4 and Chapter 5). However, no association was found between haplotypes and dementia or Alzheimer's disease (Chapter 3) (Table 1).

In both the Rotterdam Scan study (Chapter 2) and the Rotterdam study (Chapter 4), no relationship was observed between individual SNPs and risk of cerebrovascular disease, but a relationship was observed when the SNPs were combined and haplotypes were constructed. This finding suggests that haplotype analysis is a more powerful analysis because information of multiple markers is then used simultaneously.[1-2]

Surprisingly, the carriers of the FGG-H3 haplotype appear to be protected against ischemic stroke in the COCOS study (Chapter 5). In contrast, in the Rotterdam study (Chapter 4), even though the same arterial thrombotic endpoint has been used, no significant interaction was found between this haplotype and ischemic stroke. The reason for this discrepancy may be a difference in the study design, since the COCOS study is a case-control study and the Rotterdam study is a population-based cohort study. Another explanation could be the relative small population size of COCOS study that therefore has low statistical power, which increases the chance of false positive and false negative results. The SNP that is tagging FGG-H3 and not associated with stroke in the Rotterdam study, is also part of the FGG+FGA-H3 haplotype, which was associated with an increased risk of ischemic stroke (Chapter 4). This contradictory result may be explained by the effect of two other tagging SNPs that are also included in the FGG+FGA-H3.

No association between common variations in the *FGG* and *FGA* genes and risk of dementia or risk of white matter lesions could be found. Based on this finding, we propose that these genes do not modulate the risk of these diseases. Nevertheless, we cannot exclude that we have missed small haplotype effects because we used a minor allele frequency cutoff value of 1% (the Rotterdam Scan study and the Rotterdam study of

dementia) or 5% (studies of stroke). However, if we use a threshold of 1% for the minor allele frequency, the groups were very small and therefore the statistical power in the study was too low for analysis of these haplotypes.

The relationship between haplotype-tagging polymorphisms in the total fibrinogen gene cluster and fibrinogen plasma levels and the risk of cardiovascular diseases was reported.[3-5] Yet, no association between fibrinogen levels and haplotypes of the *FGG*, *FGA* plus *FGB* genes was observed in our studies. The current hypothesis is that fibrinogen levels are most strongly related to variants in the *FGB* gene since synthesis of the fibrinogen β gene is the rate limited step of fibrinogen synthesis.[6-8] However, we cannot confirm this finding. The single SNPs in the *FGB* gene explains only 1% of the total variance in plasma fibrinogen levels,[5] the remaining variations in fibrinogen levels are explained by the effects of variants in other genes and environmental factors. Our results are comparable with previous reports, which indicate that fibrinogen levels may not play a major causal role in CVD.[3,9] It remains a possibility that the effect of genetics on CVD may be through alternative pathways that are independent of fibrinogen levels. The structure of the fibrin clot is one of these possible fibrinogen-related mechanisms by which the pathogenesis of thrombosis and vascular disease can be influenced.[10-11]

Table 1. Comparative analysis of association between fibrinogen haplotypes and risk of diseases

FGG + FGA	Chapter 2		Chapter 3			Chapter 4		
Haplotype	Dementia	Alzheimer's disease	Silent brain infarcts	Periventricular white matter lesions	Subcortical white matter lesions	Stroke	Ischemic stroke	Hemorrhagic stroke
H1 (GGT-GGTA)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
H2 (GAT-AGTG)	0.91	0.86	1.41*	1.29	0.9	1.12	1.2	0.98
H3 (GGC-GATA)	0.89	0.83	1.33	1.40*	0.87	1.02	1.36*	0.71
H4 (GGT-GGCA)	0.89	0.93	1.39	1.25	0.95	1.02	1.18	1.25
H5 (GGC-AGTA)	0.89	0.84	1.3	1.08	0.76	0.94	1.14	0.9
H6 (AGTG-GTA)	1.02	1.11	0.43	0.99	0.66	N/A	N/A	N/A
H7 (GGT-AGTG)	1.13	0.89	1.03	2.08	0.98	N/A	N/A	N/A

Presented are odds ratio and 95% confidence intervals (OR, 95%CI)
N/A, data not available; * Significant different from the reference group

Recently, several genome-wide association (GWA) studies of fibrinogen levels and fibrinogen γ′ levels in relation to CVD were performed.[8,12-15] In GWA studies, numerous loci are scanned for unsuspected genetic determinants. These studies suggested that the fibrinogen levels are related with the inflammatory cascade.[8,14] There is one GWA study that investigated determinants of fibrinogen γ′, which reported that the γ′ levels are associated with SNPs nearby the fibrinogen gene.[15] Unfortunately, fibrinogen γ′ levels are not available in the Rotterdam Study, hence it is difficult to elucidate the underlying mechanism between the genetic variations and risk of CVD via γ levels. The GWA approach reveals that genetic variations that influence the levels and risk of CVD may provide us the novel biological insights in our understanding of diseases, but further replications or functional research of genetic variations is needed to verify these findings.

FIBRINOGEN γ′ LEVELS IN CARDIOVASCULAR DISEASES

The fibrinogen levels were significantly higher in patients with ischemic stroke (IS, Chapter 5, 6, 7), acute pulmonary embolism (PE, Chapter 6), refractory unstable angina pectoris (UAP, Chapter 6) and intracerebral hemorrhage (ICH, Chapter 8) compared to those in healthy controls. The fibrinogen γ′ levels and fibrinogen γ′/total fibrinogen ratios (γ′ ratios) were also elevated in patients with various cardiovascular diseases (IS, PE, UAP or ICH) compared with those in healthy controls (Table 2).

Fibrinogen γ′ is one of the natural occurring isoforms of the fibrinogen molecule, and it may be expected that during an acute phase reaction, all isoforms of fibrinogen increase in parallel. If this assumption is correct, an increase in the total amount of fibrinogen molecules will give a parallel increase in the number of fibrinogen γ′ molecules and the γ′/total fibrinogen ratio will remain unchanged. We were interested to see whether this parallel increase occurred or not. Interestingly, fibrinogen γ′ levels and ratios were significantly decreased in the IS patients during the convalescent phase (Chapter 5) and significantly increased in the stabilized UAP patients compared to healthy controls (Chapter 6), while the plasma fibrinogen levels were similar among these patients and controls. This finding indicates that the alternative mRNA processing of fibrinogen γ′ is at least partly independent of fibrinogen levels, which is in accordance with results from a previous report.[16] Furthermore, levels of fibrinogen γ′ were still significantly increased in IS patients after adjustment for cardiovascular risk factors, although the γ′ ratio was comparable in patients and controls (Chapter 7). This result is in agreement with an earlier study that the fibrinogen γ′ levels, but not the γ′ ratios, were significantly higher in patients with myocardial infarction than those in controls.[17] In addition, the absolute γ′ level in IS

patients had a stronger association with outcome than the γ′/total fibrinogen ratio, which suggest that the absolute level of fibrinogen γ′ may be more important than the γ′/total fibrinogen ratio (Chapter 7).

Table 2. Summative results of fibrinogen levels, fibrinogen γ′ levels, and fibrinogen γ′/fibrinogen ratios in various studies described in this thesis

Chapter	Group	Subgroup	N	Total fibrinogen (g/L)	Fibrinogen γ′ (g/L)	γ′ /total fibrinogen ratio
Chapter 5	Controls		119	3.41 (0.61)	0.34 (0.10)	0.10 (0.03)
	Ischemic stroke patients	Acute Phase	114	3.69 (1.06)	0.42 (0.17)	0.11 (0.03)
		Convalescent Phase	47	3.34 (0.76)	0.29 (0.09)	0.09 (0.02)
Chapter 6	Controls		173	3.30 (0.59)	0.33 (0.10)	0.10 (0.03)
	Patients	Ischemic stroke	53	3.87 (1.19)	0.49 (0.18)	0.13 (0.02)
		Pulmonary embolism				
		Non-acute phase	13	2.69 (1.42)	0.53 (0.27)	0.20 (0.06)
		Acute phase	16	4.39 (2.30)	0.79 (0.33)	0.24 (0.19)
		Unstable angina pectoris				
		Stabilized	130	3.36 (0.76)	0.46 (0.15)	0.14 (0.04)
		Refractory	72	3.82 (1.00)	0.53 (0.23)	0.14 (0.04)
Chapter 7	Controls		156	3.37 (0.72)	0.32 (0.13)	0.10 (0.03)
	Ischemic stroke patients	All	200	3.69 (0.96)	0.37 (0.15)	0.10 (0.04)
		0–7 days	100	3.76 (1.11)	0.40 (0.17)	0.11 (0.04)
		≥8 days	100	3.62 (0.78)	0.34 (0.12)	0.10 (0.03)
		Favourable outcome	173	3.64 (0.89)	0.36 (0.14)	0.10 (0.03)
		Unfavourable outcome	27	3.97 (1.34)	0.47 (0.18)	0.13 (0.06)
Chapter 8	Controls		156	3.37 (0.72)	0.32 (0.13)	0.10 (0.03)
	Intracerebral hemorrhage patients	All	55	4.41 (1.21)	0.40 (0.14)	0.09 (0.03)
		No IVE	47	4.41 (1.23)	0.38 (0.14)	0.09 (0.03)
		IVE present	8	4.40 (1.13)	0.51 (0.13)	0.12 (0.03)

IVE, intraventricular extension. Data presented are means (SD)

Compared to healthy controls, the fibrinogen γ′ levels and ratios were strongly increased in IS patients (Chapter 5, 6, 7) during the acute phase. They were comparable to those of controls in the subacute phase (≥8 days after the event, Chapter 7), and even significantly decreased in the convalescent phase (3 months after the event, Chapter 5). The increase of the levels during the acute phase is probably a consequence rather than a cause of the event since we included patients after the event occurred. The decrease in the convalescent phase may reflect the prestroke levels, which would suggest that the antithrombotic properties of fibrinogen γ′ are more important than its prothrombotic properties. One of the explanations for these findings could be that the alternative processing mechanism is affected by the acute phase of disease, since a relationship between acute-phase reactions and alternative mRNA processing have been suggested previously for other genes.[18-20] It is also possible that the clearance of fibrinogen γA and γ′ in the plasma is different during various stages of disease.[21]

Apart from the association between increased fibrinogen γ′ levels and unfavorable short-term outcome in the early phase after IS (Chapter 7), there is a nonsignificant trend toward unfavorable outcome in patients with ICH (Chapter 8). However, the fibrinogen γ′ levels or ratio do not predict short- or long-term outcome in patients with UAP (Chapter 6). The discrepancy between these results may be explained by differences in etiology and pathogenesis of these diseases. It may also partly be due to medication.

FIBRINOGEN γ′ IN WOUND HEALING

Fibrin plays an important role in haemostasis, but it also provides a temporary, degradable matrix for tissue repair. In Chapter 9, we investigated the effects of fibrinogen γ variants on the in vitro endothelial cell characteristics and in vivo wound healing.

Angiogenesis, one of the important processes of wound healing, depends highly on fibrin structure and the interactions between fibrin and cellular receptors. We evaluated angiogenesis by the *in vitro* tube formation, and say that this was more pronounced in γA than in γ′ fibrin matrices, and thus this was accompanied with an increased endothelial cell-mediated fibrinolysis. This result suggests that the γA fibrin matrix may play an important role in allowing the endothelial cell to penetrate into the three-dimensional matrix while structural stability of the supporting network is not damaged. Furthermore, the endothelial cell-mediated fibrinolytic activity was inhibited during the angiogenesis in the γ′ fibrin matrix, which was in line with previous studies that the γ′ fibrin clot were mechanically stiffer than the γA fibrin clot and resistant to fibrinolysis.[22-23] Yet, the clot structure of fibrinogen γ′ is still under debate.[24-26]

The dynamic balance between proangiogenic and antiangiogenic factors is the key determinant for angiogenesis. The special characteristics of fibrinogen γ′ include a high affinity binding site for thrombin and an extra binding site for the FXIII B subunit. Furthermore, thrombin and factor XIII (FXIII) are angiogenic factors that contribute to new vessel formation.[27-28] Therefore, it was surprising that the angiogenic effect in fibrin γ′ matrices is not as strong as in the γA fibrin matrix. We speculate that the thrombin binding site, which served as antithrombin I,[29-30] inhibits thrombin activity, as well as the loss of platelet integrin $\alpha_{IIb}\beta_3$ binding site are not in favor of angiogenic activity,[31] therefore the angiogenic effect of fibrinogen γ′ was decreased.

There were no significant differences between γA and γ′ fibrinogen coating when we studied the intrinsic endothelial cells (EC) functions that contribute to angiogenesis (adhesion, proliferation and migration). Since we examined the EC behavior on a two-dimensional (2D) coating while the *in vitro* tube formation was performed in the three-dimensional (3D) matrix, this may explain the discrepancy between EC behavior in functional assays and the angiogenesis assay. Moreover, in agreement with previous studies we demonstrated that the proliferation was strongly inhibited in human microvascular endothelial cells (HMVEC) but not in human umbilical vein endothelial cells (HUVEC),[32] which suggested that angiogenesis is mainly driven by HMVEC.[33] Therefore, no major effect was observed while we performed the adhesion, proliferation and migration assays in the 2D coating using HUVEC.

The *in vivo* full-thickness wound healing model suggests that γA promotes wound healing slightly, but not significantly, better than γ′ fibrinogen. The wound healing process is very rapid in healthy normal rats, and this may be the reason that no clear improvement could be made with fibrin matrices. The diabetic rodents with impaired repairing ability may be a good model for the wound healing study.[34] It may also have greater clinical relevance since most of wound healing disturbances are linked to diseases, such as diabetes and infection.

Furthermore, we examined the vascularization on day 14 and both vascularization and immunohistochemical structure on day 21. The nonsignificant difference may also simply result from the non-optimal time points, since the vascularization may already taken place, and the earlier time point could give better distinctions. For this reason, after the study described in Chapter 9, we conducted a pilot study with 12 rats using the same model, focusing on the first week. On day 7, the rats were sacrificed for the immunohistochemical examination. The result showed that the wound healing was improved with the fibrin and its variants applications, which was in good agreement with previous observations on the

improvement of wound healing by fibrin.[35-38] However, vascularization was comparable in both γA and γ′ fibrin, suggesting that γA and γ′ fibrin have a similar contribution in the wound healing processes during the first 7 days. On the other hand, we cannot exclude the possibility that day 7 is still a non-optimal time points concerning the rapid wound healing in the healthy rats. In summary, the increased *in vitro* tube formation and *in vivo* wound healing in γA fibrin showed the same trend, which implies a beneficial effect for scaffold vascularization during wound healing. Nevertheless, more research is warranted to confirm this finding.

FIBRINOGEN ELASTASE DEGRADATION PRODUCTS AND CARDIOVASCULAR DISEASES

In Chapter 10, the fibrinogen elastase degradation products (FgEDP) levels in patients with IS were evaluated using a newly developed assay, which enables the assessment of elastase proteolytic activity. In the COCOS study, FgEDP levels were significantly higher in cases than in controls, both in the acute phase and in the convalescent phase. The results indicate that the FgEDP levels are a good marker for stroke and independent of acute phase response, since we observed a significant association after adjustment for CRP levels and when we analyzed the association in the convalescent phase 3 months after the event.

Apart from being a predictor for stroke, elastase activity, as reflected by FgEDP levels, also appears to be involved in stroke pathogenesis, just like other inflammatory markers.[39-40] An association has been established between levels of leukocytes and the risk of cardiovascular events and IS.[41] Additionally, elastase might also directly contribute to stroke pathogenesis because of its pro-atherogenic properties[41] or through activation of coagulation pathways.[42-43] While the mechanism remains unknown, more research is needed to fully elucidate the causal role of elastase in stroke.

FUTURE PERSPECTIVES

Nowadays, one of the most important challenges facing the biomedical research is the gain the basic mechanistic knowledge, and translates this information into effective clinical therapeutics. However, with respect to genetic contributions and diseases, the results are often not straightforward and numerous research questions still remain unanswered.

Large scale prospective studies are required to explore the causal role of fibrinogen γ′ or elastase in thrombotic diseases. These studies will need baseline values and several

time points before and after the thrombotic event. Only using this approach, and then it can be determined at what time point the fibrinogen γ′ levels or FgEDP levels are elevated and how they contribute to CVD.

GWA studies are not only a good approach to unveil the genetic variations that influence the fibrinogen or fibrinogen γ′ levels and risk of CVD, [8,12-15] it also provides a step towards building predictive models of disease processes and the selection of new biomarkers and potential drug targets. Eventually, the ultimate goal of personalized medication (pharmacogenetics) can be achieved. Hence the understanding of genetic profiles is of great interest. Once a strong association has been established between genetic variations and CVD, high risk persons can be identified and preventive treatment could be started.

In addition to studies on variations in the fibrinogen genes, we attempted to explore the potential clinical applications of fibrin matrices. In this thesis, the effects of fibrinogen γ variants on the *in vitro* endothelial cell characteristics and *in vivo* wound healing were described. However, the wound healing process is dynamic and complex, and more knowledge is needed on the structure of γA and γ′ fibrin matrices, especially regarding the interaction with various blood cells, platelets and growth factors. Another challenge is to fully understand the interplay between EMC, cellular responses and intracellular signaling during wound healing. This knowledge might encourage the future development of the best fibrin composition to achieve haemostasis and accelerate wound healing.

In conclusion, the results described in this thesis may improve the understanding of the important role of fibrinogen variants in CVD and wound healing. The accumulation of insights in fibrinogen studies can overcome medical challenges, which can develop into individualized therapeutic interventions to manage the CVD and facilitate the development of regenerative fibrin matrices for tissue engineering. Expectantly, these new treatment can provide great benefit to the patients in the future.

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SUMMARY

Fibrinogen is a plasma glycoprotein that plays a key role in the haemostatic system. Its plasma levels are a well-known independent risk predictor for cardiovascular diseases (CVD). Plasma fibrinogen levels are modulated by both genetic and environmental factors. In order to fulfill its multiple functions, a large number of fibrinogen variants are present in the plasma of healthy individuals. The heterogeneity in fibrinogen influences the plasma fibrinogen concentration and the clot structure, and therefore it may also affect the risk of cardiovascular events and the process of wound healing. In this thesis, we investigated the role of fibrinogen variants and fibrinogen elastase degradation products (FgEDP) in CVD, and the role of the fibrinogen γA and γ′ variants in wound healing.

Recently, the relationship between haplotype-tagging polymorphisms in the total fibrinogen gene cluster and fibrinogen plasma levels and the risk of cardiovascular diseases was reported. We hypothesized that common genetic variation in the *FGG* and *FGA* genes play a role in regulating fibrin clot structure and may therefore be involved in the pathogenesis of vascular diseases and cerebrovascular events. We tested this hypothesis in **Chapters 2-4.** In the studies described in these chapters the plasma fibrinogen levels were measured, and in addition seven haplotype-tagging single nucleotide polymorphisms (SNPs) that cover the total common variations in the *FGG* and *FGA* genes were determined and seven haplotypes (frequency >0.01) were constructed.

In **Chapter 2**, we investigated the association between plasma fibrinogen levels and the presence of cerebral small vessel disease (SVD), as determined with magnetic resonance imaging (MRI) of the brain. In addition, the relationship between common variations in *FGG*, *FGA* and *FGB* genes and SVD was examined. We performed the study as part of the Rotterdam Scan Study, a population based imaging study in 1077 elderly patients undergoing cerebral MRI. Haplotype 2 was associated with the presence of silent brain infarcts compared with the most frequent haplotype (OR 1.41, 95% CI 1.03 to 1.94). Haplotype 3 was associated with periventricular white matter lesions in the highest tertile of the distribution (OR 1.40, 95% CI 1.01 to 1.92). Our study provides evidence for an association of common variation in the *FGG* and *FGA* genes with cerebral SVD. It is therefore possible that the structure of the fibrin clot rather than plasma fibrinogen levels plays a role in the pathogenesis of cerebral SVD.

In **Chapter 3**, we examined the association between variation in *FGG* and *FGA* genes and dementia within the Rotterdam Study, a prospective population-based study. This study included 7,983 persons aged 55 years and over, and is ongoing since 1990.

In this study, 743 dementia patients were identified. No association was found between haplotypes and dementia or Alzheimer's disease, suggesting that common variations in the *FGG* and *FGA* genes are not important risk factors for dementia.

Furthermore, we investigated the relationship between common variations in *FGG* and *FGA* genes with ischemic stroke (IS) and hemorrhagic stroke in **Chapter 4**. The study included 6,275 participants from the Rotterdam Study, who were free from stroke, and had successful assessment of at least one SNP. We observed 668 strokes, of which 393 were ischemic and 62 hemorrhagic, during a median follow-up time of 10.1 years. *FGG+FGA* haplotype 3 (H3) was associated with an increased risk of IS (OR 1.36, 95% CI 1.09–1.69) and the risk estimate for hemorrhagic stroke was 0.71 (95% CI 0.46–1.09) compared to the most frequent H1. The results suggested that opposite associations for H3 may exist for ischemic and hemorrhagic stroke.

Recent studies have reported associations between the plasma fibrinogen γ′ levels, the fibrinogen γ′/total fibrinogen ratio and deep venous thrombosis, myocardial infarction and coronary artery disease. These results indicate that *FGG* gene variations, which are a main determinant of the plasma fibrinogen γ′ levels, may contribute to the pathology of thrombotic disease and influence the risk of CVD. In **Chapter 5**, we conducted the COCOS study, a case-control study including 124 IS patients and 125 population controls. The fibrinogen γ′/total fibrinogen ratio is associated with IS, especially in the acute phase of the disease. In addition, the FGG-H3 haplotype appears to be protective against IS (OR 0.60; 95% CI, 0.38 to 0.94).

In **Chapter 6**, we tested this hypothesis that the acute phase reaction alters the mRNA processing of fibrinogen γ′, thereby increasing the γ′/total fibrinogen ratio in patients with IS, pulmonary embolism (PE) and unstable angina pectoris (UAP). The fibrinogen γ′/total fibrinogen ratios are elevated in patients with various cardiovascular diseases (IS, PE, or UAP) compared with the γ′ ratios in healthy controls. An altered mRNA processing of fibrinogen γ′ during the acute phase may contribute to this observation. However, the fibrinogen γ′/total fibrinogen ratio does not predict short- or long-term outcome in patients with UAP.

Subsequently, we compared the differences in fibrinogen γ′ levels and γ′/total fibrinogen ratio between 200 patients with IS (**Chapter 7**), 55 patients with intracerebral hemorrhage (ICH, **Chapter 8**) and 156 population-based controls from the Erasmus Stroke Study. Our study shows that fibrinogen γ′ levels were significantly higher in patients with IS than in controls ($p<0.001$), and similar results were observed in patients with ICH. Furthermore, increased fibrinogen γ′/total fibrinogen ratios are associated with unfavorable short-term

outcome in the early phase after IS, and a trend toward unfavorable outcome in patients with ICH.

We hypothesized that the structural and functional differences between γA and γ′ fibrin are associated with an altered matrix structure, and that may influence angiogenesis and wound healing. In **Chapter 9,** we investigated the effects of fibrinogen γ variants on the *in vitro* endothelial cell characteristics and *in vivo* wound healing. The *in vitro* tube formation was more pronounced in γA than in γ′ fibrin matrices and this was accompanied with an increased fibrinolysis. The *in vivo* wound healing model suggested that γA fibrin was promoting wound healing slightly better than γ′ fibrin. However, more research is warranted before conclusions can be drawn.

Previously, an association has been found between levels of leucocyte elastase and the risk of cardiovascular events and IS. In **Chapter 10**, the FgEDP levels in patients with IS was evaluated using a newly developed assay, which enables the assessment of elastase proteolytic activity. In a case-control study, significantly higher FgEDP levels were observed in cases than in controls, both in the acute phase and in the convalescent phase. Our findings suggest that FgEDP might be involved in the pathogenesis of stroke.

In **Chapter 11**, the results of our studies are discussed regarding the current fibrinogen research. Further investigation is needed for understand the mechanism, which can develop therapeutic interventions to manage the CVD and promote wound healing in the future.

NEDERLANDSE SAMENVATTING

Fibrinogeen is een plasma glycoproteine dat een sleutelrol speelt in het hemostase systeem. De plasma spiegels zijn een bekende, onafhankelijke voorspeller van risico op hart- en vaatziekten (HVZ). Plasma fibrinogeen spiegels worden gereguleerd door zowel genetische als omgevingsfactoren. Om de grote hoeveelheid aan functies te kunnen vervullen, is er een groot aantal fibrinogeen varianten aanwezig in het plasma van gezonde personen. De heterogeniteit van fibrinogeen beïnvloedt de plasma fibrinogeen concentratie en de stolselstructuur, en kan daardoor een effect hebben op het risico van HVZ en het wondgenezingsproces. In dit proefschrift hebben we rol van fibrinogeen varianten en fibrinogeen elastase degradatie producten (FgEDP) in HVZ onderzocht, en de rol van fibrinogeen γA en γ′ varianten in wondgenezing.

Recent is de relatie tussen haplotype bepalende polymorfismen in het totale fibrinogeen cluster met fibrinogeen plasma spiegels en met het risico op HVZ gerapporteerd. Onze hypothese is dat veelvoorkomende genetische variatie in de *FGG* en *FGA* genen een rol speelt bij de regulatie van de fibrine stolselstructuur and daardoor betrokken kan zijn bij de pathogenese van HVZ. Wij hebben deze hypothese getest in **Hoofdstukken 2-4.** In de studies die in deze hoofdstukken worden beschreven zijn de spiegels van fibrinogeen in plasma gemeten, en daarnaast zijn zeven haplotype-bepalende single nucleotide polymorphismen (SNPs) bepaald die de totale veelvoorkomende variatie in de *FGG* en *FGA* genen beschrijven, en zijn zeven haplotypes (frequentie >0.01) samengesteld.

In **Hoofdstuk 2** hebben we de relatie tussen plasma fibrinogeen spiegels en de aanwezigheid van ziekte van de kleine vaten van de hersenen (SVD), zoals bepaald met magnetische resonantie beeldvorming (MRI) van het brein. Daarnaast is de relatie tussen veelvoorkomende varianten in de *FGG, FGA* en *FGB* genen met SVD bestudeerd. We hebben een studie uitgevoerd als onderdeel van de Rotterdam Scan Studie, een populatie imaging studie in 1077 oudere patiënten die een MRI van de hersenen ondergingen. Haplotype 2 was geassocieerd met een verhoogd voorkomen van stille herseninfarcten, vergeleken met het meest voorkomende haplotype 1 (OR 1.41, 95% CI 1.03 to 1.94). Haplotype 3 was geassocieerd met periventriculaire witte stof laesies in het hoogste tertiel van de verdeling (OR 1.40, 95% CI 1.01 to 1.92). Onze studie levert bewijs voor een associatie tussen veelvoorkomende variatie in de *FGG* en *FGA* genen met cerebrale SVD. Het is daarom mogelijk dat de structuur van het fibrine netwerk, eerder dan de plasma fibrinogeen spiegel, een rol speelt bij de pathogenese van hersen SVD.

In **Hoofdstuk 3** hebben we de relatie tussen variatie in de *FGG* en *FGA* genen en dementie onderzocht binnen de Rotterdam Studie, een prospectieve populatie studie. In deze studie zijn sinds de start in 1990, 7983 personen geïncludeerd met een leeftijd van 55 jaar en ouder. In deze studie zijn 743 patiënten geïdentificeerd met dementie. Geen associatie werd gevonden tussen de haplotypes en dementie of Alzheimer, hetgeen suggereert dat veelvoorkomende varianten in de *FGG* en *FGA* genen geen belangrijke risicofactoren zijn voor dementie.

Daarnaast hebben we de relatie bestudeerd tussen veelvoorkomende varianten in de *FGG* en *FGA* genen met ischemische beroerte (IS) en hersenbloeding in **Hoofdstuk 4**. De studie vond plaats in 6275 deelnemers van de Rotterdam studie die geen beroerte hadden gehad en in wie we minstens 1 SNP konden bepalen. We zagen 668 beroertes, waarvan 393 ischemisch en 62 hemorrhagisch tijdens de mediaan follow-up van 10.1 jaren. Het *FGG+FGA* haplotype 3 (H3) was geassocieerd met een verhoogd risico op IS (OR 1.36, 95% CI 1.09–1.69) en het risico op een hersenbloeding was 0.71 (95% CI 0.46–1.09), vergeleken met het meest voorkomende H1. Deze resultaten suggereren tegenovergestelde associaties tussen H3 met ischemische en hemorrhagische beroerte.

Recente studies hebben associaties gerapporteerd tussen plasma fibrinogeen γ′ spiegels, de fibrinogeen γ′/total fibrinogeen ratio en diep veneuze trombose, hartinfarct en coronaire hartziekte. Deze resultaten suggereren dat *FGG* genvariaties, die de belangrijkste determinant zijn van plasma fibrinogeen γ′ spiegels, bij zouden kunnen dragen aan de pathologie van trombose en het risico op HVZ kunnen beïnvloeden. In **Hoofdstuk 5** beschrijven we de resultaten van onze substudie in de COCOS studie, een case controle studie met 124 IS patiënten en 125 populatie controles. De fibrinogeen γ′/totaal fibrinogeen ratio is geassocieerd met IS, vooral in de acute fase van de ziekte. Daarnaast blijkt het FGG-H3 haplotype beschermend te zijn tegen IS (OR 0.60; 95% CI, 0.38 tot 0.94).

In **Hoofstuk 6** hebben we de hypothese getest dat de acute fase reactie de mRNA processing van fibrinogeen γ′ verandert, en daarbij de fibrinogeen γ′ / total fibrinogeen ratio verhoogd in patiënten met IS, longembolie (PE) en onstabiele angina pectoris (UAP). De fibrinogeen γ′ totaal fibrinogeen ratio is verhoogd in patiënten met de verschillende HVZ (IS, PE en UAP) vergeleken met de γ′ ratios in gezonde controles. Een veranderde mRNA processing van fibrinogeen γ′ tijdens de acute fase zou kunnen bijdragen aan deze observatie. Daarentegen voorspelt de fibrinogeen γ′ /totaal fibrinogeen ratio niet de korte of lange termijn uitkomst in patiënten met UAP.

Vervolgens hebben we de verschillen in fibrinogeen γ′ spiegels en γ′ / totaal

fibrinogeen ratio vergeleken tussen 200 patiënten met IS (**Hoofdstuk 7**), 55 patiënten met hersenbloeding (ICH, **Hoofdstuk 8**) en 156 populatie controles uit de Erasmus Stroke Studie. Onze studie laat zien dat fibrinogeen γ′ spiegels significant hoger zijn in patiënten met IS dan in controles ($p<0.001$), en vergelijkbare resultaten werden gezien in patiënten met ICH. Verder waren verhoogde fibrinogeen γ′/totaal fibrinogeen ratio geassocieerd met ongunstige korte termijn uikomst in de vroege fase na IS, en er was een trend naar een ongunstige uitkomst in patiënten met ICH.

Wij hypothetiseerden dat de structurele en functionele verschillen tussen γA en γ′ fibrine geassocieerd zouden zijn met een veranderde matrix structuur, en dat dit angiogenese en wondherstel zou beïnvloeden. In **Hoofdstuk 9** hebben we de effecten van fibrinogeen γ varianten op de *in vitro* endotheelceleigenschappen en het *in vivo* wondherstel bestudeerd. The *in vitro* vaatvorming was sterker in γA dan in γ′ fibrine matrices en dit ging samen met een verhoogde fibrinolyse. Het *in vivo* wondgenezingsmodel suggereerde dat γA fibrine wondgenezing enigszins beter stimuleerde dan γ′ fibrine. Maar meer onderzoek is nodig voor conclusies kunnen worden getrokken.

Eerder is een associatie beschreven tussen de spiegels van leukocyten elastase en het risico op HVZ. In **Hoofdstuk 10** zijn de FgEDP spiegels in patiënten met IS gemeten met een nieuw ontwikkelde meetmethode voor het bepalen van de elastase proteolytische activiteit. In een case controle studie werden significant hogere FgEDP spiegels gezien in patiënten dan in controles, zowel in de acute fase als in de stabiele fase. Onze bevindingen suggereren dat FgEDP betrokken kan zijn bij de pathogenese van beroerte.

In **Hoofdstuk 11** worden de resultaten van onze studies bediscussieerd in relatie tot het huidige fibrinogeen onderzoek. Verdere onderzoeken zijn nodig om alle mechanismen te begrijpen, hetgeen kan leiden tot toekomstige therapeutische interventies bij HVZ en het stimuleren van wondgenezing.

ABBREVIATIONS

γ′	Fibrinogen gamma prime variant
αE	Fibrinogen extended Aα variant
ANOVA	Analysis of variance
CI	Confidence interval
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular diseases
DVT	Deep venous thrombosis
EC	Endothelial cells
ECG	Electrocardiogram
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EM	Expectation-maximization
ESS	Erasmus Stroke Study
FE	Fibrinogen equivalents
FGA	Fibrinogen alpha gene
FGB	Fibrinogen beta gene
FgEDP	Fibrinogen elastase degradation products
FGF-2	Fibroblast growth factor-2
FGG	Fibrinogen gamma gene
FITC	Fluorescein isothiocyanate isomer I
FpA	Fibrinopeptides A
FpB	Fibrinopeptides B
FXIII	Factor XIII
γ′ ratio	Fibrinogen γ′/total fibrinogen ratio
GP1bα	Platelet glycoprotein Ib-α
GWA	Genome-wide association
H	Haplotype
HDL	High-density lipoprotein
HMVEC	Human microvascular endothelial cells
HMW	High molecular weight fibrinogen
HSA	Human serum albumin
HSi	Heat-inactivated human serum
HUVEC	Human umbilical vein endothelial cells
I-CAM	Intercellular adhesion molecule

ICH	Intracerebral hemorrhage
IL-6	Interleukin 6
IS	Ischemic stroke
IVE	Intraventricular extension
kb	Kilobases
kDa	Kilo Dalton
LDPM	Laser Doppler perfusion measurements
LMW	Low molecular weight fibrinogen
MI	Myocardial infarction
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mRS	Modified Rankin Scale
MW	Molecular weight
NBCSi	Heat-inactivated newborn calf serum
NIHSS	National Institutes of Health Stroke Scale
OR	Odds ratio
PA	Plasminogen activators
PAI-1	Plasminogen activator inhibitor 1
PE	Pulmonary embolism
PMNs	Polymorphonuclear leucocytes
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERPINS	Serine protease inhibitors
SNPs	Single Nucleotide Polymorphisms
SVD	Small vessel disease
TAFI	Thrombin-activatable fibrinolysis inhibitor
TIA	Transient ischemic attack
TNFα	Tumor necrosis factor-a
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
t-PA	Tissue-type plasminogen activator
UAP	Unstable angina pectoris
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
VE	Vascular Endothelial
VEGF	Vascular endothelial growth factor
α2-AP	α2-antiplasmin

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CURRICULUM VITAE

Elim Cheung was born on 26th February 1977 in Hong Kong. In 2000, she graduated from the Hong Kong University of Science and Technology with a major in biology. Subsequently, she pursued a master degree at the University of Hong Kong with the objective to identify and characterize the putative molecular targets of *Penicillium marneffei*. In 2003, she obtained the Master of Philosophy degree in Microbiology. Afterwards, she moved on to work for the Department of Paediatrics & Adolescent Medicine at the same university, and her research has focused on the primary Epstein-Barr virus infection in children. Since August 2005, she has started the doctoral studies described in this thesis under the supervision of Prof. Frank W. Leebeek and Dr. Moniek P.M. de Maat.

PHD PORTFOLIO

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Summary of PhD training and teaching activities

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PhD period: August 2005 - December 2009
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Research School: COEUR
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1. PhD training	Year	Workload (Hours/ECTS)
General academic skills		
• Biomedical English Writing and Communication	2006	3.0 ECTS
• Laboratory animal science	2008	3.0 ECTS
Research skills		
• Statistics (NIHES)	2006	5.7 ECTS
In-depth courses (e.g. Research school, Medical Training)		
• PhD courses at COEUR (2x) and MolMed (4x)	2005-2006	9.0 ECTS
• PhD-training course from Netherlands Heart Foundation	2008	2.0 ECTS
• Electronic Microscopy Training	2006	1.5 ECTS
Presentations		
• Oral (8x)	2005-2009	5.6 ECTS
• Posters (5x)	2005-2009	2.5 ECTS
International conferences (6x)	2006-2009	6.9 ECTS
Seminars and workshops		
• COEUR Research seminars and lectures (5x)	2006-2008	2.0 ECTS
• Symposia of the Dutch Society of Thrombosis and Haemostasis (3x)	2007-2009	1.5 ECTS
• Other symposia and lectures (10x)	2005-2008	2.6 ECTS
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Lecturing		
Supervising practicals and excursions		
Supervising Master's theses		
• 1 student (24 weeks)	2009	2.4 ECTS
Supervising Bachelor's theses		
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• 1 student in the third year (20 weeks)	2009	2.0 ECTS

PUBLICATIONS

van den Herik EG, **Cheung EY**, de Lau LM, den Hertog HM, Leebeek FW, Dippel DW, Koudstaal PJ, de Maat MP. Fibrinogen γ' levels in patients with intracerebral hemorrhage. *Thromb Res*. 2012 (in press)

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van Oijen M, **Cheung EY**, de Jong FJ, Hofman A, Koudstaal PJ, de Maat MP, Breteler MM. Haplotypes in the fibrinogen gene and dementia. (Submitted)

Cheung EY*, Weijers EM*, Tong M, Scheffer RJ, van Neck JW, Leebeek FW, Koolwijk P, de Maat MP. The effect of fibrinogen γ variants on wound healing. (Submitted)

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AWARDS

Student Award
The XXIst International Fibrinogen Workshop, Rotterdam, the Netherlands. 2010.

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Travel Award
The XXth International Fibrinogen Workshop, Venice, Italy. 2008.