

From the Department of Medicine, Solna  
Atherosclerosis Research Unit  
Karolinska Institutet

# **FIBRINOGEN AND SUSCEPTIBILITY TO MYOCARDIAL INFARCTION**

**Role of gene-gene and gene-  
environment interactions**

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*“Nog finns det mål och mening i vår färd-  
men det är vägen, som är mödan värd.”*

*“Yes, there is goal and meaning in our path -  
but it's the way that is the labour's worth.”*

Karin Boye

*To my parents and grandparents*

## ABSTRACT

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Fibrinogen, the precursor of fibrin, is a glycoprotein synthesized in the liver and maintained in plasma at concentrations normally ranging between 2-4 g/L. The fibrinogen molecule consists of two sets of three non-identical polypeptide chains, which are encoded by the fibrinogen gamma (FGG), fibrinogen alpha (FGA) and fibrinogen beta (FGB) genes clustered on chromosome 4. Elevated plasma fibrinogen concentration is considered an independent predictor of myocardial infarction (MI), while the role of the less abundant fibrinogen  $\gamma'$  chain variant has not as yet been explored in this context. The aim of the present thesis was to study the impact of genetic and environmental factors on total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations, fibrin gel structure and the risk of MI, using well defined clinical cohorts and biochemical, molecular biological and molecular genetic techniques.

The results presented in this thesis are based on findings from three case-control studies comprising survivors of a first MI and population-based controls. The Hypercoagulability and Impaired Fibrinolytic function MECHANisms (HIFMECH) study was designed to identify genetic and environmental factors underlying differences in risk of MI between high-risk (Stockholm and London) and low-risk (Marseille and San Giovanni Rotondo) centres in the North and in the South of Europe. The Stockholm Coronary Atherosclerosis Risk Factor (SCARF) and the Stockholm Heart Epidemiology Program (SHEEP) studies are two independent case-control studies undertaken to investigate genetic, biochemical and environmental factors predisposing to precocious MI.

Both elevated total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations related to MI. However, the former entity appeared to contribute differently to MI in the European centres participating in the HIFMECH study, and was an independent discriminator between cases and controls only in London. In general, IL6, smoking and BMI seem to contribute to the variation in total plasma fibrinogen concentration, while fibrinogen and the FGG 9340T>C and FGA 2224G>A haplotype tag single nucleotide polymorphisms (htSNPs) contribute to the plasma fibrinogen  $\gamma'$  concentration.

Several SNPs were detected in candidate regions in the fibrinogen genes, presumed to play a role in the regulation of the plasma fibrinogen concentration and the fibrin clot structure and therefore to influence the risk of MI. Neither individual fibrinogen SNPs nor FGB haplotypes appeared to influence the risk of MI. On the other hand, fibrinogen haplotypes inferred using genotype data from the FGG 9340T>C and FGA 2224G>A htSNPs seemed to contribute to the risk of MI, independently of the plasma fibrinogen concentration.

Effects on fibrin clot porosity appeared to partly explain the lowered risk of MI conferred by the haplotype consisting of the minor FGG 9340C and FGA 2224A alleles. Furthermore, the fibrinogen haplotypes seem to exert pleiotropic effects on the serum IL6 concentration that are consistent with their impact on the risk of MI, i.e. the haplotype that conferred an increased risk (containing the major FGG 9340T and FGA 2224G alleles) was associated with significantly higher IL6 concentrations than the seemingly protective haplotype (containing the minor FGG 9340C and FGA 2224A alleles).

In addition, gene-gene and gene-environment interaction analyses were performed. Risk factors such as dyslipidemia and high waist-to-hip ratio were stronger predictors of MI than the SNPs included in these analyses. However, a high-order interaction between the total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations and the FGG 9340T>C and FGA 2224G>A htSNPs was noted, yielding a ~3 fold increase in the risk of MI.

**In conclusion**, total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations are related to MI. Also, genetic variation in the fibrinogen genes contribute to the risk of MI, and this relationship seems to be mediated via effects on plasma  $\gamma'$  fibrinogen concentration and fibrin clot structure, and pleiotropic effects on serum IL6 concentration.

**Keywords:** atherothrombosis, epistasis, fibrinogen, fibrinogen  $\gamma'$ , fibrin clot structure, haplotypes, myocardial infarction, pleiotropy, SNPs

## LIST OF PUBLICATIONS

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This thesis is based on the following original articles and manuscripts, which will be referred to in the text by their Roman numerals.

- I. **Mannila MN**, Silveira A, Hawe E, Eriksson P, Aillaud MF, Juhan-Vague I, Yudkin J, Margaglione M, di Minno G, Mussoni L, Tremoli E, Humphries S, Hamsten A. The HIFMECH Study Group. Plasma fibrinogen concentration predicts the risk of myocardial infarction differently in various parts of Europe: effects of  $\beta$ -fibrinogen genotype and environmental factors. The HIFMECH Study. *Thromb Haemost.* 2004; 92: 1240-1249.
- II. **Mannila MN**, Eriksson P, Lundman P, Samnegård A, Boquist S, Ericsson C-G, Tornvall P, Hamsten A, Silveira, A. Contribution of haplotypes across the fibrinogen gene cluster to variation in risk of myocardial infarction. *Thromb Haemost.* 2005; 93: 570-577.
- III. **Mannila MN**, Eriksson P, Ericsson C-G, Hamsten A, Silveira A. Epistatic and pleiotropic effects of polymorphisms in the fibrinogen and coagulation factor XIII genes on plasma fibrinogen concentration, fibrin gel structure and risk of myocardial infarction. *Thromb Haemost.* 2006; 95: 420-427.
- IV. **Mannila MN**, Lovely R, Kazmierczak SC, Eriksson P, Samnegård A, Farrell DH, Hamsten A, Silveira A. Elevated plasma fibrinogen  $\gamma'$  concentration increases the risk of myocardial infarction: effects of genetic variation in fibrinogen genes and environmental factors. *Manuscript*.
- V. **Mannila MN**, Eriksson P, Leander K, Wiman B, de Faire U, Hamsten A, Silveira A. The association between fibrinogen haplotypes and myocardial infarction in men is partly mediated through pleiotropic effects on the serum IL6 concentration. *Manuscript*.

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## LIST OF ABBREVIATIONS

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ASA	acetylsalicylic acid
BMI	body mass index
CAD	coronary artery disease
CHD	coronary heart disease
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
F	(coagulation) factor
F13A1	coagulation factor XIII gene
FDP	fibrin degradation product
FGA	alpha fibrinogen gene
FGB	beta fibrinogen gene
FGG	gamma fibrinogen gene
FPA	fibrinopeptide A
FPB	fibrinopeptide B
GP	glycoprotein
HDL	high density lipoprotein
ht	haplotype tag
ICAM-1	intercellular adhesion molecule 1
IL6	interleukin 6
LD	linkage disequilibrium
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
MCP-1	monocyte chemoattractant protein 1
MDR	multifactor dimensionality reduction
MI	myocardial infarction
NF- $\kappa$ B	nuclear factor $\kappa$ B
OR	odds ratio
PAI	plasminogen activator inhibitor
PAR	protease-activated receptor
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SOR	standardized odds ratio
TAFI	thrombin-activatable fibrinolysis inhibitor
TF	tissue factor
TFPI	TF pathway inhibitor
t-PA	tissue plasminogen activator
VLDL	very low density lipoprotein
vWF	von Willebrand factor

## INTRODUCTION

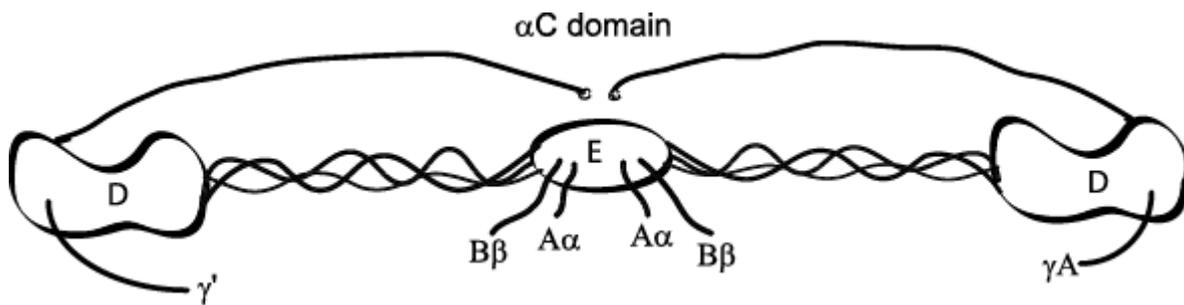
Myocardial infarction (MI) is a severe clinical manifestation of atherothrombosis, i.e. thrombus formation superimposed upon a ruptured or eroded atherosclerotic plaque. It is the most common cause of death in both men and women in Sweden, and the number of deaths from cardiovascular disease increases worldwide. Potentially modifiable lifestyle factors (i.e. smoking, dietary habits and physical inactivity) and complex traits such as hypertension, dyslipidemia, obesity and diabetes mellitus are major contributors to the etiology of atherothrombosis.<sup>1</sup> However, despite unequivocal success, neither lifestyle modifications nor treatment with acetylsalicylic acid, antihypertensive agents and statins seem to be able to eradicate the incidence of the majority of cardiovascular events.<sup>2</sup> This failure may be ascribed to lack of specificity for most interventions in relation to pro-inflammatory (e.g. interleukin 6) and pro-coagulant (e.g. fibrinogen, coagulation factor XIII, tissue factor) components, which also play critical roles in the etiology of atherothrombosis.<sup>3,4</sup> Furthermore, it may reflect that genetic heritability, i.e. the proportion of the phenotypic variation that can be attributed to genetic factors, makes a substantial contribution to the risk of atherothrombosis as evidenced by twin,<sup>5</sup> family<sup>6</sup> and genome wide<sup>7</sup> studies. In fact, most of the risk factors for atherothrombosis display a considerable genetic component,<sup>8,9</sup> and gene-gene interactions or epistasis<sup>10</sup> in concert with gene-environment interactions<sup>11</sup> may also play a key role in the etiology of this disease. Unless we clarify all pieces of the atherothrombosis puzzle we will continue, in all likelihood, to fail in eliminating the majority of the cardiovascular events, especially now as we are facing an increased longevity and obesity epidemics worldwide. Therefore, a better understanding of the molecular and cellular mechanisms involved in the etiology of atherothrombosis may pave the way for improved diagnostic and treatment strategies that in concert with adequate preventive approaches could be of significant benefit for people worldwide. Fibrinogen is one piece of this complex puzzle that has gained recognition as an independent risk factor for MI, which constituted the incentive for the studies performed within the framework of the present thesis.

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### Fibrinogen

Fibrinogen, or coagulation factor I, is a large (340 kDa) fibrous glycoprotein present in plasma at concentrations normally ranging between 2-4 g/L. The fibrinogen molecule is an elongated 45 nm structure composed of three pairs of polypeptides with the stoichiometry (A $\alpha$ B $\beta$  $\gamma$ )<sub>2</sub>.<sup>12</sup> The predominant fibrinogen A $\alpha$ , B $\beta$  and  $\gamma$  chains contain 610, 461 and 411 amino acids (aa), respectively. However, as a result of alternative splicing there are two fibrinogen  $\gamma$  chain variants present in plasma, namely the  $\gamma$ A (411aa)

and  $\gamma'$  (427aa) chains.<sup>12</sup> The majority of the fibrinogen molecules (approximately 85%) are homodimeric, i.e. both halves contain  $\gamma$ A chains (denoted  $\gamma$ A/ $\gamma$ A), whereas a minor fraction (about 15%) is heterodimeric, i.e. it contains one  $\gamma$ A chain and one  $\gamma'$  chain (denoted  $\gamma$ A/ $\gamma'$ ) and less than 1% is homodimeric, i.e. it contains two  $\gamma'$  chains (denoted  $\gamma'/\gamma'$ ). The polypeptide chains are held together by disulfide bonds, yielding two symmetrically arranged halves with a central E domain linked by coiled-coil segments to the outer D domains (Figure 1).<sup>13</sup> From the central E domain



**Figure 1. Simplified illustration of the fibrinogen molecule.** The presented structure is a heterodimer ( $A\alpha B\beta\gamma$ )( $A\alpha B\beta\gamma'$ ). The central E domain is linked by coiled-coil segments to the outer D domains from where the  $\alpha C$  domains emerge (Adapted from Mosesson 2003).

emerge the amino-terminal sequences of the fibrinogen  $A\alpha$  and  $B\beta$  chains, containing the fibrinopeptides A and B (FPA and FPB), which are cleaved by thrombin in the final step of the coagulation pathway. From the outer D domains emerge the carboxyl-terminal regions of the fibrinogen  $A\alpha$  chains, termed the  $\alpha C$  domains, which are non-covalently connected to the central E domain.

Three distinct genes clustered in a 50kb region on chromosome 4q28 encode the fibrinogen polypeptides: fibrinogen gamma (FGG), alpha (FGA) and beta (FGB) (Figure 2). The fibrinogen genes, which probably have evolved from a common ancestral gene,<sup>14</sup> contain similar regulatory

*cis*-elements<sup>15-17</sup> and are transcribed in a tightly coordinated manner<sup>18</sup> with the FGB gene in opposite transcriptional direction toward the FGG and FGA genes.<sup>14</sup> Hormones (e.g. glucocorticoid and thyroid hormones),<sup>18,19</sup> interleukin 6 (IL6)<sup>20</sup> and transcription factors (e.g. peroxisome proliferator activated receptor (PPAR)- $\alpha$ )<sup>21</sup> have been implicated in regulating the synthesis of fibrinogen during basal and/or inflammatory conditions taking place predominantly in the liver. The polypeptides undergo several post-translational modifications<sup>22</sup> and are assembled in a stepwise manner in the rough endoplasmic reticulum in hepatocytes.<sup>23</sup> Fully assembled fibrinogen molecules are then secreted into the blood.<sup>23</sup>

#### FGG



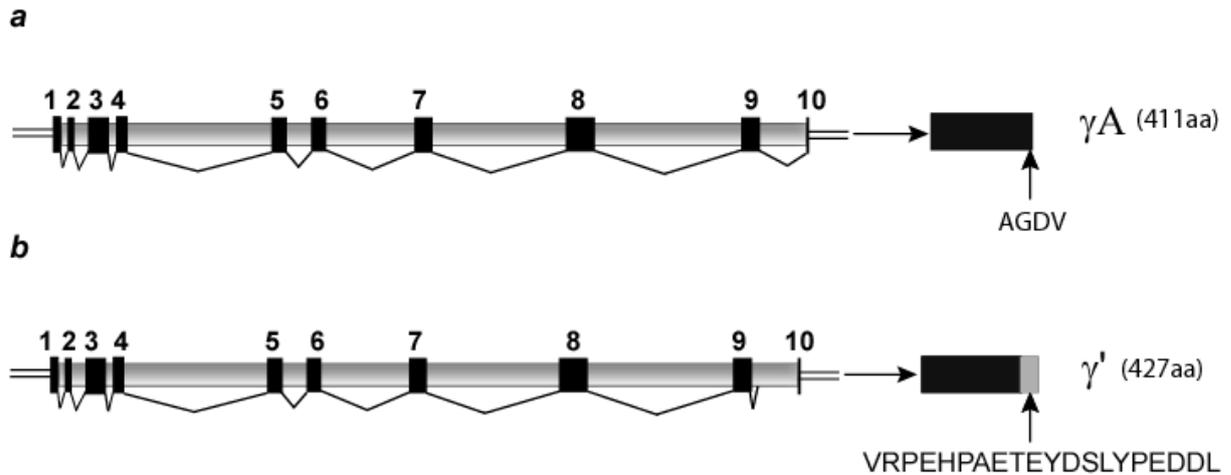
#### FGA



#### FGB



**Figure 2. Schematic illustration of the fibrinogen gamma (FGG), alpha (FGA) and beta (FGB) genes, clustered in a 50kB region on chromosome 4q28.** The dark shaded boxes indicate the exons in each gene, whereas the intronic regions are grey shaded.



**Figure 3. RNA splicing in the FGG gene. (a)** RNA processing generating the fibrinogen  $\gamma$ A chain. **(b)** Alternative RNA processing giving rise to the fibrinogen  $\gamma'$  chain. aa, amino acid

### Fibrinogen heterogeneity

More than one million different fibrinogen molecules have been estimated to be present in the blood.<sup>22</sup> Most of these molecules arise as a consequence of alternative mRNA splicing (e.g. the fibrinogen  $\gamma'$  chain), post-translational modifications, enzymatic degradation and presence of non-synonymous single nucleotide polymorphisms (SNPs), e.g. FGA Thr312Ala and FGB Arg448Lys.<sup>24</sup>

The fibrinogen  $\gamma'$  chain variant arises as a consequence of alternative splicing in intron 9 of the FGG gene (Figure 3).<sup>25,26</sup> This fibrinogen variant and its major counterpart, the fibrinogen  $\gamma$ A chain, are two structurally and functionally unique molecules. The basis for these differences is the replacement of the last four residues of the  $\gamma$ A chain with an anionic twenty amino acid sequence (VRPEHPAET EYDSL YPEDDL).<sup>27</sup> The last five carboxyl-terminal QAGDV residues of the  $\gamma$ A chain are critical for binding to the glycoprotein (GP) IIb/IIIa receptor (also known as  $\alpha_{IIb}\beta_3$ ), thereupon promoting platelet aggregation. In contrast, the fibrinogen  $\gamma'$  chain is significantly less efficient than the  $\gamma$ A chain in promoting platelet aggregation,<sup>28</sup> but it contains high affinity binding sites for thrombin<sup>29</sup> and

coagulation factor (F) XIII.<sup>30,31</sup> Presence of the fibrinogen  $\gamma'$  chain has been implicated in formation of fibrin clots with altered structure and function.<sup>32,33</sup>

Also as a result of alternative splicing, a longer fibrinogen A $\alpha$  chain variant (denoted  $\alpha$ E, 420 kDa) is present in the blood.<sup>34</sup> The fibrinogen  $\alpha$ E chain amounts approximately 1-2% of the total plasma fibrinogen and has been reported to influence the fibrin clot structure<sup>35</sup> and to participate in cellular interactions.<sup>36</sup> Additional variation, that has also been suggested to have an impact on the formation of the fibrin clot, is brought about by partial degradation of fibrinogen A $\alpha$  chains in plasma.<sup>37</sup> Yet another contributor to the fibrinogen heterogeneity is the FGA Thr312Ala polymorphism which is quite common in European populations (prevalence 20-30%) and has been associated with increased poststroke mortality in individuals with atrial fibrillation.<sup>38</sup> Further heterogeneity is conferred by posttranslational modifications such as phosphorylation of fibrinogen A $\alpha$  chains<sup>39</sup> and glycosylation of fibrinogen  $\gamma$  chains.<sup>40</sup>

Dysfibrinogenemias encompass a plethora of structurally abnormal fibrinogen

molecules,<sup>41</sup> which are known to impair the fibrin clot formation and/or dissolution.<sup>42</sup> Some forms of dysfibrinogenemias display functional defects of clinical significance (bleeding and/or thrombosis tendency), but the majority of the carriers are asymptomatic.<sup>41</sup>

### **Environmental determinants**

Various non-modifiable and modifiable factors, most of which are well-established risk factors for cardiovascular disease, influence the plasma fibrinogen concentration.<sup>43,44</sup> An association between low birth weight, a risk factor for coronary heart disease,<sup>45</sup> and higher plasma fibrinogen concentration, that may be partly explained by genetic factors, has been reported.<sup>46</sup> Fibrinogen increases gradually with age in both men and women.<sup>47</sup> Seasonal variations in plasma fibrinogen concentrations have been reported.<sup>48</sup> During winter, the plasma fibrinogen concentrations are higher amongst elderly, and may account for 15% of the increase in risk of ischemic heart disease.<sup>49</sup> Relevant in this context is that fibrinogen is an acute phase reactant which increases in response to various challenges (e.g. trauma, infection, MI) and an increased incidence in infections during winter could be a likely explanation for the seasonal variations in plasma fibrinogen concentration.<sup>49</sup>

Smoking, a major risk factor for cardiovascular disease, is one of the strongest environmental determinants of plasma fibrinogen concentration.<sup>50</sup> Dietary components such as fish oil and fibers seem to have a modest effect on plasma fibrinogen.<sup>51</sup> Alcohol consumption,<sup>52,53</sup> physical activity<sup>54</sup> and HDL-cholesterol<sup>55</sup> are inversely associated with plasma fibrinogen concentration, whereas the opposite has been reported for overweight.<sup>52</sup> Whether fibrinogen is associated with hypertension is doubtful as conflicting

results have been reported so far.<sup>56,57</sup> In diabetics, the plasma fibrinogen is affected both quantitatively (i.e. increased plasma concentration)<sup>58</sup> and qualitatively (i.e. glycosylation).<sup>59</sup>

Lipid-lowering treatment with fenofibrate<sup>60</sup> and bezafibrate,<sup>61</sup> but not with statins,<sup>62</sup> has been reported to reduce plasma fibrinogen concentrations. In a randomized controlled study of postinfarction patients, both acetylsalicylic acid (ASA) and clopidogrel appeared to lower the plasma fibrinogen concentration.<sup>63</sup> Oral contraceptives are known to increase the plasma fibrinogen concentration,<sup>64</sup> whereas hormone replacement therapy in healthy postmenopausal women has been associated with reduced concentrations.<sup>65</sup>

### **Genetic determinants**

Based on data from family and twin studies approximately 20-50% of the variation in plasma fibrinogen concentration has been ascribed to genetic heritability.<sup>44,66,67</sup> Presence of a quantitative trait locus implicating one of the FGA promoter polymorphisms as a potential contributor to the variation in plasma fibrinogen concentration has been reported.<sup>68</sup> Moreover, several genetic variants confined to the FGB gene that may explain up to 15% of the phenotypic variation in plasma fibrinogen concentration have been identified.<sup>69,70</sup>

However, as a consequence of the linkage disequilibrium (LD) pattern in the fibrinogen genes<sup>71,72</sup> it is difficult to discern the effects of individual SNPs. Functional studies have indicated that some FGB promoter polymorphisms are implicated in differential binding of nuclear proteins<sup>73,74</sup> which may be a plausible explanation for their impact on plasma fibrinogen concentration. However, recent data from an *in vitro* study employing small

interfering RNA technology has implicated yet another FGB promoter polymorphism as a potential functional determinant of plasma fibrinogen concentration.<sup>75</sup> This particular FGB polymorphism is located in a regulatory sequence, critical for the IL6 induced expression of fibrinogen. Since IL6 is one of the strongest endogenous determinants of plasma fibrinogen concentration,<sup>20</sup> particularly important during acute phase reactions, presence of this polymorphism may influence the fibrinogen response to various environmental stimuli (e.g. smoking).

Gender-specific effects of fibrinogen polymorphisms<sup>76-78</sup> as well as gene-environment interactions<sup>79</sup> seem to influence the plasma fibrinogen concentration. Fibrinogen polymorphisms have also been implicated in genotype specific changes in plasma fibrinogen concentration in response to physical exercise.<sup>80,81</sup> Moreover, there are ethnicity related differences in plasma fibrinogen concentration to which certain fibrinogen gene variants may contribute.<sup>82</sup>

Lack of association between fibrinogen gene polymorphisms across the entire fibrinogen gene cluster and plasma fibrinogen concentration has also been reported.<sup>83-85</sup> Moreover, the genome wide studies published so far have failed to detect any linkage peak corresponding to the fibrinogen gene cluster.<sup>86,87</sup> Notably, results from a survey of genetic and epigenetic variation influencing the human gene expression have indicated that the expression of the FGB gene is both random and monoallelic (i.e. either allele in heterozygotes is randomly expressed).<sup>88</sup> These data along with the possibility that some fibrinogen gene variants may exert epistatic effects on plasma fibrinogen concentration in the absence of individual main effects may partly explain the

difficulties and inconsistencies encountered so far in genotype-phenotype association studies. To this end, as the plasma fibrinogen concentration is a complex polygenic trait, the role of plausible biological determinants encoded by genes outside the fibrinogen gene cluster (e.g. IL6) needs to be clarified. Given the reported heritability estimates<sup>44,66,67</sup> supported by the notion that between 25-35% of the phenotypic variation may be explained by *cis*-acting factors<sup>89</sup> it is likely that there are genetic contributors (located both within and outside the fibrinogen gene cluster) to the plasma fibrinogen concentration yet to be discovered.<sup>90</sup>

## **Fibrin(ogen) functions**

Fibrinogen, the precursor of fibrin, plays a major role in hemostasis, a process that could be lifesaving when sealing of an injured vessel. Conversely, inappropriate activation of hemostatic processes within the vasculature may endanger life if vessels such as the coronary or cerebral arteries are occluded by a thrombus.

Upon damage to the vascular integrity, platelets adhere and aggregate at the site of injury resulting in formation of a primary hemostatic plug. This process is mediated by platelet-von Willebrand factor (vWF) and platelet-fibrinogen interactions via the integrin GP1b and GPIIb/IIIa receptors. The hemostatic plug is fragile and easily dissolved unless it is stabilized by a fibrin clot network, which is the end product of the coagulation pathway. The fibrin clot is critical not only for firmly sealing of the injury, but it also provides an adhesive scaffold for the cellular and molecular interactions necessary for adequate tissue repair.

As the survival of the damaged tissue is dependent on oxygen and nutritional supply, it is essential that the vascular

patency is re-established. The means by which the clot is dissolved and the vascular patency restored employs the fibrinolytic system and fibrin(ogen) is one of the key partakers in this process as well.

In addition, fibrinogen has a pleiotropic nature, i.e. it is implicated in (patho)physiological processes such as angiogenesis,<sup>91</sup> atherogenesis,<sup>92</sup> embryonic development,<sup>93</sup> inflammation<sup>94</sup> and neoplasia<sup>95</sup> that reach beyond hemostasis. The fibrinogen functions in hemostasis and some of its pleiotropic effects are briefly presented in the next sections.

### **Blood coagulation**

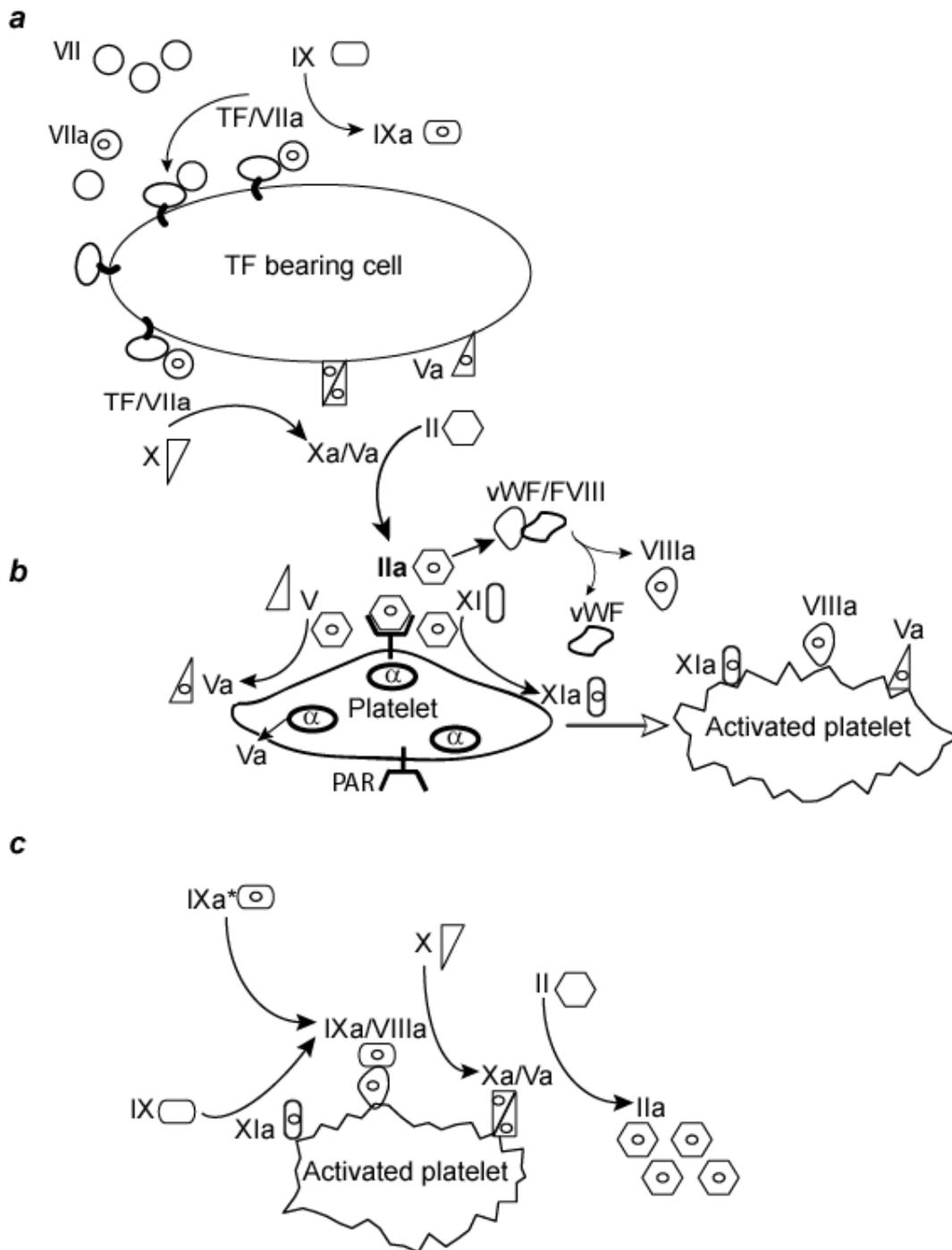
The coagulation “cascade” was originally considered to involve two mutually exclusive pathways: the extrinsic and the intrinsic pathway. The activation of the extrinsic or tissue factor (TF) pathway is usually attributed to exposure of TF to blood upon vascular injury. Binding of TF to FVIIa results in formation a complex (TF/FVIIa) that converts FX to FXa, either directly or indirectly by activating FIX to FIXa. The triggering event of the intrinsic or contact pathway has been ascribed to the autoactivation of FXII (or Hageman factor) caused by interactions with negatively charged surfaces. FXIIa converts prekallikrein to kallikrein and activates FXI to yield FXIa in the presence of high molecular weight kininogen. The FXIa activates factor FIX to FIXa, which forms then a complex with FVIIIa (FIXa/VIIIa, the tenase complex) that activates FX. Upon generation of FXa the two pathways merge into a common pathway. The FXa forms a complex with its cofactor Va (FXa/Va, the prothrombinase complex) that converts prothrombin (FII) to thrombin (FIIa), which in the final step of the coagulation pathway cleaves fibrinogen to fibrin, the main component of the blood clot.

Although this model seemed to adequately explain *in vitro* hemostatic processes it appeared to be insufficient as a model for *in vivo* situations. For instance, it cannot explain why deficiency in FVIII, FIX or FXI is associated with a bleeding tendency, since a failing intrinsic pathway would be expected to be compensated for by the extrinsic pathway. A conceptually new model, i.e. the cell-based model of coagulation, evolved gradually, according to which the hemostatic processes occur on specific cell surfaces in three overlapping and highly orchestrated steps: the initiation, amplification and propagation phases (Figure 4a-c).<sup>96-98</sup>

### ***The cell-based coagulation pathway***

#### The initiation phase

The physiological trigger of the initiation phase is exposure of TF to circulating blood upon vascular injury. TF is a transmembrane glycoprotein normally residing on the surface of TF bearing cells in the extravascular space. TF binds to FVIIa, a serine protease normally present in small amounts (i.e. 1-2% of the total FVII) in the blood (Figure 4a). The TF/FVIIa complex amplifies the initial trigger by activating more FVII, hence generating additional TF/FVIIa complexes that activate small amounts of FIX and FX. The FV, which circulates in plasma and in platelets,<sup>99</sup> is then activated to yield FVa either by FXa<sup>100</sup> or by non-coagulant proteases (e.g. elastases).<sup>101</sup> Additional FVa is supplied by partially activated platelets that adhere to the site of injury, whereupon the contents of their  $\alpha$ -granules are released.<sup>102</sup> The FVa is a cofactor for FXa, and together they form the FXa/Va complex (the prothrombinase complex) that catalyses the conversion of small amounts of prothrombin to thrombin.



**Figure 4. Schematic illustration of the cell-based coagulation pathway, which occurs in three steps: (a) the initiation phase, (b) the amplification phase and (c) the propagation phase. \*FIXa generated during the initiation phase; PAR, protease-activated receptor (Adapted after Hoffman 2003).**

### The amplification phase

Although only small amounts of thrombin are generated on the surface of TF bearing cells during the initiation phase, these are pivotal in the amplification phase. This step is localized on the surface of platelets, of which thrombin is a powerful agonist.<sup>103</sup> Thrombin activates the platelets via

protease-activated receptors (PARs)<sup>104</sup> that induce a series of intracellular signalling networks substantiated via profound structural changes (Figure 4b).<sup>105</sup> Phosphatidylserines (PSs) are redistributed on the surface of activated platelets and act as docking sites permitting assembly of coagulation factors and their cofactors.

Moreover, platelets become degranulated, and fibrinogen, vWF and FV are some of the components of the  $\alpha$ -granules that are released into the extracellular space. On the surface of activated platelets thrombin then activates the FV, FVIII and FXI. The FVIII, which is a cofactor for FIXa, circulates in plasma predominantly as a non-covalent complex with vWF, but upon activation by thrombin it dissociates from this complex and becomes attached to the activated platelets.

### The propagation phase

During the propagation phase large numbers of platelets adhere and become fully activated at the site of injury, a process facilitated by the presence of vWF. The FIXa, that is either generated during the initiation phase or activated by FXIa,<sup>106</sup> forms a complex with the FVIIIa (the tenase complex) followed by activation of FX on the platelet surface (Figure 4c). This activation is critical for the continuation of the coagulation process, since FXa cannot move efficiently from the TF bearing cells as it is efficiently inhibited by the TF pathway inhibitor (TFPI) or antithrombin. The FXa then forms a complex with FVa (the prothrombinase complex) that fuels a burst of thrombin generation followed by formation of a fibrin/platelet clot.

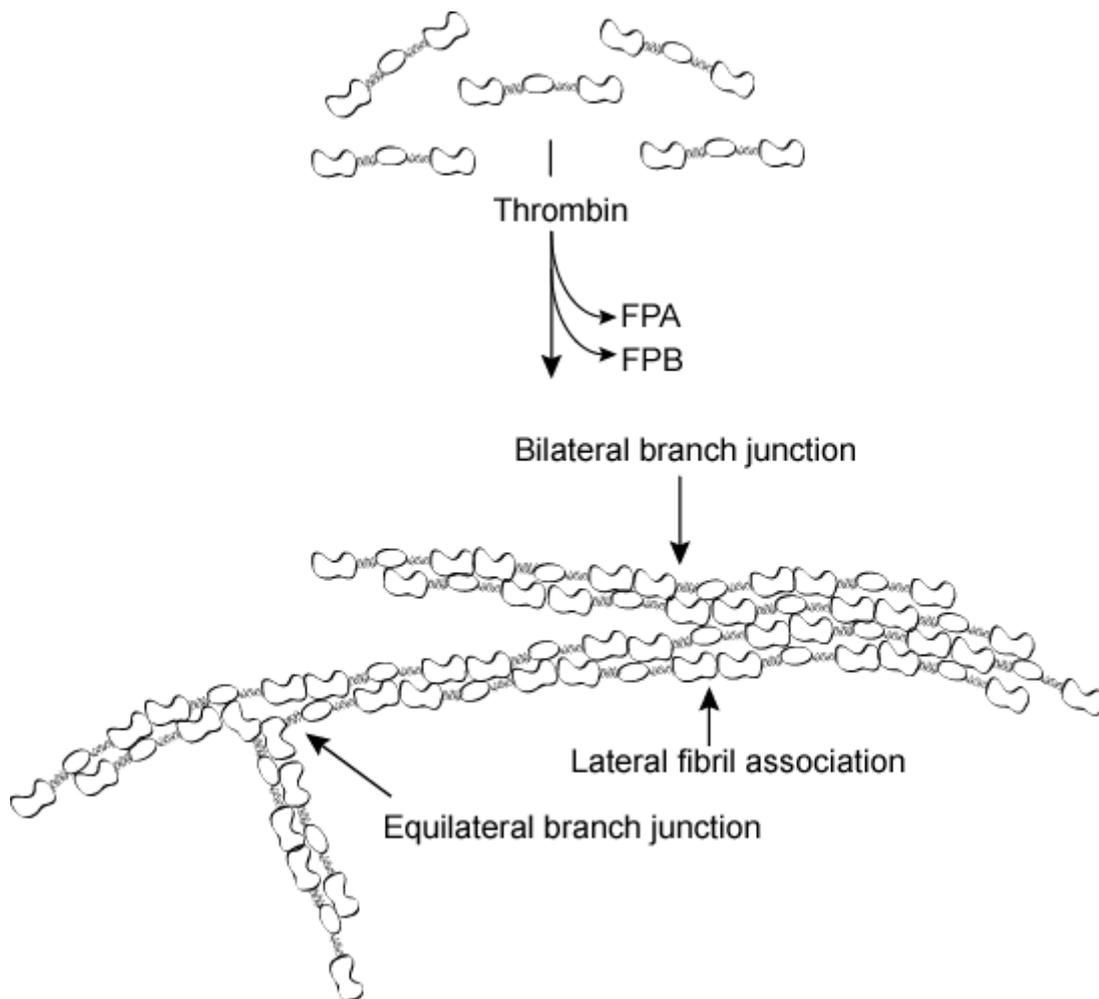
### ***Formation and stabilization of the fibrin clot***

The formation of the fibrin clot is the final step in the coagulation pathway, providing a protective seal to the injured tissue and a scaffold for wound healing. It is a highly regulated process initiated by thrombin which cleaves the amino-terminal FPA of the fibrinogen A $\alpha$  chains thereby exposing the polymerization sites (denoted E<sub>A</sub>-sites).<sup>107</sup> The E<sub>A</sub>-sites interact with complementary pockets (D<sub>a</sub>) localized in the D domain of adjacent fibrin molecules, generating double-stranded fibrils assembled in an overlapping end-to-middle

domain manner. Double-stranded fibrils converge, resulting in formation of two different types of branch junctions. Bilateral branch junctions are formed by lateral convergence of double-stranded fibrils generating four-stranded fibrils, whereas equilateral branch junctions are formed by interactions between three fibrin molecules generating three double-stranded fibrils (Figure 5).<sup>108</sup>

The fibrinogen B $\beta$  chain amino-terminal FPB is also cleaved by thrombin.<sup>107</sup> This is a slower process that results in exposure of a second polymerization site (denoted E<sub>B</sub>-site), which interacts with a complementary site (D<sub>b</sub>) localized in the D domain of the fibrinogen B $\beta$  chain.<sup>109</sup> These associations result in formation of intermolecular contacts between the carboxyl-terminal region of the B $\beta$  chain ( $\beta_C:\beta_C$ ),<sup>110</sup> thereby contributing to the lateral association of fibrils (Figure 5). The  $\alpha_C$  domain of the fibrinogen A $\alpha$  chain, that emerges from the D domain and is non-covalently associated to the central E domain, also contributes to the fibrin clot formation. Upon release of the FPB it dissociates from the E domain and interacts with other  $\alpha_C$  domains, hence promoting lateral aggregation of fibrils. Moreover, interactions between self-association sites confined to the fibrinogen  $\gamma$  chain region of the D domain, facilitate the FXIII mediated cross-linking (the  $\gamma_{XL}$ -sites) and are necessary for accurate assembly of the fibrin molecules (the D:D-sites).

The fibrin clot is stabilised by FXIII, a transglutaminase circulating in plasma as an A<sub>2</sub>B<sub>2</sub>-tetramer bound to fibrinogen  $\gamma$ ' chains via the B-subunits.<sup>30</sup> The activation of FXIII is triggered by thrombin, which cleaves the peptide bonds between the Arg37 and Gly38 residues, confined to the A-subunit, which dissociates from the B-subunit in the presence of Ca<sup>2+</sup> and becomes activated. The FXIIIa catalyses



**Figure 5. Simplified illustration of fibrin assembly, branching and lateral fibril association.** FPA, fibrinopeptide A; FPB, fibrinopeptide B (Adapted after Mosseson 2001).

formation of  $\epsilon$ -( $\gamma$ -glutamyl)lysine bonds between the  $\gamma$ 406 lysine of one fibrinogen  $\gamma$  chain and a glutamine at position  $\gamma$ 398/ $\gamma$ 399 of another fibrinogen  $\gamma$  chain. Additional cross-linking reactions occur between fibrinogen A $\alpha$  chains<sup>111</sup> as well as between fibrinogen A $\alpha$  and  $\gamma$  chains.<sup>112</sup> Moreover, proteins such as  $\alpha$ 2-antiplasmin,<sup>113</sup> vWF<sup>114</sup> and trombospondin<sup>115</sup> are incorporated into the fibrin clot by formation of cross-links, which are also mediated by FXIIIa. The cross-linking of the fibrin network along with the incorporation of plasma proteins gives strength to the clot against mechanical challenges and it confers resistance to proteolytic cleavage by plasmin.

#### ***Anticoagulant mechanisms***

The coagulation pathway is tightly regulated in order to avoid massive activation and to allow formation of sufficient amounts of fibrin to seal the injured tissue. The regulation is orchestrated at several levels. Firstly, TF, the prerequisite for the initiation phase, is under normal circumstances separated from the circulating blood. TF is usually harboured on the surface of TF bearing cells and becomes exposed to the blood mainly upon disruption of the vasculature. Secondly, the coagulation pathway is constrained at sites of injury where the sequential activation of coagulation factors is actually a limiting factor. Thirdly, powerful endogenous anticoagulant proteins, such as the TFPI, the

protein C pathway and antithrombin, normally preclude any inaccurate propagation of the coagulation pathway.

TFPI is a complex protein that is released from endothelial cells. TFPI forms a complex with any FXa that has detached from the surface on which it was generated, followed by binding to the TF/FVIIa complex and formation of a quaternary complex.<sup>96</sup> Hence, both the FXa and the TF/FVIIa complex are inhibited and the coagulation pathway can only ensue provided that sufficient amounts of FVIII, FIX and FXI have been activated downstream.

Protein C is a vitamin K-dependent protein that circulates in plasma as an inactive zymogen.<sup>116</sup> Protein C binds to the endothelial cell protein C receptor (EPCR). The EPCR facilitates the transfer of protein C to a complex formed by thrombin and thrombomodulin, which is a transmembrane protein expressed by endothelial cells. Protein C is then activated upon proteolytic cleavage by thrombin yielding activated protein C (APC). The latter dissociates from the thrombin-thrombomodulin complex and binds to its cofactor, protein S, which is also a vitamin K-dependent plasma protein. The APC/protein S complex inhibits efficiently the cofactors Va and VIIIa hence suppressing further generation of thrombin. Thus, thrombin is a key partaker in its own inhibition.

Antithrombin is a glycoprotein that inactivates several components of the activated coagulation pathway: thrombin, FIXa, FXa and to a lesser extent the FXIa, FXIIa and FVIIa. Binding of antithrombin to thrombin leads to formation of stable thrombin-antithrombin complexes which are useful markers of thrombin generation.<sup>117</sup> The antithrombin mediated inhibition of activated coagulation factors is

markedly enhanced in the presence of heparin or heparan sulphate.

### **Fibrinolysis**

The fibrinolytic system provides an important mechanism by which blood clots are dissolved and vascular patency is restored. Fibrin plays a key role in this process as it (i) facilitates and enhances the activation of plasminogen and (ii) acts as a substrate for plasmin, the main effector of the fibrinolytic system.

Plasminogen is a glycoprotein that upon conversion to the active enzyme plasmin efficiently degrades the fibrin clot network. Tissue plasminogen activator (t-PA) is the main activator of plasminogen. Both plasminogen and t-PA bind to fibrin forming a cyclic ternary complex.<sup>118,119</sup> Consequently, the affinity of t-PA for plasminogen is increased and the conversion to plasmin is accelerated. Proteolytic cleavage of fibrin by plasmin generates additional binding sites for plasminogen, which is enriched and activated in the clot, thereby strengthening the fibrinolytic capacity.

There are several mechanisms by which the fibrinolytic process is constrained at sites of fibrin clot formation: (i) fibrinogen lacks more or less completely the ability to contribute to the activation of plasminogen since the sites that are involved in this process are cryptic in fibrinogen but not in fibrin, (ii) t-PA is a poor enzyme in the absence of fibrin, and (iii) free plasmin is rapidly inactivated by  $\alpha_2$ -antiplasmin in contrast to the plasmin that is bound to fibrin.

### ***Fibrin degradation***

The fibrin clot is dissolved upon proteolytic cleavage by plasmin resulting in formation of fibrin degradation products (FDPs) of various sizes.<sup>120</sup> The fragments derived

from the amino-terminal region are usually referred to as fragment E, whereas those from the carboxyl-terminal region as fragment D. Interestingly, fibrin D and E dimers seem to stimulate fibrinogen synthesis in hepatocytes via an indirect leukocyte mediated pathway.<sup>121</sup> D-dimers are markers of cross-linked fibrin turnover, clinically used for diagnostic purposes (e.g. venous thromboembolism).<sup>122</sup>

### ***Inhibitors of the fibrinolytic system***

Serine protease inhibitors (serpins) contribute to the inhibition of the fibrinolytic system that occurs on several levels. Amongst these,  $\alpha_2$ -antiplasmin is one of the key inhibitors of plasmin. Plasminogen activator inhibitor 1 (PAI-1), of which endothelial cells, adipose tissue and hepatocytes are the major sources, is a serpine that efficiently inhibits t-PA.<sup>123</sup> Plasminogen activator inhibitor 2 (PAI-2) is a placental-tissue derived serpin that also inhibits t-PA, but less efficiently than PAI-1. On the other hand, PAI-2 seems to exert some important intracellular functions i.e., it alters gene expression, the rate of cell proliferation and differentiation, and inhibits apoptosis.<sup>124</sup>

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasmin inhibitor that becomes activated by the thrombin-thrombomodulin complex.<sup>125</sup> Activated TAFI removes the carboxyl-terminal lysine and arginine residues from fibrin, hence obstructing the activation of plasminogen to plasmin resulting in reduced fibrinolysis rate.<sup>126</sup>

Lipoprotein(a) (Lp(a)) consists of low density lipoprotein (LDL) and apolipoprotein (apo) B100 to which apo(a) is attached. Apo(a) displays an extensive structural homology with plasminogen<sup>127</sup> and can therefore compete with the latter for binding sites on fibrin, hence inhibiting the fibrinolytic process.<sup>128</sup>

### **Pleiotropic effects**

Fibrinogen exerts significant effects on various cells via molecular interactions with integrins (e.g. GPIIb/IIIa,  $\alpha$ M $\beta$ 2,  $\alpha$ V $\beta$ 3) and adhesion molecules such as the intercellular adhesion molecule 1 (ICAM-1). Many of these pleiotropic functions are decrypted upon conversion of fibrinogen to fibrin,<sup>129</sup> which may be provoked by tissue damage brought about by various conditions (e.g. infection, trauma, neoplasia and atherosclerosis). As exposure of certain residues (e.g. the B $\beta$ 15-42 residues) is a prerequisite for fibrin to support platelet spreading,<sup>130</sup> endothelial cell and fibroblast proliferation,<sup>131</sup> it also provides a regulatory mechanism governed by specific structural features of fibrin(ogen).

In response to inflammatory challenges, fibrin(ogen) promotes the migration and adhesion of leukocytes, induces cytokine and chemokine synthesis and initiates tissue repair processes.<sup>94,132,133</sup> Moreover, fibrinogen binding to the integrin  $\alpha$ M $\beta$ 2 receptor on leukocytes<sup>134</sup> results in clearance of bacteria<sup>135</sup> at sites of infection. The importance of the fibrinogen leukocyte interactions was evidenced in transgenic mice in which mutation of the fibrinogen sequence that is the prerequisite for binding to the integrin  $\alpha$ M $\beta$ 2 receptor resulted in a severely compromised inflammatory response in the infected mice.<sup>135</sup> Moreover, fibrin(ogen) deficient mice display increased mortality upon peritoneal infection with an intracellular pathogen, further suggesting that fibrin(ogen) may have important host-protective functions.<sup>136</sup> However, fibrin(ogen) can also exacerbate certain infectious diseases by means of enhanced bacterial gene expression leading to increased pathogenic burden and host mortality.<sup>137</sup>

Formation of fibrin is probably the first step in wound healing, a process involving chemotaxis, synthesis of matrix proteins

and angiogenesis.<sup>138</sup> Via interactions with the integrin  $\alpha$ M $\beta$ 2 receptor, the fibrin network promotes adhesion of leukocytes to endothelial cells<sup>139</sup> and to the extracellular matrix.<sup>140</sup> The former involves binding of fibrin(ogen) to ICAM-1 expressed on endothelial cells.<sup>139</sup> Efficient clearance of foreign particles and other pathogens by neutrophils as well as presence of growth factors (e.g. fibroblast growth factor 2, insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor) are required for proper wound healing. Fibrin(ogen) binding to insulin-like growth factor binding protein 3 causes enrichment of IGF-1 which stimulates stromal cell function and proliferation at sites of injury.<sup>141</sup> In addition, fibrin(ogen) contributes to the revascularisation of the damaged tissue by stimulating angiogenesis<sup>91</sup> via interactions with vascular endothelial cadherin on endothelial cells.<sup>142</sup>

Angiogenesis is not only critical in wound healing but also in neoplasia and atherogenesis. Integrin  $\alpha$ V $\beta$ 3 mediated melanoma cell adhesion to fibrinogen fuels significant cell spreading, which may have severe implications for tumour development and metastasis.<sup>95</sup> Indeed, fibrinogen seems to be a powerful determinant of the metastatic potential of certain tumours, as revealed by studies in fibrinogen knockout mice in which both lymphogenous and hematogenous tumour dissemination is diminished.<sup>143</sup>

### **Fibrin clot structure**

A complex interplay between environmental and genetic factors contributes to fibrin clot structure and function,<sup>144-146</sup> some of which will be briefly presented in the following sections.

### **Environmental determinants**

Fibrinogen, the precursor of fibrin, is one of the major determinants of fibrin clot structure.<sup>147</sup> Therefore, factors that influence fibrinogen (quantitatively or qualitatively) may indirectly influence the fibrin clot structure. Elevated concentrations of acute phase proteins (e.g. orosomucoid and C-reactive protein (CRP)) including fibrinogen, elicited by inflammatory stimuli (e.g. infection, neoplasia), have been associated with lower fibrin clot porosity and impaired fibrinolysis.<sup>148,149</sup>

In healthy individuals, the fibrin clot porosity was reported to be positively associated with high density lipoprotein (HDL)-cholesterol, whereas inverse correlations were found with body mass index (BMI) and very low density lipoprotein (VLDL)-triglycerides.<sup>150</sup> In diabetics, qualitative changes of the fibrinogen molecule (e.g. glycation),<sup>59</sup> that influence the fibrin clot structure, occur in a dose dependent manner. Fibrin clots formed in the presence of fibrinogen from type 1 and type 2 diabetics are denser and less porous as compared with those from healthy individuals,<sup>151,152</sup> and these changes are inversely correlated with the glycemic control.

In addition, medication with metformin<sup>153</sup> and ASA<sup>154</sup> is also known to influence the fibrin clots structure.

### **Genetic determinants**

Based on data from a twin study it has been estimated that genetic heritability accounts for approximately 39% of the phenotypic variation in fibrin clot porosity.<sup>145</sup> Several fibrinogen gene variants have been reported to influence the plasma fibrinogen concentration<sup>69,72,78</sup> and therefore one might also expect an indirect effect on the fibrin clot structure. However, these data have been challenged, not least by genome wide

studies<sup>86,87</sup> and thus far none of the polymorphisms previously reported to influence plasma fibrinogen concentration have been found to influence the fibrin clot structure.

On the other hand, the FGA Thr312Ala polymorphism seems to influence the stability of the fibrin clot via mechanisms that are unrelated to the plasma fibrinogen concentration. This polymorphism is located close to the fibrinogen A $\alpha$ 303 and A $\alpha$ 328 residues where FXIIIa cross-links  $\alpha$ 2-antiplasmin<sup>113,155</sup> and fibrinogen A $\alpha$  chains,<sup>156</sup> respectively. The formation of fibrinogen A $\alpha$ -A $\alpha$  cross-links and incorporation of  $\alpha$ 2-antiplasmin, which gives strength to the clot and confers protection against proteolytic cleavage by plasmin,<sup>157,158</sup> might differ in the presence of the FGA Thr312Ala genotypes. Moreover, the FGA Thr312Ala polymorphism may interfere with the activation of FXIII, since it is localized in a region known to reduce the Ca<sup>2+</sup> concentration that is necessary for the dissociation of the A- and B-subunits at physiological levels.<sup>159</sup>

The rate of FXIII activation varies according to the FXIII Val34Leu polymorphism, located close to the thrombin cleavage site. In the presence of the FXIII 34Leu allele the activation rate increases<sup>160</sup> and fibrin clots with reduced fiber mass to length ratio and porosity are formed.<sup>161</sup> The FXIII Val34Leu polymorphism has been associated with risk of MI,<sup>162</sup> venous thrombosis<sup>163</sup> and intracranial hemorrhage.<sup>164</sup> Interestingly, a stepwise decrease in the rate of change of fibrin clot porosity with increasing plasma fibrinogen concentration has been noted in the presence of an increasing number of FXIII 34Leu alleles.<sup>165</sup> This observation may explain the puzzling protective effect of the FXIII 34Leu allele despite its

association with formation of an unfavourable fibrin clot network at low fibrinogen concentrations.<sup>165</sup>

## **Role of fibrin(ogen) in atherothrombosis**

### **Epidemiological evidence**

The epidemiological studies providing evidence to suggest that fibrinogen is an independent predictor of cardiovascular disease are remarkably consistent. Already in the 1950s it was found that the plasma fibrinogen concentration was higher in patients with ischemic heart disease than in healthy individuals<sup>166</sup> and that it may be a valuable index of acute MI.<sup>167</sup> Since the beginning of the 1980s compelling evidence has accumulated on this theme, mainly from prospective studies and meta-analyses, of which only a few will be briefly summarized.

The Northwick Park Heart Study (NPHS)<sup>168</sup> was one of the first studies undertaken to explore the relationship between haemostatic factors (e.g. fibrinogen, factor VII) and cholesterol with cardiovascular disease and death. A total of 1510 white men aged between 40-64 years and who had no history of cardiovascular disease were recruited for this purpose and after 4 years of follow-up it was reported that fibrinogen was independently associated with cardiovascular death.<sup>168</sup> After about 10 years of follow-up, the NPHS study reported that high concentrations of fibrinogen were associated, at least as strongly as cholesterol, with non-fatal and fatal MI.<sup>169</sup>

In the Gothenburg study a total of 792 men born in 1913 (54 years old at recruitment) were included.<sup>170</sup> After 13.5 years of follow-up it was reported, based on results from univariate analyses, that fibrinogen was a significant risk factor for MI.

However, the magnitude of this relationship was reduced when the effect of potential confounders (blood pressure, smoking habits and cholesterol concentration) was accounted for in multivariate analyses.

Also prospective data from the Caerphilly and Speedwell collaborative heart disease studies based on a combined cohort of 4860 middle-aged men from the general population in Caerphilly and Speedwell, indicated (after a follow-up period of 5.1 and 3.2 years, respectively) that fibrinogen was an independent risk factor for MI and the strength of this association was comparable to that of traditional risk factors.<sup>171</sup> A subsequent study based on the same cohort and performed after 10 years of follow-up, provided further evidence to suggest that hemostatic/inflammatory risk factors are at least as powerful as plasma lipids in predicting risk of MI.<sup>172</sup>

Until now, surprisingly few studies have addressed the potential role of plasma fibrinogen concentration as a risk predictor of MI in women. The Framingham study was one of the first to include women and after 20 years of follow-up it was reported that fibrinogen was an independent predictor of MI in both sexes, but was related to recurrent events only in men.<sup>173</sup> However, fibrinogen may be an important risk factor both for fatal and non-fatal coronary heart disease (CHD) in women, as evidenced by data derived from several other studies such as the Atherosclerosis Risk in Communities Study,<sup>174</sup> and the Scottish Heart Health Study.<sup>175</sup>

All the major meta-analyses published so far have unequivocally suggested that fibrinogen is an independent predictor of MI.<sup>176-180</sup> A total of 154 211 participants from 31 prospective studies were included in the most recent and comprehensive meta-analysis.<sup>180</sup> This particular study is robust

for several reasons: (1) the large sample size, (2) the results were based on individual participant data, (3) studies that recruited individuals having previous cardiovascular disease and individuals with known pre-existing CHD or stroke were excluded, thereby restraining any potential influence of clinical disease on plasma fibrinogen concentration, and (4) correction for the variation in plasma fibrinogen concentration was performed. According to the reported data, a 1g/L increase in plasma fibrinogen concentration yields an age- and sex- adjusted hazard ratio of 2.42 95% confidence interval (CI): (2.24, 2.69) for CHD. The magnitude of this association was attenuated, but remained significant, when the effect of potential confounders was accounted for [adjusted hazard ratio (95%CI): 1.82 (1.60, 2.06)]. Interestingly, the relationship between plasma fibrinogen concentration and CHD did not vary substantially according to the baseline levels of classical risk factors (e.g. smoking, blood pressure and serum lipid levels). These data strongly suggest that the association between fibrinogen and CHD is not simply a reflection of a relationship with other risk factors.

### **Clinical studies**

The plasma fibrinogen concentration has been associated with the presence and extent of silent atherosclerosis.<sup>181,182</sup> Moreover, a substantial increase in plasma fibrinogen concentration occurs during acute MI events<sup>167</sup> which are characterized by complex inflammatory responses.<sup>183</sup> Therefore, it has been argued that the elevated plasma fibrinogen concentrations observed in post-infarction patients may be simply a reflection of coronary artery disease (CAD) or of ongoing inflammatory processes, the latter being a hallmark of atherosclerosis.<sup>184</sup> Nevertheless, even if a raised plasma fibrinogen concentration might be a consequence of clinically overt

disease, it does not imply lack of contribution to its progression and/or exacerbation.

Notably, the extent of coronary atheromatosis in young male survivors of MI (aged <45 years) was more pronounced in those individuals with higher plasma fibrinogen concentrations.<sup>185</sup> As fibrinogen has a fairly high degree of genetic heritability<sup>44,66</sup> it is likely that a raised plasma fibrinogen concentration in individuals with CAD as evidenced by coronary angiography<sup>185</sup> is not only a reflection of the atheromatosis burden. Also, it is possible that in genetically predisposed individuals a more prominent raise in plasma fibrinogen concentration may occur in response to environmental challenges or disease related stimuli, which may be more important for the outcome than the basal concentrations. Worth mentioning is that already about three decades ago it was reported that re-infarctions tend to occur mainly in those individuals with the highest increase in plasma fibrinogen concentration (>7.5 g/L) during an acute MI event.<sup>186</sup> These data imply that some individuals have a higher propensity to plasma fibrinogen elevations and subsequently to MI. Moreover, both in patients with stable and unstable CAD, raised plasma fibrinogen concentrations have been associated with increased risk of future non-fatal and fatal cardiac events.<sup>186-188</sup> In addition, as fibrinogen is the main component of the fibrin clot, it plays a key role during acute MI events. *In vitro* formation of tight and rigid fibrin clot networks, most likely a consequence of elevated plasma fibrinogen concentrations, has *in vivo* been associated with myocardial infarction at a young age.<sup>150</sup>

In summary, despite the different time scales and the various study settings, the results from epidemiological and clinical

studies support the notion that fibrinogen is an independent risk factor for future cardiovascular events. Yet, the nagging question of causality remains to be addressed in proper epidemiological, clinical and/or experimental settings.

## Mechanisms

During the last decades a paradigm shift has occurred in relation to atherothrombosis as the critical role of pro-coagulant and pro-inflammatory factors in the context of atherosclerotic plaque formation and rupture gained recognition. Accordingly, a more comprehensive understanding of the etiology of this complex condition has been achieved.

Atherosclerotic lesion formation is preferentially initiated at sites of predilection,<sup>189</sup> i.e. where the morphology of the endothelial cells is altered due to unfavourable hemodynamic forces (e.g. shear stress and turbulent blood flow at arterial branches) and raised concentrations of blood lipids and inflammatory factors. Shear stress is proportional to the blood flow viscosity (Poiseuille's Law), of which fibrinogen is a major determinant. Therefore, increased plasma fibrinogen concentrations contribute to an increased hemodynamic strain on the vessel wall. Consequently, the endothelial cells express cell adhesion molecules (e.g. ICAM-1, vascular cell adhesion molecule 1),<sup>190</sup> chemotactic factors (e.g. monocyte chemoattractant protein 1 (MCP-1))<sup>191</sup> and growth factors (e.g. platelet-derived growth factor)<sup>192</sup> and lose their anticoagulant functions<sup>193</sup> in favour of an increased thrombogenicity. Fibrinogen binds to endothelial cells via ICAM-1 and elicits vasomotor responses, in a concentration dependent manner, probably by triggering signalling pathways that fuels the synthesis of vasoactive substances.<sup>194</sup> Moreover, fibrinogen is deposited in the subendothelial

extracellular matrix, also in a concentration dependent manner,<sup>195</sup> a process that has been shown *in vivo* to precede the fatty streak formation.<sup>196</sup>

In the subendothelial extracellular matrix, fibrinogen augments the shear stress induced nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation, thereby enhancing the endothelial cell response to perturbed blood flow.<sup>196</sup> Based on *in vitro* experiments it was demonstrated that fibrinogen induces the expression of MCP-1 and IL8 in endothelial cells through NF- $\kappa$ B activation.<sup>197</sup> MCP-1 plays a key role during atherogenesis since it recruits monocytes to the vessel wall. Altered endothelial cells express TF,<sup>198</sup> a key modulator of atherosclerotic plaque thrombogenicity,<sup>199</sup> which promotes the conversion of fibrinogen to fibrin within the vessel wall. Fibrin(ogen) stimulates endothelial cells to secrete vWF from Weibel-Palade bodies<sup>200</sup> and to proliferate.<sup>131</sup> Also, fibrin(ogen) may exert other profound effects on the vasculature as demonstrated in a transgenic mouse model of hyperfibrinogenemia<sup>201</sup> that enables studies of cause-effect relationships between raised plasma fibrinogen concentrations and vascular disease in the absence of underlying inflammation.<sup>202</sup> In these mice, a modest increase in plasma fibrinogen concentration was associated with increased fibrin deposition within the arterial tree and with marked neointimal hyperplasia.<sup>201</sup>

Increased endothelial cell permeability allows LDL particles to diffuse into the subendothelial space<sup>203</sup> whereupon they undergo several modifications (oxidation, aggregation and proteolysis). Fibrin(ogen) contributes to the sequestration of LDL<sup>204</sup> and in particular of Lp(a), a highly atherogenic molecule.<sup>205</sup> The tendency of Lp(a) to accumulate in the arterial wall is

highly dependent on its binding to fibrin as evidenced by an *in vivo* study in mice.<sup>206</sup> In this study it was demonstrated that in the presence of altered binding sites for fibrin the accumulation of Lp(a) in the vessel wall is significantly reduced. The pro-atherogenic effects of Lp(a) have been ascribed to the competitive inhibition of plasminogen<sup>207</sup> with impaired fibrin clot lysis<sup>208</sup> and smooth muscle cell (SMC) proliferation<sup>209</sup> as a result.

Oxidized LDL stimulates the endothelial cells to produce more adhesion molecules, chemotactic factors and growth factors, hence promoting recruitment of monocytes to the subendothelial space. Molecular interactions between fibrin(ogen) and the integrin  $\alpha$ M $\beta$ 2 receptor<sup>139,210</sup> facilitate the accumulation of monocytes in the vessel wall, whereupon they differentiate into macrophages which express toll-like receptors and scavenger receptors. Fibrin(ogen) stimulates macrophages to produce more chemokines such as MCP-1 by binding to toll-like receptors,<sup>132</sup> and may therefore contribute to the exacerbation of the inflammation in the vessel wall. Oxidized LDL increases further the thrombogenicity of the lesion by stimulating production of TF in endothelial cells<sup>211</sup> and SMCs<sup>212</sup> and by contributing with phospholipid surfaces that support the activities of the tenase<sup>213</sup> and prothrombinase<sup>214</sup> complexes. Macrophages engulf modified LDL particles via scavenger receptors and are gradually transformed into foam cells leading to formation of fatty streaks.

SMCs play a critical role in plaque progression. SMCs are responsible for the production of collagen, which is the major component of the extracellular matrix and of the fibrous cap. In addition, *in vitro* experiments have demonstrated that SMCs produce PAI-1 in response to platelet-

derived growth factor and transforming growth factor- $\beta$ .<sup>215</sup> Also SMCs express TF on their surface,<sup>198</sup> hence promoting both pro-coagulant and anti-fibrinolytic forces within the vessel wall. Fibrin(ogen) has the ability to stimulate SMC migration and proliferation,<sup>216,217</sup> and may therefore contribute to the enlargement of the atherosclerotic plaque. The nature of the fibrin scaffold seems to influence the migration of the SMCs as suggested by *in vitro* data showing that SMCs migrate much faster into cross-linked fibrin clots as compared to non cross-linked clots.<sup>218</sup>

Fibrin(ogen) promotes angiogenesis,<sup>91</sup> and it may be presumed that the new vessels facilitate further accumulation of pro-atherogenic molecules and cells in the atherosclerotic plaque. Cytokines such as IL6 are produced during all stages of atherosclerotic plaque formation. IL6 amplifies the systemic inflammatory response by stimulating production of fibrinogen and CRP. Locally, IL6 contributes to endothelial dysfunction<sup>219</sup> and exacerbates the development of the atherosclerotic plaque.<sup>220</sup> Fibrin(ogen) might contribute to the local enrichment of IL6 by facilitating the vascular endothelial growth factor mediated production of this pro-inflammatory cytokine in monocytes.<sup>221</sup> The accumulation of pro-inflammatory and pro-thrombotic components leads to a progressive loss of the stability of the plaque. Macrophage-derived collagenases (e.g. matrix metalloproteinase 1 and 13), produced in response to pro-inflammatory cytokines, render the plaque even more vulnerable by degrading the extracellular matrix, thus leading to collagen loss in the fibrous cap.<sup>222</sup> Notably, an elevated plasma fibrinogen concentration was found to be significantly associated with high numbers of inflammatory cells in advanced atherosclerotic plaques and with reduced thickness of the fibrous cap.<sup>223</sup>

Apoptotic endothelial cells, SMCs and foam cells provide a PS-rich lipid core that supports pro-coagulant processes and are major sources of TF<sup>224</sup> in advanced atherosclerotic lesions. Thus, the thrombogenicity of the atherosclerotic plaque increases across all stages of lesion formation and also reflects the loss of plaque stability as evidenced by higher TF concentrations in coronary atherectomy specimens from patients with unstable angina than in those with stable angina pectoris.<sup>225</sup> Moreover, the TF expression is co-localized with the distribution of fibrin in advanced coronary plaques in patients with unstable angina pectoris.<sup>226</sup> Rupture prone plaques have a thin fibrous cap, numerous macrophages and a prominent thrombogenic lipid core.

When plaque rupture or endothelial erosion occurs, platelets adhere and are rapidly activated at the affected site. Fibrinogen promotes platelet aggregation via interactions with the integrin GPIIb/IIIa receptor and augments their degranulation,<sup>227</sup> resulting in release vWF, fibrinogen, FV, PAI-1 and adenosine diphosphate at the site of injury. Exposure of TF to the blood triggers the formation of a thrombus that may occlude the lumen of the affected vessel. Thrombus formation on a ruptured or eroded coronary plaque (i.e. atherothrombosis) plays a critical role in acute coronary syndromes, i.e. unstable angina pectoris, non-fatal and fatal MI. Fibrinogen and fibrinogen-derived products seem to influence the infarct size and the ischemia induced reperfusion injury as evidenced by studies in fibrinogen knockout mice which display substantially smaller infarct sizes than wild-type specimens.<sup>228</sup>

The structure of the thrombus of which fibrin is a major component is essential for the outcome of acute coronary events. Increased plasma fibrinogen concentration

in young male survivors of a first MI has been associated with formation of a tight and rigid fibrin clot structure with reduced porosity<sup>148</sup> that seems to be less susceptible to fibrinolysis.<sup>229</sup> The fibrinogen  $\gamma'$  chain variant has been demonstrated to form fibrin clots characterized by reduced susceptibility to fibrinolysis,<sup>32,230</sup> which may explain the observed association between elevated plasma fibrinogen  $\gamma'$  concentration and CAD.<sup>231</sup>

Altogether, the results derived from *in vitro* experiments, various animal models and clinical studies unequivocally implicate fibrinogen in the pathophysiology of atherothrombosis. Since even in the best of circumstances sustainable lifestyle changes are difficult to accomplish this knowledge might equip us with alternative and novel treatment strategies specifically targeted against the molecular and cellular mediators of risk factors such as smoking.

### **Treatment**

Unhealthy lifestyle factors (i.e. smoking, poor dietary habits and physical inactivity) together with risk factors such as hypertension, diabetes mellitus, dyslipidemia, obesity and psychosocial stress, are major contributors to the risk of MI worldwide.<sup>1</sup> Most likely, these risk factors have a higher impact on genetically predisposed individuals. Luckily enough, the majority of these factors are reasonably amenable to control and the evidence to suggest that significant benefit can be achieved by preventive lifestyle interventions is both convincing and encouraging.

The means by which the various risk factors influence the risk of MI are as yet not fully understood. However, lifestyle factors such as smoking and unhealthy dietary habits lead to unfavourable blood rheology and lipid profiles, inculcating factors such as blood viscosity, fibrinogen and LDL-

cholesterol in the etiology of MI. Conversely, adequate lifestyle changes exert favourable effects on risk factors for MI such as the plasma fibrinogen concentration.<sup>232</sup>

A substantial proportion of the increase in risk of cardiovascular disease in smokers has been ascribed to the effect of this habit on the plasma fibrinogen concentration.<sup>50,233</sup> Upon smoking cessation a gradual decrease in plasma fibrinogen concentration occurs<sup>50</sup> that parallels a reduction in the risk of cardiovascular disease and an increased life time expectancy.<sup>234</sup> More prudent dietary habits such as the Mediterranean diet,<sup>235</sup> which appeared to reduce the risk of recurrence after a first MI,<sup>236</sup> and weight reduction<sup>50</sup> may also reduce the plasma fibrinogen concentration. However, the fibrinogen lowering effects of the Mediterranean diet have been recently questioned in patients with CAD.<sup>237</sup> A decrease in plasma fibrinogen concentration has been observed in individuals that exercise regularly.<sup>52,54</sup> These data imply that the benefit of physical activity on CAD may be partly ascribed to the reduction in hemostatic factors, including fibrinogen.<sup>54</sup> Moderate consumption of alcohol has also been associated with lowered plasma fibrinogen concentration.<sup>52,53</sup> It has been suggested that fibrinogen may be the link between the protective effect of moderate alcohol consumption and cardiovascular disease.<sup>238</sup> Nevertheless, it could be risky to encourage moderate consumption of alcohol as a means of preventing MI, since the number of heavy drinkers might increase.

Antihypertensive medication has been demonstrated to reduce the risk of atherothrombotic events.<sup>239</sup> In addition to lowering the blood pressure, angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers seem to

decrease the TF expression and to hinder the accumulation of monocytes in atherosclerotic plaques.<sup>240,241</sup> In a very modestly sized cohort (54 patients) of previously untreated hypertensives, a significant reduction in plasma fibrinogen concentration was observed in individuals treated with either a  $\beta_1$ -receptor blocker (atenolol) or an angiotensin II receptor blocker (irbesartan).<sup>242</sup> However, these data remain inconclusive since conflicting results have also been reported<sup>243,244</sup> and the means by which antihypertensive therapy may influence the plasma fibrinogen concentration are elusive in absence of any experimental support.

The evidence, derived from both primary and secondary studies, suggesting that therapy with statins significantly reduces the risk of cardiovascular events is compelling.<sup>245-247</sup> It is notable in this context that a reduction of the risk of a first coronary event occurs even in individuals with average concentrations of LDL-cholesterol<sup>246</sup> lending support to the view that “the lower the better”. However, statins do not seem to influence the plasma fibrinogen concentration.<sup>62</sup> On the other hand, treatment with another lipid-lowering drug, namely bezafibrate,<sup>60,61</sup> has been observed to reduce the plasma fibrinogen concentration. In young male (age <45 years) survivors of precocious MI, treatment with bezafibrate resulted in a 12% decrease in plasma fibrinogen concentration and appeared to hamper the progression of CAD.<sup>248</sup> Experimental data indicate that the mechanism by which fibrates reduce plasma fibrinogen concentration involves PPAR- $\alpha$ .<sup>21,249</sup> Of note, a significant interaction between PPAR- $\alpha$  genotypes and baseline plasma fibrinogen concentrations in determining the decrease of fibrinogen caused by treatment has been reported<sup>250</sup> emphasizing the hidden genetic complexity of these type of effects.

Needless to say, good metabolic control, in particular in individuals with overt diabetes mellitus, is crucial in preventing the cardiovascular complications associated with this disease. Poor metabolic control has been associated with increased plasma fibrinogen concentration<sup>251</sup> and abnormal fibrin clot structure.<sup>151,252</sup> Notably, the latter observation has been reported to occur in diabetics despite normal plasma fibrinogen concentrations<sup>151</sup> and may involve glycosylation of fibrinogen<sup>59</sup> yielding an altered functionality of the protein. Medication with metformin, which has been shown to be cardioprotective,<sup>253</sup> has been associated with decreased plasma fibrinogen concentration.<sup>254</sup> However, conflicting data have also been reported,<sup>255,256</sup> and recent data indicate that an intensive lifestyle intervention in individuals with impaired glucose tolerance has a significantly higher fibrinogen lowering effect as compared to treatment with metformin.<sup>257</sup> On the other hand, metformin seems to interfere with fibrin polymerization and to decrease the FXIII cross-linking activity resulting in formation of clots that may be more susceptible to fibrinolysis.<sup>153</sup> Also, treatment with insulin has been reported to result in formation of fibrin clots with a favourable structure, i.e. increased porosity, which seemed to be related to changes in the blood lipid profile rather than to improvement of the glycemic control.<sup>258</sup>

Antithrombotic treatment with ASA has been associated with a significant decrease in risk of non-fatal and fatal MI both in primary<sup>259</sup> and secondary<sup>260</sup> prevention trials and plays a key role during acute coronary events. The beneficial effect of ASA has been ascribed to inhibition of cyclooxygenase-1, which prevents the formation of thromboxane A<sub>2</sub> resulting in reduced platelet aggregation. In addition, ASA acetylates plasma proteins, including

fibrinogen,<sup>261</sup> and this modification has been associated with formation of fibrin clots with increased porosity<sup>262</sup> and reduced clot lysis time.<sup>263</sup> The adenosine diphosphate receptor inhibitor, clopidogrel, which also has a given place in the prevention and treatment of atherothrombotic events, seems to lower the plasma fibrinogen concentration to a similar extent as ASA.<sup>63</sup>

The cornerstones of reperfusion therapy during acute atherothrombotic events

involve the administration of antiplatelet treatment (i.e. ASA and/or the glycoprotein GPIIb/IIIa receptor inhibitor such as Abciximab) and fibrin-selective or non-fibrin-selective thrombolytic agents (i.e. recombinant t-PA, streptokinase and alteplase, respectively). However, percutaneous coronary interventions have been associated with better clinical outcomes than pharmacological reperfusion<sup>264</sup> and are increasingly used in coronary intensive care units.

## AIMS OF THE THESIS

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- (I) to investigate the contribution of plasma fibrinogen and fibrinogen  $\gamma'$  concentrations to the variation in risk of MI
- (II) to identify genetic variants in the fibrinogen gene cluster that may contribute to the variation in plasma fibrinogen and fibrinogen  $\gamma'$  concentrations and fibrin clot structure, and therefore to the risk of MI
- (III) to study gene-gene and gene-environment interactions in relation to MI

## MATERIAL AND METHODS

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### Subjects

#### **The HIFMECH study (paper I)**

The Hypercoagulability and Impaired Fibrinolytic function MECHANisms predisposing to myocardial infarction (HIFMECH) study, is a multi-centre case-control study designed to identify genetic and environmental factors that may contribute to the differences in risk of MI between the North and the South of Europe. The HIFMECH cohort comprises male, Caucasian survivors of a first MI aged under 60 years (excluding patients with familial hypercholesterolaemia and insulin-dependent diabetes mellitus) and age-matched healthy individuals from the same catchment areas recruited from: Stockholm (STO) and London (LON) representing the North of Europe and Marseille (MAR) and San Giovanni Rotondo (SGR) representing the South of Europe. The participation rate was 79% for eligible patients and 84% for controls. A total of 533 postinfarction patients and 575 controls were included in the present study. Postinfarction patients were examined 3 to 6 months after the acute event. Both patients and controls were investigated in parallel in the early morning after an overnight fast. The participants completed a questionnaire regarding lifestyle (e.g. smoking habits, alcohol consumption) and weight, height and systolic and diastolic blood pressure were measured.

#### **The SCARF study (papers II-IV)**

The Stockholm Coronary Atherosclerosis Risk Factor (SCARF) study is a case-control study, designed to form the basis for studies of genetic and biochemical factors predisposing to precocious MI. A total of

387 survivors of a first MI aged less than 60 years who had been admitted to the coronary care units of the three hospitals in the northern part of Stockholm (Danderyd Hospital, Karolinska University Hospital Solna and Norrtälje Hospital) were included. Briefly, unselected patients meeting the inclusion criteria were enrolled, and the exclusion criteria were type 1 diabetes mellitus, renal insufficiency (defined as plasma creatinine  $>200$   $\mu\text{mol/L}$ ), any chronic inflammatory disease, drug addiction, psychiatric disease or inability to comply with the protocol. For each postinfarction patient a sex- and age-matched control person was recruited from the general population (response rate 79%). Three months after the index cardiac event, both patients and controls underwent medical examination and blood samples were drawn following an overnight fast. Background data (e.g. social situation, lifestyle, medical history and medication) were collected by means of a structured interview. Ethnicity was recorded on the basis of self-reported origin as far as 3 generations back and more than 99% of the participants in the study were considered Caucasians.

#### **Coronary angiography**

All patients included at two of the hospitals ( $n=269$ ) were offered routine coronary angiography. A total of 243 postinfarction patients (90%) agreed to be included in the coronary angiography substudy. Coronary angiography was performed during the initial admission, if needed for clinical reasons ( $n=35$ ), or otherwise three months later ( $n=208$ ). Angiograms divided into 15 coronary segments were analyzed by quantitative coronary angiography (QCA)

using the Medis QCA-CMS system. In each segment, reference diameter, minimum lumen diameter (MLD), percentage diameter stenosis, mean segment diameter (MSD), segment length, plaque area, segment area and number of significant (>50%) stenoses were measured.

### **The SHEEP study (paper V)**

The Stockholm Heart Epidemiology Program (SHEEP) study is a large population-based case-control study aiming to investigate genetic, biochemical and environmental factors predisposing to MI. Potential study participants (age range 45-70 years) were all Swedish citizens living in Stockholm County without a previous clinical diagnosis of MI. Male cases were recruited between 1992-1993 and female cases between 1992-1994. The criteria for MI diagnosis were based on guidelines issued by the Swedish Society of Cardiology in 1991 and included: (1) typical symptoms; (2) marked elevation of the enzymes serum creatine kinase (S-CK) and lactate dehydrogenase (LDH) and (3) characteristic electrocardiogram changes. If two of the three criteria were fulfilled, the patient was diagnosed with MI. For each postinfarction patient a randomly selected healthy individual was recruited within two days of the case event, after matching for age, sex and catchment area. The present sub-study was based on a database and biobank comprising a total of 1213 cases (852 men and 361 women) and 1561 controls (1054 men and 507 women). Blood samples were collected approximately three months after the index cardiac event in the patients and all participants underwent physical examination.

### **Biochemical analyses**

Plasma fibrinogen concentration was determined by the Clauss method<sup>265</sup> (papers I-IV) and as described by Vermylen<sup>266</sup> (paper V). Plasma fibrinogen  $\gamma'$  concentra-

tion was measured by an ELISA assay using a monoclonal antibody (2.G2.H9) directed against  $\gamma'$  chains that does not cross-react with  $\gamma$ A chains (modification of the assay described by Lovely et al<sup>231</sup>) and for comparison purpose with a second ELISA essentially as described by de Willige et al<sup>267</sup> (paper IV). Plasma cholesterol and triglyceride concentrations were determined by enzymatic methods (paper I and V), whereas a combination of preparative ultracentrifugation and precipitation of apolipoprotein B-containing lipoproteins followed by lipid analyses was used for analyses of plasma lipoproteins (papers II-IV). Insulin concentration was determined by a specific in-house 2-site immuno-enzymometric assay (paper I),<sup>268</sup> by ELISA (paper II-IV) and by radioimmunoassay (paper V). Serum IL6 and Lp(a) concentrations were measured by using ELISA (papers I-V and paper I, respectively). CRP was determined by using an immunonephelometric assay (papers I-IV).

### **Genetic analyses**

Genomic DNA was extracted by using the salting-out method (paper I),<sup>269</sup> the QIAGEN Blood and Cell Culture DNA kit (QIAGEN Ltd, Crawly, UK) (papers II-IV) and the RapidPrep Macro Genomic DNA Isolation Kit (Pharmacia Biotech, Sweden) (paper V).

### **Sequencing**

The following gene segments were sequenced in a total of 34 survivors of a first MI (age <45 years):<sup>150</sup> in the FGG gene the promoter region, exons 1-4 and 9-10, and the 3' end, in the FGA gene the promoter region and exons 1, 2 and 5, and in the FGB gene exon 1 (paper II). The sequencing was performed using the Taq DyeDeoxy Terminator Sequencing System (Perkin Elmer, Applied Biosystems Division, Foster City, CA) or the Big Dye

**Table 1. Reference sequences and SNP IDs**

Gene symbol <sup>1</sup>	GenBank		Position <sup>3</sup>	Alleles	dbSNP ID	SNP type	Published name
	Accession number	Position <sup>2</sup>					
FGA	AF361104	2224	-58	G>A	rs2070011	Promoter	-3G/A
		6534	991	A>G	rs6050	Codon	Thr312Ala
FGB	AF388026	1744	-165	C>T	rs1800787	Promoter	-148C/T
		1643	-257	C>T	rs1800788	Promoter	-249C/T
		1437	-463	G>A	rs1800790	Promoter	-455G/A
		1038	-862	G>A	rs1800791	Promoter	-854G/A
		899	-1001	C>T	rs2227389	Promoter	-993G/A
		472	-1428	G>A	rs1800789	Promoter	-1420G/A
FGG	AF350245	902	-647	A>G	rs1800792	Promoter	-
		9340	1299+79	T>C	rs1049636	Intron	7792T/C
		9615	1300-189	T>C	rs2066864	Intron	-
		F13A1	AF418272	4377	103	G>T	rs5985
IL6	AF372214	1510	-174	G>C	rs1800795	Promoter	-174G/C

<sup>1</sup>Gene symbol approved by the HUGO Gene Nomenclature Committee. <sup>2</sup>Nucleotide numbering according to the Seattle SNPs database; <sup>3</sup>Nucleotide numbering using cDNA as reference sequence, nucleotide +1 being nucleotide A of the ATG-translation initiation codon.

Terminator v3.1 cycle sequencing kit (3100 Genetic Analyzer, Applied Biosystems).

Numbering of the SNPs detected in paper II was performed according to nomenclature recommendations,<sup>270</sup> using cDNA as reference sequence, nucleotide +1 being the A of the ATG-translation initiation codon (paper II).

### Genotyping

The SNPs that have been determined are presented in Table 1.

In paper I, genotyping for the  $\beta$ -fibrinogen -455G/A polymorphism with the correct name according to the nomenclature recommendations: FGB -463G>A (FGB 1437 G>A [rs1800790] SNP; Table 1), was performed by restriction fragment length polymorphism (RFLP) analysis as described.<sup>70</sup> Genotyping for the other FGB SNPs included in paper I and for the polymorphisms included in papers II-IV

was performed using the Taqman PCR method (Applied Biosystems). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry technology (SEQUENOM Inc., San Diego, CA) was used in paper V.<sup>271</sup>

### Determination of fibrin clot structure.

In paper III, the physical properties of the fibrin clot structure, formed *in vitro* from plasma samples, were determined in 60 control subjects selected based on homozygosity for either the minor FGG 9340C allele (n=30) or the major FGG 9340T allele (n=30). None was taking acetylsalicylic acid, anticoagulants or glucose-lowering compounds. The permeability coefficient (Ks, cm<sup>2</sup>), reflecting fibrin clot porosity, was determined as described.<sup>147</sup> Briefly, plasma samples were dialyzed against TNE-buffer (0.05 mol/L Tris, 0.1 mol/L NaCl, 1mmol/L EDTA-buffer with aprotinin 1 KIU/mL, pH=7.4). 1.5 mL of the dialyzed plasma was recalcified and thrombin was

added giving final concentrations of 20 mM for CaCl<sub>2</sub> and 0.25 NIH U/mL for thrombin. The gels were allowed to mature in cuvettes for 18-24 hours at room temperature. Flow measurements were then performed using a Tris-Imidazol buffer (0.02 mol/L Tris, 0.02 mol/L Imidazole, aprotinin 5 KIU/mL, 0.1 mol/L NaCl, pH=7.4) that was percolated through the gels for a given time and at different hydrostatic pressures (dyne/cm). The Ks value was calculated according to the formula:  $Ks=Q \cdot L \cdot \eta / (T \cdot A \cdot \Delta P)$ , where Q is the volume of liquid (cm<sup>3</sup>) with the viscosity  $\eta$  (poise) flowing through a clot of length L (cm) and an area A (cm<sup>2</sup>) in time T (s) under a pressure gradient  $\Delta P$  (dyne/cm<sup>2</sup>). The inter-assay coefficient of variation for Ks was 11.3%.

### Statistical analyses

Statistical analyses were conducted using the STATA package (Intercooled Stata 6.0, STATA Corp., College Station, TX, USA) (paper I) and the StatView package (SAS Institute, Inc, version 5.0.1 for Windows) (papers II-V).

Measurements with a skewed distribution are presented as median and interquartile range (IQR) or as geometric means with either standard deviations (SD) or 95% confidence intervals (CI) and were normalized by square root or logarithmic transformation before entering statistical analysis. The Mann-Whitney U test, t-tests, Kruskal-Wallis test, analysis of variance (ANOVA) and  $\chi^2$  tests were used for group comparisons.

The Hardy-Weinberg equilibrium was assessed using  $\chi^2$  tests. Multilocus Hardy-Weinberg equilibrium analyses were performed using the Genetic Data Analysis program.<sup>272</sup> Comparisons of genotype and allele frequency distributions between groups were made by using  $\chi^2$  tests and/or the Metropolitan method.<sup>273</sup> The normalized

pairwise LD coefficients ( $|D'|$ ) were calculated by the method of Chakravarti et al<sup>274</sup> or the ASSOCIATE software<sup>275</sup> and visualized using the Graphical Overview of Linkage Disequilibrium (GOLD) software. Haplotypes were inferred using the PHASE package, version 2.0.2<sup>276,277</sup> and the THESIAS programme, version 2.<sup>278</sup> Recombination rates between consecutive pairs of SNPs were estimated using the PHASE package, version 2.0.2.<sup>276,277</sup>

Correlations between variables were estimated by calculation of Pearson correlation or Spearman rank correlation coefficients. Multiple stepwise regression analysis was performed in order to identify the determinants of total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations and fibrin clot porosity. The proportion of variation accounted for by individual variables was derived by calculation of adjusted R<sup>2</sup>. Differences between regression lines were assessed using extended analysis of covariance (paper III).

In paper I, standardized odds ratios (SORs) for a 1SD increase in plasma fibrinogen were calculated using conditional logistic regression analysis, hence considering the matching of cases to controls on both centre and age, and the corresponding probability values are from likelihood ratio tests. In papers II-V, ORs and SORs for a 1SD increase in plasma fibrinogen and fibrinogen  $\gamma'$  concentration were calculated using unconditional logistic regression analysis. Analyses in which the effect of potential confounders was accounted for were also performed.

Pair-wise gene-gene interactions on intermediate phenotypes (total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations) were evaluated by ANOVA and as described by Cheverud and Routman.<sup>279</sup>

Gene-gene and gene-environment interactions on risk of MI were evaluated using the multifactor dimensionality reduction (MDR) method.<sup>280</sup> MDR is a nonparametric and genetic-free approach that reduces high dimensional genetic and environmental data into a single dimension, thus circumventing the limited ability of logistic regression analysis to detect high-order interactions due to sparseness of data in high dimensions. The data was analysed 10 times with different random seeds, each time using 10-fold cross-validation intervals in order to ensure that the analysis was not influenced by chance division of the data. The average cross-validation consistency (CVC) and the average prediction error (PE) across all runs are presented in the final model. Statistical significance was determined by the permutation and sign tests implemented in the MDR software.

## RESULTS

### Fibrinogen and MI in a cross-cultural context (paper I)

The present study was undertaken in order to assess whether fibrinogen contributes to the differences in propensity to MI between individuals recruited from STO and LON (North of Europe) and MAR and SGR (South of Europe).

Taken as a group, the patients had a greater cardiovascular risk factor burden, that was accounted for by higher BMI, a higher proportion of current and ex smokers, elevated plasma triglyceride levels and a substantial proportion of persons with type 2 diabetes compared with controls. The concentrations of IL6 and CRP were significantly higher in cases than in controls. As a consequence of risk factor modification (27% were on lipid-lowering compounds and 84% on medication influencing blood pressure), similar values for blood pressure and plasma cholesterol concentration were observed in postinfarction patients and controls. However, statistically signifi-

cant differences between the centres were observed for the use of lipid-lowering drugs and beta-blockers (results not shown). Moreover, the distribution of risk factors differed between centres both among cases and controls.

The plasma fibrinogen concentration was significantly higher in cases compared with controls ( $p < 0.001$ ) and differed between centers in controls ( $p = 0.005$ ) but not in cases ( $p = 0.17$ ) (Table 2). Patients had significantly higher plasma fibrinogen concentration than controls in STO ( $p = 0.006$ ), LON ( $p < 0.001$ ) and SGR ( $p < 0.001$ ) but not in MAR ( $p = 0.60$ ). There was no evidence of a North-to-South difference in plasma fibrinogen in either cases ( $p = 0.16$ ) or controls ( $p = 0.55$ ). The unadjusted SOR for plasma fibrinogen concentration differed significantly between the centers ( $p = 0.006$ ) but not between the two regions ( $p = 0.58$ ). The SOR remained significant in LON, but not in the other centers, after adjustment for BMI, smoking, triglycerides, Lp(a)

**Table 2. Plasma fibrinogen concentrations in postinfarction patients and healthy individuals grouped according to centre and region in Europe with standardized odds ratios**

Centre	Fibrinogen (mg/dl)			SOR (95% CI), p-value <sup>3</sup>			
	Cases	Controls	p-value	Unadjusted	Adjusted for traditional risk factors <sup>4</sup>	Adjusted for traditional risk factors <sup>4</sup> and CRP	Adjusted for traditional risk factors <sup>4</sup> and IL-6
STO	371 (82)	347 (76)	0.006	1.41 (1.10;1.81), 0.005	1.33 (0.98;1.79), 0.06	1.13 (0.74;1.73), 0.57	1.29 (0.91;1.83), 0.15
LON	397 (88)	334 (58)	<0.001	2.92 (1.57;5.42), <0.001	3.58 (1.31;9.83), 0.002	3.89 (1.27;11.88), 0.002	3.08 (1.08;8.77), 0.01
MAR	363 (106)	356 (75)	0.60	1.10 (0.81;1.47), 0.54	0.82 (0.47;1.44), 0.49	0.81 (0.46;1.45), 0.48	0.54 (0.28;1.05), 0.06
SGR	367 (94)	329 (63)	<0.001	1.82 (1.41;2.33), <0.001	1.32 (0.92;1.91), 0.13	1.20 (0.82;1.77), 0.34	1.22 (0.80;1.85), 0.35
North	377 (84)	343 (71)	<0.001	1.64 (1.30;2.06), <0.001	1.50 (1.15;1.96), 0.002	1.60 (1.11;2.30), 0.007	1.47 (1.09;2.00), 0.009
South	366 (98)	339 (68)	<0.001	1.51 (1.25;1.82), <0.001	1.14 (0.85;1.53), 0.37	1.09 (0.81;1.47), 0.56	1.02 (0.73;1.42), 0.93
All	371 (92)	341 (69)	<0.001	1.56 (1.35;1.80), <0.001	1.27 (1.06;1.52), 0.009	1.22 (0.99;1.50), 0.06	1.16 (0.95;1.42), 0.14
p-value	0.17 <sup>1</sup>	0.005 <sup>1</sup>		0.006 <sup>5</sup>	0.02 <sup>5</sup>	0.06 <sup>5</sup>	0.06 <sup>5</sup>
	0.16 <sup>2</sup>	0.55 <sup>2</sup>		0.58 <sup>6</sup>	0.25 <sup>6</sup>	0.15 <sup>6</sup>	0.41 <sup>6</sup>

STO, Stockholm; LON, London; MAR, Marseille; SGR, San Giovanni Rotondo. Values are mean (SD) in milligram per deciliter. P-values for differences between cases and controls <sup>1</sup>by centre and <sup>2</sup>by region. <sup>3</sup>Standardized odds ratio (SOR, odds ratio based on a 1SD increase in square root transformed fibrinogen level) and p-value from conditional logistic regression. <sup>4</sup>Adjusted for smoking, BMI, Tg, Lp (a) and insulin. <sup>5</sup>p-value for interaction between centre status and fibrinogen on risk, <sup>6</sup>p-value for interaction between region status and fibrinogen on risk of MI.

**Table 3. Stepwise regression analysis of determinants of plasma fibrinogen concentration in postinfarction patients and healthy individuals, conducted for individual centres in Europe**

Variable	Cases				Controls			
	STO	LON	MAR	SGR	STO	LON	MAR	SGR
BMI <sup>1</sup>	4.2*						4.5*	
IL6 <sup>1</sup>	10.0***	29.3***	5.3*	3.0	12.0***	4.8	16.6***	15.2**
-455G/A	3.0*	15.7**						
Multiple adjusted R <sup>2</sup>	19.0	35.6	4.2	1.9	12.0	3.3	20.0	14.3

STO, Stockholm; LON, London; MAR, Marseille; SGR, San Giovanni Rotondo. BMI, body mass index; IL6, interleukin 6. <sup>1</sup>Partial R<sup>2</sup> for a 1 SD increase in the transformed variable. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

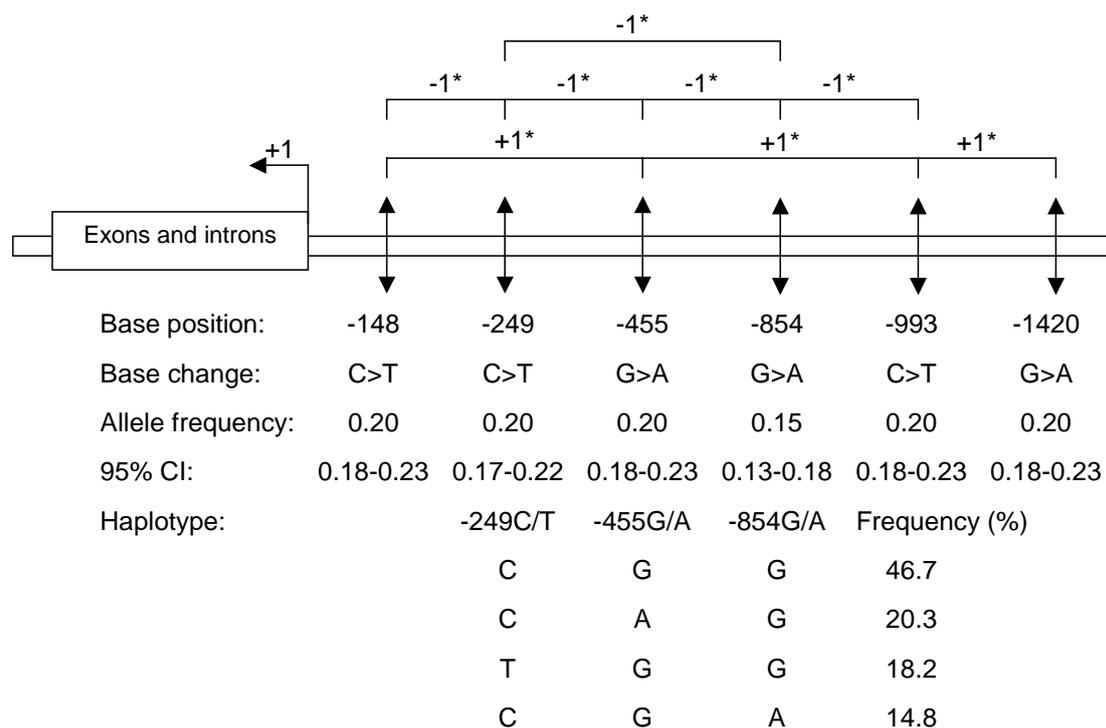
and insulin [SOR (95%CI): 3.58 (1.31, 9.83)] and CRP [3.89 (1.27, 11.88)] or IL6 [3.08 (1.08, 8.77)] (Table 2).

Six FGB promoter SNPs were determined in this study: -148C/T [rs1800787], -249C/T [rs1800788], -455G/A [rs1800790], -854G/A [rs1800791], -993C/T [rs2227389] and -1420G/A [rs1800789], GenBank accession number AF388026. All SNPs were in Hardy-Weinberg equilibrium. There were no significant differences in genotype frequency distribution between cases and controls or between the centers. The -148C/T, -455G/A, -993C/T and -1420G/A SNPs were in perfect LD with each other and in negative LD with the -249C/T and -854G/A SNPs. Therefore, only three of the SNPs (-249C/T, -455G/A and -854G/A) were considered further in the statistical and haplotype analyses.

The -455G/A SNP appeared to influence the plasma fibrinogen concentration in patients (p<0.001) but not in controls (p=0.06). No significant effect on the plasma fibrinogen concentration was observed for the -249C/T and -854G/A SNPs, either in cases (p=0.47 and p=0.90, respectively) or in controls (p=0.14 and p=0.91, respectively). The plasma fibrinogen concentration differed

significantly according to smoking status in controls, being higher in current smokers than in ex smokers and non-smokers (p<0.001), but not in patients (p=0.19). Smoking status did not influence the plasma fibrinogen concentrations differently across the centres in either cases (p=0.66) or controls (p=0.95).

In this study, potential determinants of plasma fibrinogen concentration were searched for. In patients, IL6 (8.6%), centre (3.6%), and the -455G/A genotype (2.4%) emerged as independent determinants, accounting for 14.6 % (11.5 % adjusted) of the variation in plasma fibrinogen concentration. In controls, IL6 (8.9%), centre (3.1%), smoking (1.9%) and BMI (1.2%) were independent predictors, together accounting for 15.1 % (15.7% adjusted) of the variation in plasma fibrinogen concentration. The contribution of IL6 to the plasma fibrinogen concentration in the controls was significant in STO, MAR and SGR, but not in LON (Table 3). In patients, IL6 contributed almost to one third of the variation of fibrinogen in LON, 10% in STO and only 5.3% and 3.0% respectively in MAR and SGR. The



**Figure 6. Schematic presentation of all polymorphisms studied in the FGB promoter.** Allelic associations (\*delta',  $p < 0.005$ ) between polymorphic sites in HIFMECH control subjects are given. Haplotypes with the respective frequencies are also presented.

contribution of IL6 to the plasma fibrinogen concentration in patients from STO and LON was accompanied by a significant effect of the -455G/A polymorphism in these centres.

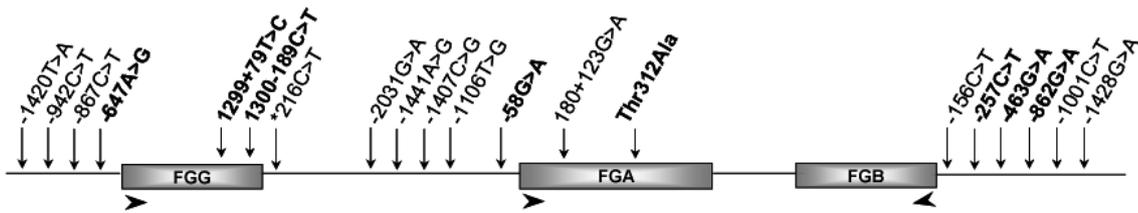
Haplotype analyses using genotype data from the -249C/T, -455G/A and -854G/A polymorphisms revealed the presence of four common haplotypes in the HIFMECH sample: CGG (46.7%), CAG (20.3%), TGG (18.2%) and CGA (14.8%; Figure 6). Across all haplotypes, the plasma fibrinogen concentration was significantly higher in patients compared with controls. A significant haplotype effect on the plasma fibrinogen concentration was observed in patients ( $p < 0.001$ ), carriers of the CAG haplotype having the highest plasma fibrinogen concentration ( $395 \pm 94$  mg/dL). The haplotype effect on the plasma fibrinogen concentration was independent of centre in cases ( $p = 0.17$ ) and in controls ( $p = 0.84$ ).

Moreover, none of the haplotypes had a different effect on the risk of MI according to centre ( $p = 0.80$ ).

In conclusion, in the individual centres different factors contribute to the plasma fibrinogen concentration, LON and STO being the only centres in which genetic predisposition, i.e. the -455G/A polymorphism, appeared to be an independent predictor. The relationship between the plasma fibrinogen concentration and MI differed between the centres, whereas genetic variation in the FGB promoter was not associated with risk of MI.

### Fibrinogen haplotypes and MI (paper II)

The main objective of this study was to screen for SNPs in specific regions in the fibrinogen gene cluster, presumed to be implicated in the regulation of the plasma fibrinogen concentration or to influence the fibrin clot structure and the



**Figure 7. Schematic presentation of SNPs in the fibrinogen gene cluster.** FGG, gamma fibrinogen gene; FGA, alpha fibrinogen gene; FGB, beta fibrinogen gene. The SNPs presented in bold represent the SNPs that have been genotyped in the entire study population. Arrowheads indicate the direction of transcription.

risk of MI. The SCARF study sample was used in order to evaluate potential case-control differences according to the genetic variants encountered in the fibrinogen genes.

In general, patients had more cardiovascular risk factors (e.g. higher BMI and concentration of insulin, triglycerides, IL6 and CRP, were more likely to be smokers and to suffer from type 2 diabetes and hypertension) than controls. The plasma fibrinogen concentration was significantly higher in patients, and appeared to be associated with a modest increase in risk of MI [unadjusted SOR (95%CI): 1.43 (1.24, 1.64)], that remained significant after adjustment for cardiovascular risk factors [adjusted SOR (95%CI): 1.22 (1.03, 1.45)].

The sequencing analyses resulted in detection of several SNPs in the FGG and FGA genes in addition to the previously published FGG 1299+79T>C [rs1049636] and FGA Thr312Ala [rs6050] SNPs (Figure 7). The SNPs were numbered using cDNA as the reference sequence, nucleotide +1 being the A of the ATG-translation initiation codon.<sup>270</sup> SNPs with a minor allele frequency <5% were not considered further in the present study. As a consequence of the LD pattern, genotyping was carried out for eight of

the SNPs: FGG -647A>G [rs1800792], 1299+79T>C [rs1049636] and 1300-189C>T [rs2066864], FGA -58G>A [rs2070011] and Thr312Ala [rs6050], and FGB -257C>T [rs1800788], -463G>A [rs1800790] and -862G>A [rs1800791]. There was a significant case-control difference in the allele frequency distribution for the FGG 1299+79T>C SNP ( $p < 0.05$ ), but not for the other SNPs.

The SNPs within each gene were in complete LD. The FGB -862G>A SNP was in strong LD with the SNPs in the FGG and FGA genes. In contrast, the FGG 1299+79T>C SNP appeared to be independent of the FGA -58G>A SNP and only weak allelic associations were detected between the FGB -463G>A and the SNPs in the FGG and FGA genes. As a consequence, the recombination rates appeared to be increased between the FGA and the FGB genes and also between the FGG and the FGA genes, relative to the average background recombination rate across the 50kb fibrinogen gene cluster.

Haplotype analyses were performed using genotype data from each fibrinogen gene and also combining genotype data from the different fibrinogen genes. Three haplotypes were detected in the FGG gene (denoted FGG\*1-\*3), three in the FGA gene

**Table 4. Odds ratios (ORs) for myocardial infarction in relation to fibrinogen gene haplotypes**

Haplotype	OR (95% CI), P-value		
	Unadjusted	Adjusted <sup>1</sup>	Adjusted <sup>2</sup>
FGG-FGA*1	1.51 (1.18, 1.93); <0.001	1.50 (1.17, 1.93); 0.002	1.66 (1.25, 2.22); <0.001
FGG-FGA*2	1.03 (0.83, 1.27); 0.81	0.99 (0.79, 1.23); 0.90	0.87 (0.68, 1.12); 0.28
FGG-FGA*3	0.82 (0.65, 1.03); 0.09	0.85 (0.67, 1.07); 0.17	0.83 (0.63, 1.08); 0.15
FGG-FGA*4	0.79 (0.64, 0.98); 0.03	0.75 (0.60, 0.93); 0.009	0.69 (0.53, 0.88); 0.003
FGG-FGA-FGB*1a	1.33 (1.08, 1.64); 0.007	1.40 (1.13, 1.73); 0.002	1.49 (1.17, 1.90); 0.001
FGG-FGA-FGB*2a	1.04 (0.85, 1.27); 0.72	1.00 (0.81, 1.23); 0.99	0.98 (0.77, 1.24); 0.84
FGG-FGA-FGB*3a	0.89 (0.72, 1.09); 0.25	0.89 (0.72, 1.10); 0.30	0.93 (0.73, 1.18); 0.53
FGG-FGA-FGB*4a	0.94 (0.76, 1.17); 0.59	0.90 (0.72, 1.13); 0.37	0.84 (0.65, 1.08); 0.16
FGG-FGA-FGB*5a	0.73 (0.54, 0.96); 0.03	0.67 (0.50, 0.90); 0.008	0.53 (0.38, 0.74); <0.001
FGG-FGA-FGB*1b	1.35 (1.11, 1.66); 0.003	1.43 (1.16, 1.76); <0.001	1.49 (1.18, 1.89); <0.001
FGG-FGA-FGB*2b	1.00 (0.80, 1.24); 0.99	1.00 (0.80, 1.25); 0.99	0.98 (0.76, 1.27); 0.89
FGG-FGA-FGB*3b	1.06 (0.85, 1.33); 0.58	1.03 (0.82, 1.30); 0.80	1.04 (0.80, 1.34); 0.79
FGG-FGA-FGB*4b	0.76 (0.61, 0.96); 0.02	0.74 (0.58, 0.94); 0.01	0.70 (0.54, 0.92); 0.01
FGG-FGA-FGB*5b	0.59 (0.45, 0.76); < 0.001	0.55 (0.42, 0.73); <0.001	0.44 (0.32, 0.61); < 0.001

ORs and p-values from logistic regression analysis. The reference level is noncarriers of the haplotypes. <sup>1</sup>ORs adjusted for plasma fibrinogen concentration. <sup>2</sup>ORs adjusted for plasma fibrinogen concentration, BMI, smoking, IL6, insulin, HDL-cholesterol and triglycerides.

(denoted FGA\*1-3) and four in the FGB gene (denoted FGB\*1-4). Four haplotypes, FGG-FGA\*1-4, were inferred using genotype data from the FGG 1299+79T>C and FGA -58G>A SNPs. Eight haplotypes, FGG-FGA-FGB\*1a-8a, were detected when the FGB -463G>A SNP was added to the haplotype analysis and five of these (FGG-FGA-FGB\*1a-5a) accounted for 94.2% of all haplotypes. When considering all eight SNPs, 23 haplotypes were detected, five of which (FGG-FGA-FGB\*1b-5b) represented 81.4% of all haplotypes identified.

Potential determinants of plasma fibrinogen concentration were searched for. In controls, IL6 (5.3%), smoking (1.0%) and insulin (2.6%) were independent predictors, explaining 8.9% of the variation in plasma fibrinogen concentration. In patients, IL6 (21.7%), LDL-cholesterol (1.7%), BMI (3.7%) and smoking (3.5%) were independent predictors, together accounting for

30.6% of the variation in plasma fibrinogen concentration. None of the fibrinogen SNPs appeared to influence the plasma fibrinogen concentration. Amongst controls, presence of the FGG\*3 and FGA\*2 haplotypes was associated with significantly higher plasma fibrinogen concentration compared to noncarriers ( $3.7 \pm 0.8$  g/L vs.  $3.5 \pm 0.7$  g/L;  $p=0.006$  and  $3.7 \pm 0.8$  g/L vs.  $3.5 \pm 0.7$  g/L;  $p=0.007$ , respectively).

No significant associations between individual SNPs and risk of MI were detected. On the other hand, the FGG\*2 and FGA\*3 haplotypes seemed to be protective [OR (95%CI): 0.79 (0.64, 0.96) and 0.59 (0.46, 0.75), respectively], whereas the FGB haplotypes were not associated with risk of MI. Moreover, the FGG-FGA\*1, FGG-FGA-FGB\*1a and \*1b haplotypes were associated with increased risk of MI [OR (95%CI): 1.51 (1.18, 1.93), 1.33 (1.08, 1.64) and 1.35 (1.11, 1.66),

respectively; Table 4]. Conversely, presence of the FGG-FGA\*4, FGG-FGA-FGB\*5a and \*5b haplotypes seemed to be protective [OR (95%CI): 0.79 (0.64, 0.98), 0.73 (0.54, 0.96) and 0.59 (0.45, 0.76), respectively]. These associations remained significant after adjustment for plasma fibrinogen concentration as well as after further adjustment for BMI, smoking, IL6, insulin, HDL-cholesterol and triglycerides (Table 4). There were no consistent associations between fibrinogen haplotypes and severity of CAD as determined by QCA.

Thus, fibrinogen haplotypes inferred using genotype data from the FGG 1299+79T>C and FGA -58G>A SNPs are associated with variation in risk of MI, independently of plasma fibrinogen concentration and other risk factors.

### **Fibrinogen, fibrinogen SNPs, fibrin clot structure and MI (paper III)**

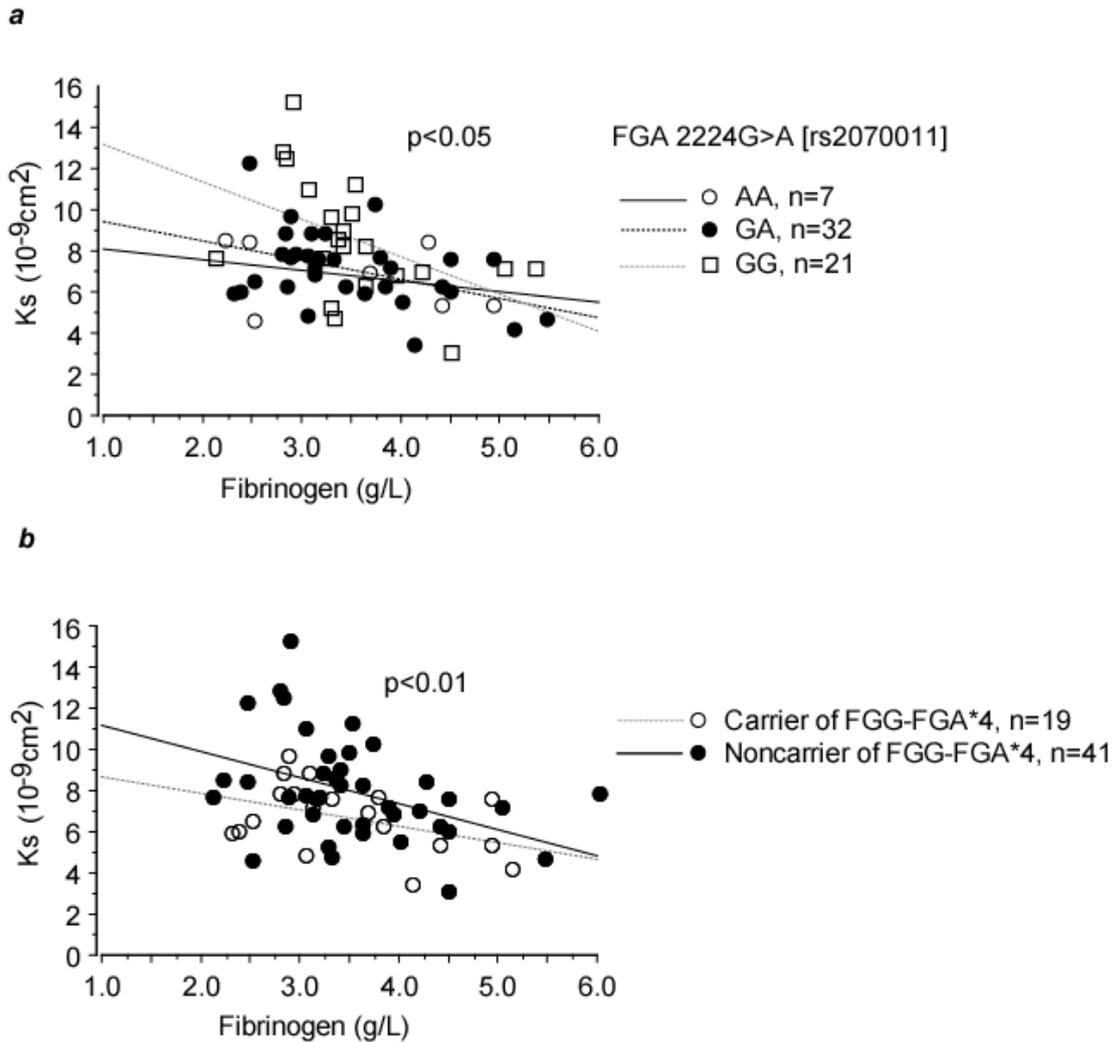
According to the results derived from the SCARF study sample, there was evidence to suggest that fibrinogen haplotypes are related to risk of MI, independently of plasma fibrinogen concentration. Fibrinogen is the precursor of fibrin, which is the main component of the fibrin clot. An abnormal fibrin clot structure has been associated with precocious MI.<sup>150</sup> Therefore, we hypothesized that potential effects on the fibrin clot structure may be an intermediate link between the fibrinogen haplotypes and MI. The SCARF study population was used to test this hypothesis and to study gene-gene and gene-environment interactions on the risk of MI.

The FGG 9340T>C [rs1049636], FGA 2224G>A [rs2070011], FGB 1038G>A

[1800791] (previously referred to as FGG 1299+79T>C, FGA -58G>A and -862G>A, respectively; Table 1), F13A1 Val34Leu [rs5985] and IL6 1510G>C [rs1800795] (known as the -174G>C polymorphism; Table 1) SNPs were included in the present study. Sixty healthy individuals were selected according to the FGG 9340T>C SNP for analyses of fibrin clot structure. There were no significant differences in basic characteristics according to the FGG 9340T>C genotypes.

The potential contributions of genetic and environmental factors to the fibrin clot porosity were evaluated. These analyses were restricted to fibrinogen SNPs that are independent of the FGG 9340T>C SNP used for the selection of the sixty healthy individuals (i.e. the FGA 2224G>A and F13A1 Val34Leu SNPs). Fibrinogen (13.1%), the FGA 2224G>A genotype (9.2%) and age (8.1%) together explained 30.4% of the variation in fibrin clot porosity. The fibrinogen haplotypes, previously inferred using genotype data from the FGG 9340T>C and FGA 2224G>A SNPs, were also related to the fibrin clot porosity. The FGG-FGA\*4 haplotype explained 8.8% of the variation in Ks, along with age (7.7%) and plasma fibrinogen concentration (13.9%).

The FGA 2224G>A SNP appeared to influence the relationship between plasma fibrinogen concentration and fibrin clot porosity (Figure 8a). The correlation between plasma fibrinogen concentration and fibrin clot porosity was strongest in homozygotes for the major G allele ( $r=-0.47$ ,  $p=0.03$ ), intermediate in heterozygotes ( $r=-0.42$ ,  $p=0.01$ ) and not significant homozygotes for the minor A allele ( $r=-0.34$ ,  $p=0.48$ ). The regression coefficients (designated



**Figure 8.** Scatter plots with regression lines of Ks on plasma fibrinogen concentration according to the FGA 2224G>A [rs2070011] SNP (a) and FGG-FGA\*4 haplotype (b). The p-values refer to the differences between the slopes.

b) differed significantly between the FGA 2224G>A genotypes ( $p=0.048$ ), homozygotes for the G allele having the steepest decrease in fibrin clot porosity with increasing plasma fibrinogen concentration ( $b=-1.8$ ) compared with heterozygotes ( $b=-0.9$ ) and homozygotes for the A allele ( $b=-0.5$ ). Similarly, a significantly lower rate of change in Ks at increasing plasma fibrinogen concentration was observed in carriers of the FGG-FGA\*4 haplotype compared with noncarriers ( $b=-0.8$  vs.  $b=-1.3$ ,  $p=0.005$ ; Figure 8b).

Potential epistatic effects on the plasma fibrinogen concentration were searched

for amongst control subjects in whom confounding factors were considered to be less prominent than amongst patients. A significant interaction ( $p<0.001$ ) on the plasma fibrinogen concentration was detected between the F13A1 Val34Leu and FGA 2224G>A SNPs, homozygotes for the minor alleles (T and A, respectively) having the highest concentrations. No epistatic effects were observed between the fibrinogen SNPs, nor did the IL6 1510G>C SNP seem to have an individual main effect or to interact with any SNPs on plasma fibrinogen concentration. The lack of association between the IL6 1510G>C SNP and plasma fibrinogen concentra-

tion is in agreement with data from the HIFMECH study.<sup>281</sup>

Multilocus Hardy-Weinberg equilibrium analyses were performed separately in patients and controls. These analyses indicated that certain SNPs occur together in patients more frequently than what would be expected by chance. Therefore, MDR analyses were performed aiming to identify the SNPs that either in isolation or through interaction with other SNPs confer a higher or lower risk of MI. A significant interaction on the risk of MI risk was detected between the FGG 9340T>C and FGB 1038G>A SNPs [OR<sub>MDR</sub> (95%CI): 1.83 (1.33, 2.52)]. These results were replicated using logistic regression analysis (p=0.02 for interaction). In addition, haplotypes inferred using genotype data from these two SNPs appeared to be associated with variation in risk of MI. The FGG-FGB\*1 haplotype (TG, prevalence 65.2%, in controls) appeared to be associated with increased risk of MI [OR (95%CI): 1.49 (1.07, 2.08)], whereas the FGG-FGB\*2 haplotype (CG, prevalence 17.5%, in controls) appeared to be protective [OR (95%CI): 0.62 (0.49, 0.77)] when non-carriers of the respective haplotype were used as reference groups.

The effect of SNPs was further evaluated through addition of environmental variables to the analysis. The most parsimonious model that offered a low PE (23.7%, p<0.001) included the joint effects of a dyslipidemic phenotype and increased waist-to-hip circumference ratio. The effect of the SNPs was observed in a six-way interaction model with a PE of 27.2% (p<0.001), which contained the FGG 9340T>C, FGA 2224G>A and IL6 1510G>C SNPs

along with dyslipidemia, hypertension and CRP.

In conclusion, fibrinogen, age and the FGA 2224G>A SNP are independent determinants of the fibrin gel porosity and haplotypic effects on the latter may partly explain the relationship between the haplotypes inferred using the FGG 9340T>C and FGA 2224G>A htSNPs and overt MI.

### **Plasma fibrinogen $\gamma'$ concentration and MI (paper IV)**

The main objective of the present study was to assess whether the plasma fibrinogen  $\gamma'$  concentration contributes to the variation in risk of MI in the SCARF study sample. Also genetic and environmental determinants of the plasma fibrinogen  $\gamma'$  concentration were searched for and interaction analyses on MI risk were performed.

The plasma fibrinogen  $\gamma'$  concentration was significantly higher in patients than in controls ( $0.277 \pm 0.122$  vs.  $0.250 \pm 0.107$  g/L, p=0.001). However, this difference was confined to men. Significantly higher plasma fibrinogen  $\gamma'$  concentrations were also observed in patients with a total plasma fibrinogen concentration in the top quartile, who were smokers, overweight or had an HDL-cholesterol concentration  $\geq 1.0$  mmol/L.

The FGG 9340T>C and FGA 2224G>A SNPs influenced the plasma fibrinogen  $\gamma'$  concentration in both patients and controls, increasing number of the minor FGG 9340C allele or of the major FGA 2224G allele being associated with higher concentrations (Table 5). The plasma fibrinogen  $\gamma'$  concentration varied significantly according to the FGB 1038G>A SNP in controls

**Table 5. Plasma fibrinogen  $\gamma'$  concentration according to fibrinogen SNPs in cases and controls**

SNP	Fibrinogen $\gamma'$ (g/L)				
	Case	n	Control	n	p-value
FGG 9340T>C					
CC	0.380 (0.138)	30	0.310 (0.098)	46	0.01
TC	0.286 (0.109)	156	0.269 (0.106)	169	0.18
TT	0.253 (0.121)	179	0.213 (0.096)	166	<0.001
p-value	<0.001		<0.001		
FGA 2224G>A					
AA	0.222 (0.130)	45	0.212 (0.122)	54	0.69
GA	0.271 (0.116)	172	0.253 (0.102)	178	0.11
GG	0.301 (0.122)	148	0.260 (0.104)	149	0.002
p-value	0.001		0.02		
FGB 1038G>A					
AA	0.371 (0.074)	8	0.244 (0.075)	13	0.001
GA	0.284 (0.117)	106	0.285 (0.112)	105	0.99
GG	0.272 (0.124)	251	0.236 (0.103)	263	<0.001
p-value	0.06		<0.001		

Values are mean (SD) and number (n) of subjects in each group.

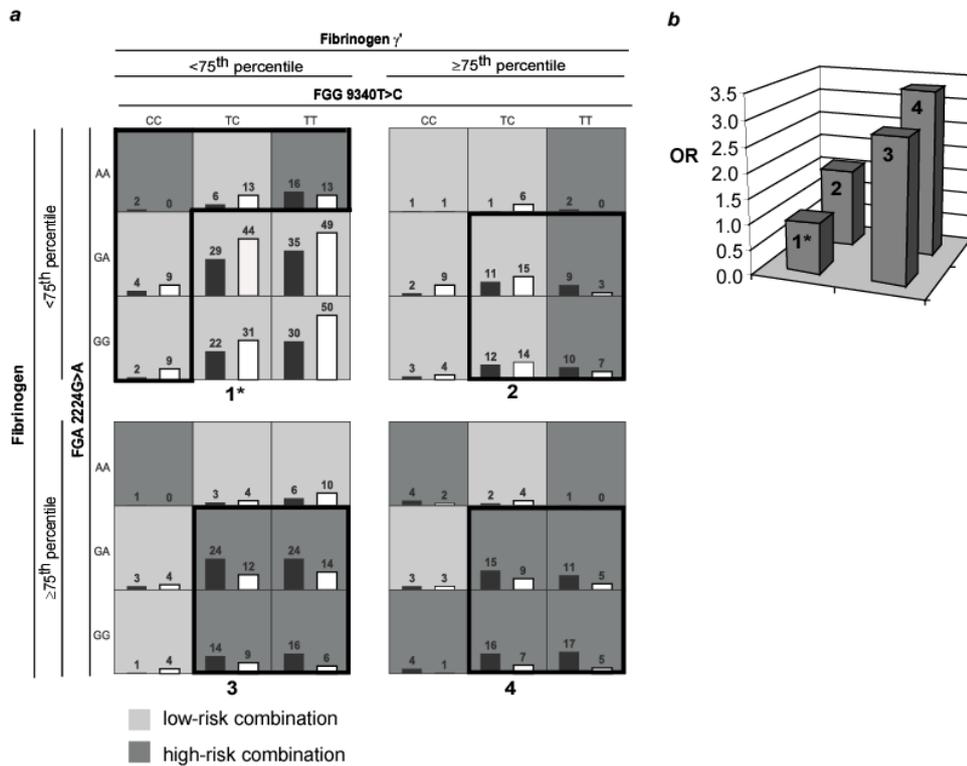
( $p < 0.001$ ), but not in patients. This SNP was excluded from the subsequent gene-gene interaction analyses on the plasma fibrinogen  $\gamma'$  concentration due to its LD with the other SNPs included in the present study (paper II).

A significant epistatic effect, involving the FGG 9340T>C and FGA 2224G>A SNPs, on the plasma fibrinogen  $\gamma'$  concentration was observed in both patients ( $p = 0.047$ ) and controls ( $p = 0.02$ ). In both groups, presence of the TT/AA haplotype was associated with significantly lower plasma fibrinogen  $\gamma'$  concentration as compared to the TT/GG haplotype ( $p < 0.001$ ).

In patients, fibrinogen (9.2%), FGG 9340T>C (5.9%), FGA 2224G>A (3.9%), HDL-cholesterol (1.4%), insulin (1.1%) and gender (0.9%) emerged as independent determinants of the plasma

fibrinogen  $\gamma'$  concentration, together accounting for 22.4% of the variation. In controls, FGG 9340T>C (10.4%), fibrinogen (3.4%) and FGA 2224G>A (2.2%) emerged as independent predictors, together accounting for 16.0% of the variation in plasma fibrinogen  $\gamma'$  concentration.

Elevated plasma fibrinogen  $\gamma'$  concentration was associated with a modest increase in risk of MI [unadjusted SOR (95%CI): 1.25 (1.09, 1.44)], that remained significant after controlling for the FGG 9340T>C SNP [SOR (95%CI): 1.37 (1.17, 1.61)] and for the effects of age, gender, smoking, alcohol consumption, BMI, fibrinogen, insulin, triglycerides and HDL-cholesterol together with the FGG 9340T>C SNP [SOR (95%CI): 1.31 (1.06, 1.62)]. In contrast, the ratio of plasma fibrinogen  $\gamma'$  to total plasma fibrinogen concentration (the  $\gamma'/\gamma A$  ratio)



**Figure 9. Best predictive interaction model associated with risk of MI. (a)** The corresponding distribution of cases (dark bars in boxes) and controls (white bars in boxes) is depicted for each multilocus genotype and variable combination. Note the shift in pattern of high-risk and low-risk cells across the different multilocus dimensions, suggesting the presence of interaction. **(b)** Odds ratios (ORs) for myocardial infarction across the different multilocus dimensions. The combined distribution of variables in the cells denoted 2, 3 and 4 (Figure 9a, within the boxes demarcated with bold lines) was used for calculation of ORs using the cells within 1\* (demarcated with bold lines) as reference category.

was not associated with risk of MI. There was no association between the plasma fibrinogen  $\gamma'$  concentration and fibrin clot structure or CAD severity scores.

The FGG 9340T>C, FGA 2224G>A and FGB 1038G>A SNPs, the plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations (dichotomized using the 75<sup>th</sup> percentile as cut-off values) were then evaluated in relation to risk of MI. A significant high-order interaction on the risk of MI ( $p=0.02$  from permutation test) was detected between the fibrinogen  $\gamma'$  concentration, the total fibrinogen concentration and the FGG 9340T>C and FGA 2224G>A SNPs. The best predictive model [ $OR_{MDR}$  (95% CI): 3.2

(2.4, 4.4)] was the result of a marked shift in the genotype frequency distributions amongst individuals with total plasma fibrinogen and/or fibrinogen  $\gamma'$  concentrations in the top quartiles (Figure 9a).

ORs for MI were then estimated using the non-carriers of the major FGG 9340T and FGA 2224G alleles who had both the plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations below the 75<sup>th</sup> percentile as a reference group (group 1\* in Figure 9a and 9b). There was no difference in risk of MI between carriers of the major FGG 9340T and FGA 2224G alleles (TC/GA plus TC/GG plus TT/GA plus TT/GG) who had a plasma fibrinogen  $\gamma'$  concentration but not a

total plasma fibrinogen concentration above the 75<sup>th</sup> percentile (Figure 9a and 9b, group 2) as compared with the reference group [OR (95%CI): 1.58 (0.84, 2.99)]. Simultaneous presence of the major FGG 9340T and FGA 2224G alleles was more common amongst patients who had a total plasma fibrinogen concentration in the top quartile ( $p < 0.0001$ ; Figure 9a, group 3) as compared to the reference group and the risk of MI in these individuals was increased [OR (95%CI): 2.79 (1.53, 5.08); Figure 9b, group 3]. Individuals who had both the total plasma fibrinogen and fibrinogen  $\gamma'$  concentrations above the 75<sup>th</sup> percentile (Figure 9a, group 4) ran a further increase in the risk of MI [OR (95%CI): 3.33 (1.73, 6.40); Figure 9b, group 4].

In conclusion, the plasma fibrinogen  $\gamma'$  concentration is related to the risk of MI, independently of other risk factors and is involved together with the total plasma fibrinogen concentration and the FGG 9340T>C and FGA 2224G>A htSNPs in a high-order interaction yielding a 3 fold increase in risk of precocious MI.

### **Pleiotropic effects on IL6 may partly explain the relationship between fibrinogen haplotypes and MI (paper V)**

Experimental evidence has indicated that fibrinogen stimulates the production of the pro-inflammatory cytokine IL6, which has been related to the risk of MI. Moreover, the F13A1 Val34Leu SNP has been shown to influence the IL6 concentration.<sup>282</sup> The main objective of the present study was to examine if genetic variation in the fibrinogen genes and the F13A1 Val34Leu SNP affects the serum IL6 concentration and whether

such an influence may be related to the risk of MI. The SHEEP study sample has been used for these purposes.

In general, the prevalence of cardiovascular risk factors was higher in patients than in controls. Gender-specific differences in risk factor distributions were observed: the female patients were older ( $p < 0.001$ ), had more frequently hypercholesterolemia ( $p < 0.01$ ), had a higher plasma fibrinogen concentration ( $p < 0.001$ ) and were less frequently smokers ( $p < 0.001$ ).

All SNPs were in Hardy-Weinberg equilibrium and there were no differences in genotype or allele frequency distributions between cases and controls grouped according to gender.

Four haplotypes were detected based on genotype data for the FGG 9340T>C and FGA 2224G>A SNPs: FGG-FGA\*1 (TG, prevalence 45%), FGG-FGA\*2 (TA, prevalence 25%), FGG-FGA\*3 (CG, prevalence 18%) and FGG-FGA\*4 (CA, prevalence 12%). In patients, the frequency distribution of the FGG-FGA\*1 haplotype differed significantly between men and women ( $p = 0.004$ ). In controls, the frequency distribution of the FGG-FGA\*2 haplotype was significantly different according to gender ( $p = 0.01$ ).

In male controls, the serum IL6 concentration differed according to the FGA 2224G>A genotype ( $p = 0.04$ ), homozygotes for the A allele having significantly lower levels compared with homozygotes for the G allele [geometric mean (95%CI): 0.70 (0.42, 1.17) vs. 1.28 (1.04, 1.58) ng/L,  $p = 0.01$ ]. Also amongst male controls, the FGG-FGA\*1 haplotype was associated with a significantly

**Table 6. Serum IL6 concentration according to FGG-FGA haplotypes in cases and controls**

Haplotype	Men		Women	
	Cases	Controls	Cases	Controls
<b>FGG-FGA*1 (TG)*</b>				
Carrier	1.64 (1.48, 1.81)	1.15 (1.02, 1.29)	1.48 (1.28, 1.71)	1.41 (1.20, 1.65)
Noncarrier	1.53 (1.25, 1.87)	0.79 (0.61, 1.02)	1.78 (1.35, 2.34)	1.78 (1.31, 2.41)
p-value	0.57	0.004	0.22	0.19
<b>FGG-FGA*2 (TA)</b>				
Carrier	1.63 (1.39, 1.90)	0.97 (0.79, 1.19)	1.51 (1.11, 1.70)	1.38 (1.21, 1.88)
Noncarrier	1.61 (1.44, 1.80)	1.10 (0.98, 1.24)	1.58 (1.28, 1.84)	1.53 (1.35, 1.86)
p-value	0.91	0.27	0.73	0.47
<b>FGG-FGA*3 (CG)</b>				
Carrier	1.52 (1.28, 1.81)	1.06 (0.88, 1.27)	1.55 (1.21, 1.99)	2.23 (1.72, 2.89)
Noncarrier	1.64 (1.48, 1.83)	1.06 (0.93, 1.20)	1.56 (1.34, 1.81)	1.28 (1.09, 1.51)
p-value	0.50	0.99	0.98	<0.001
<b>FGG-FGA*4 (CA)</b>				
Carrier	1.65 (1.39, 1.96)	0.82 (0.67, 1.00)	1.76 (1.43, 2.09)	1.33 (0.98, 1.79)
Noncarrier	1.60 (1.44, 1.79)	1.19 (1.05, 1.35)	1.46 (1.23, 1.72)	1.55 (1.33, 1.80)
p-value	0.78	0.001	0.18	0.32

Values are presented as geometric means (95%CI). \*Order of the alleles is FGG 9340T>C and FGA 2224G>A from left to right.

higher serum IL6 concentration compared with noncarriers of the haplotype [1.15 (1.02, 1.29) vs. 0.79 (0.61, 1.02) ng/L,  $p=0.004$ ; Table 6]. Conversely, carriers of the FGG-FGA\*4 haplotype had significantly lower serum IL6 concentrations compared with non-carriers of the haplotype [0.82 (0.67, 1.00) vs. 1.19 (1.05, 1.35) ng/L,  $p=0.001$ ]. Moreover, carriers of the FGG-FGA\*4 haplotype had significantly lower serum IL6 concentration than carriers of the FGG-FGA\*1 haplotype ( $p<0.001$ ). Amongst women controls, presence of the FGG-FGA\*3 haplotype was associated with significantly higher serum IL6 concentrations compared with non-carriers of this haplotype [2.23 (1.72, 2.89) vs. 1.28 (1.09, 1.51) ng/L,  $p<0.001$ ]. The F13A1 Val34Leu SNP was not associated with the serum IL6 concentration either in women or in men. There was no difference in plasma

fibrinogen concentration according to the FGG 9340T>C, FGA 2224G>A and F13A1 Val34Leu genotypes.

In isolation, the FGG 9340T>C, FGA 2224G>A and F13A1 Val34Leu SNPs were not associated with risk of MI. On the other hand, the FGG-FGA\*2 haplotype was associated with an increased risk of MI in men [OR (95%CI): 1.19 (1.04, 1.37)], and this association remained significant after adjustment for cardiovascular risk factors [adjusted OR (95%CI): 1.29 (1.06, 1.58)] (Table 7). The FGG-FGA\*1 and FGG-FGA\*4 haplotypes appeared to be associated with risk of MI after adjustment for cardiovascular risk factors [adjusted OR (95%CI): 1.29 (1.02, 1.62) and 0.70 (0.57, 0.86), respectively]. Complementary analysis using the most common FGG-FGA\*1 haplotype as reference group generated

**Table 7. Odds ratio (OR) for myocardial infarction in relation to FGG-FGA haplotypes**

Haplotype	Men			Women		
	OR (95%CI), p-value <sup>†</sup>	OR (95%CI), p-value <sup>‡</sup>	OR (95%CI), p-value <sup>‡</sup>	OR (95%CI), p-value <sup>†</sup>	OR (95%CI), p-value <sup>†</sup>	OR (95%CI), p-value <sup>‡</sup>
FGG-FGA*1	0.94 (0.80, 1.10); 0.45	1.12 (0.90, 1.39), 0.31	1.29 (1.02, 1.62); 0.03	0.76 (0.61, 0.96); 0.02	0.72 (0.53, 0.97), 0.03	0.77 (0.55, 1.08); 0.12
FGG-FGA*2	1.19 (1.04, 1.37); 0.01	1.34 (1.11, 1.61), 0.002	1.29 (1.06, 1.58); 0.01	1.05 (0.85, 1.29); 0.66	1.09 (0.84, 1.41), 0.53	1.12 (0.83, 1.52); 0.45
FGG-FGA*3	0.99 (0.85, 1.16); 0.91	0.79 (0.64, 0.97), 0.02	0.85 (0.69, 1.06); 0.16	1.11 (0.88, 1.40); 0.38	1.00 (0.75, 1.34), 0.99	0.80 (0.57, 1.12); 0.19
FGG-FGA*4	0.89 (0.77, 1.03); 0.12	0.81 (0.67, 0.99), 0.04	0.70 (0.57, 0.86); <0.001	1.08 (0.87, 1.34); 0.46	1.21 (0.93, 1.58), 0.16	1.17 (0.87, 1.58); 0.31

The reference category is all haplotypes but the one given. \*ORs adjusted for age and residential area. †ORs adjusted for age, residential area and IL6; ‡ORs adjusted for age, residential area, IL6, hypercholesterolemia, triglycerides, insulin, hypertension, waist-to-hip ratio, smoking (never vs. former and current) and physical inactivity.

similar results for the FGG-FGA\*4 haplotype [adjusted OR (95%CI): 0.68 (0.53, 0.87), p=0.002] whereas the effect of the FGG-FGA\*2 haplotype did not differ significantly from that of the most common haplotype.

In women, the FGG-FGA\*1 haplotype appeared to be protective when adjusting for age, residential area and IL6 [adjusted OR (95%CI): 0.72 (0.53, 0.97)]. However, this relationship was no longer significant when controlling for other risk factors in addition to IL6 [adjusted OR (95%CI): 0.77 (0.55, 1.08)]. Adjustment for the effects of

either hormone replacement therapy or menopause did not influence the ORs [adjusted OR (95%CI): 0.75 (0.54, 1.06) and 0.79 (0.56, 1.11), respectively].

Thus, in healthy men, fibrinogen haplotypes inferred using genotype data from the FGG 9340T>C and FGA 2224G>A htSNPs influence the serum IL6 concentrations in a manner consistent with their relationship with MI, i.e. the risk-increasing haplotype FGG-FGA\*1 (TG) was associated with the highest IL6 concentration, whereas the opposite was observed for risk-lowering haplotype FGG-FGA\*4 (CA).

## GENERAL DISCUSSION

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### Plasma fibrinogen concentration and MI

Atherothrombotic disease is by far more common in the North than in the South of Europe, and the factors underlying these differences are still unknown. Valuable insights are to be gained by studying potential risk factors such as fibrinogen amongst various ethnic groups from different geographical regions. Against this background, the relationship between the plasma fibrinogen concentration and MI has been explored in the SCARF study sample consisting of Northern European participants and in the HIFMECH study comprising individuals from four European centres: Stockholm, Sweden and London, UK (North of Europe) and Marseille, France and San Giovanni Rotondo, Italy (South of Europe).

The results from these studies are in accordance with each other and with the notion that an elevated plasma fibrinogen concentration confers an increased risk of MI.<sup>180</sup> Of note, based on results from the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study<sup>283</sup> it has been estimated that about 30% of the higher risk of CHD in Northern Ireland compared with France may be ascribed to differences in plasma fibrinogen concentration. Striking differences in plasma fibrinogen concentration were also observed among the European centres participating in the HIFMECH study. In particular, no case control differences in plasma fibrinogen concentrations were observed in MAR, the only centre in which BMI made a significant contribution to the plasma

fibrinogen concentration amongst the healthy individuals. Since IL6 is partly produced in adipose tissue<sup>284</sup> it is notable in this context that BMI is significantly higher amongst the controls from the South of Europe than amongst those from the North of Europe ( $p=0.01$ ).<sup>281</sup> Moreover, patients from the South of Europe have significantly higher serum IL6 concentration and a higher percentage were smokers as compared to those from the North of Europe ( $p=0.002$  and  $p=0.003$ , respectively).<sup>281</sup> These differences may be important since both IL6 and smoking are known to influence plasma fibrinogen concentration.<sup>20,50</sup>

Relevant in this context is also the inconsistent relationship between genetic predisposition (i.e. the FGB promoter polymorphisms) and plasma fibrinogen concentration observed across the European centres. It is possible that these SNPs may not exert significant effects during chronic low-grade inflammatory conditions, hence emphasizing the critical role of context- and time-dependency. Accordingly, it could be argued that the FGB promoter polymorphisms may exert significant effects on plasma fibrinogen concentration in response to a disease related stimuli which may be operating to a higher extent in the North than in the South of Europe. The fact that “centre” appeared to be an independent contributor to the plasma fibrinogen concentration in both patients and controls lends support to this argument. For instance, the Northern European climate is much colder than the Southern European, and since higher plasma

fibrinogen concentrations have been observed during winter,<sup>49</sup> one could speculate that it may contribute to the higher prevalence of MI in North of Europe as compared to South of Europe.

Since the cardiovascular risk factor profile and the genetic and environmental contribution to the plasma fibrinogen concentration varied across the centres, accounting for the effects of BMI, smoking and IL6 may have different effects on the strength of the relationship between plasma fibrinogen concentration and the risk of MI. Nevertheless, these data imply a causal relationship between fibrinogen and susceptibility to MI since in North of Europe the higher cardiovascular morbidity is accompanied by a significant association between the plasma fibrinogen concentration and MI, whereas the lower morbidity in South of Europe is unaccompanied by such an association (i.e. one would expect a higher morbidity in the South of Europe if plasma fibrinogen concentration would have been higher in patients than in controls after adjustment for potential confounders).

### **Plasma fibrinogen $\gamma'$ concentration and MI**

To the best of our knowledge, paper IV reports the first case-control study in which the relationship between plasma fibrinogen  $\gamma'$  concentration and risk of MI has been explored. The fibrinogen  $\gamma'$  chain variant amounts to about 15% of the plasma fibrinogen concentration, and differs both structurally and functionally from the predominant fibrinogen  $\gamma$ A chain.

The results from this study indicate that elevated plasma fibrinogen  $\gamma'$  concentration confers an increased risk of MI, independently of the total plasma

fibrinogen concentration and other cardiovascular risk factors. The previously reported<sup>231</sup> positive association between the plasma fibrinogen  $\gamma'$  concentration and coronary artery stenosis (>50% stenosis) in a much smaller cohort of CAD patients (91 CAD patients and 42 controls) lends support to the findings obtained in the SCARF study sample.

The means by which an elevated plasma fibrinogen  $\gamma'$  concentration may confer an increased risk of atherothrombotic disease have as yet not been elucidated. The predominant fibrinogen  $\gamma$ A chain is significantly more efficient in promoting platelet aggregation than the fibrinogen  $\gamma'$  chain,<sup>28</sup> a property conferred by its last carboxyl-terminal residues (QAGDV). Conversely, the carboxyl-terminal sequence of the fibrinogen  $\gamma'$  chain variant comprises twenty anionic amino acids (VRPEHPAETEDSLYPEDDL) that replaced the carboxyl-terminal residues of the fibrinogen  $\gamma$ A chain (AGDV) and that contain high affinity binding sites for thrombin<sup>29</sup> and FXIII.<sup>30</sup> Therefore, the physiological function of the fibrinogen  $\gamma$ A/ $\gamma'$  variant may be to render fibrin clots stability by acting as a biological reservoir of FXIII.<sup>32</sup> It is notable in this context that fibrin augments the activation of FXIII by thrombin.<sup>285</sup> The FXIII mediated cross-linking provides the fibrin clot stability and strength to withstand the proteolytical forces in the blood.<sup>286,287</sup> Interestingly, the fibrinogen  $\gamma$ A/ $\gamma'$  variant was observed to accelerate the activation of FXIII by thrombin,<sup>288</sup> which may explain the more extensive cross-linking of fibrin clots formed in its presence.<sup>32,289</sup> Although, it has become a matter of debate whether the fibrin clots formed by the fibrinogen  $\gamma$ A/ $\gamma'$  variant are more cross-linked or not<sup>230</sup> the observation that these particular fibrin

clots are more resistant to fibrinolysis is consistent.<sup>32,230,289</sup> Moreover, vitronectin, which is an abundant plasma glycoprotein involved in complement activation and cell adhesion, preferentially binds to fibrin(ogen)  $\gamma$ A/ $\gamma$ ' during coagulation<sup>290</sup> and mediates the binding of PAI-1 to fibrin(ogen)<sup>291</sup> and may therefore reinforce the fibrinolytic resistance of the fibrin clot.

Clinically, hypofibrinolysis has been associated with precocious MI<sup>292</sup> and venous thrombosis.<sup>42</sup> Therefore, it could be argued that the higher risk of MI conferred by elevated plasma fibrinogen  $\gamma$ ' concentration might be due to the resistance to fibrinolysis of fibrin clots formed by this variant.<sup>32,230,289</sup> Hence, the findings from this study may be clinically relevant as they suggest that individuals with an elevated plasma fibrinogen  $\gamma$ ' concentration may form fibrin clots that are more stable and which may therefore exhibit a delayed response to thrombolytic therapy. Accordingly, it is not unreasonable to envisage the fibrinogen  $\gamma$ ' variant as a potential fibrin clot specific therapeutic target. It is well-established that a rapidly restored vascular patency is essential in order to prevent or hinder further damage caused by a thrombus in a vital organ such as the heart or the brain (i.e. "time is muscle" and "time is brain"). Obviously, the novel findings herein presented need to be confirmed in independent studies.

### **Fibrinogen SNPs and MI**

Thus far, the FGB gene has gained most of the attention in genotype-phenotype association studies since early experimental data indicated that the synthesis of the B $\beta$  chain is the rate-limiting step in the production of mature fibrinogen in hepatocytes.<sup>293</sup> However,

experimental evidence to suggest that overexpression of any of the fibrinogen genes is accompanied by a similar increase in the expression of the other two genes has also been reported<sup>294,295</sup> which allowed us to hypothesize that strategically located polymorphisms may have an overall impact on intermediate (i.e. plasma fibrinogen concentration and fibrin clot structure) and possibly on clinical (i.e. MI) phenotypes. Accordingly, gene segments of presumed physiological significance were sequenced across the entire fibrinogen gene cluster and several SNPs have been detected.

None of the fibrinogen SNPs appeared to have significant individual main effects on the risk of MI. Lack of association between the FGB -455G/A SNP and risk of MI has been reported before, in the SHEEP study<sup>78</sup> and in several other studies.<sup>296,297</sup> Moreover, none of the FGB haplotypes were related to risk of MI across the four European centres that participated in the HIFMECH study (paper I) and these findings were corroborated in the SCARF study (paper II).

Conversely, in the SCARF study, the FGG\*2 haplotype (containing the minor 902A, 9340C and 9615C alleles, Table 1), and the FGA\*3 haplotype (containing the 2224A and Thr312 alleles) were found to be associated with lowered risk of MI. Furthermore, the FGG-FGA\*4 haplotype (containing the FGG 9340C and FGA 2224A alleles) also appeared to be protective and this finding was confirmed in male participants in the SHEEP study (paper V) but only after adjustment for potential confounders. The slightly inconsistent results between the SCARF and the SHEEP studies may partly reflect differences in study design

(i.e. inclusion and exclusion criteria, age range, exposures).

The plasma fibrinogen concentration did not appear to be an intermediate phenotype linking fibrinogen haplotypes and MI. Several studies have reported that fibrinogen polymorphisms influence the plasma fibrinogen concentration.<sup>69,70,74</sup> In contrast, the genome wide studies published so far have failed to detect any linkage peak corresponding to the fibrinogen gene cluster.<sup>86,87</sup> Nevertheless, considering the rather high genetic heritability estimates (20-50%) reported so far,<sup>44,66,67</sup> it cannot be excluded that there are genetic variants confined to the fibrinogen gene cluster that may influence the plasma fibrinogen concentration. The noted lack of consistency could be due to (1) lack of any effects; (2) small effects sizes that are hard to detect; (3) low penetrance and expressivity (4) epistasis and (5) pleiotropy.

The latter alludes to the existence of genes outside the fibrinogen gene cluster that may influence the plasma fibrinogen concentration. This possibility was addressed in the present thesis by exploring whether the IL6 1510G>C and the F13A1 Val34Leu SNPs influence the plasma fibrinogen concentration. The IL6 1510G>C SNP did not appear to have an individual main effect, in agreement with a previous study<sup>281</sup> or to interact with any of the fibrinogen SNPs on plasma fibrinogen concentration. Furthermore, no significant variation in plasma fibrinogen concentration was observed according to F13A1 Val34Leu genotypes. On the other hand, this SNP appeared to interact with the FGA 2224G>A SNP on plasma fibrinogen concentration in healthy individuals participating in the SCARF study. It

must be emphasized, however, that this is a novel observation which lacks experimental support. Interestingly, the activated FXIII has been shown to have biological activities, like gene regulation, reaching beyond its role in hemostasis.<sup>298</sup> Therefore, the F13A1 Val34Leu polymorphism may indeed be involved in the regulation of plasma fibrinogen concentration.

In conclusion, unless factors such as epistasis and pleiotropy are adequately taken into consideration one cannot exclude the possibility that genetic variation within and outside the fibrinogen gene cluster may influence the plasma fibrinogen concentration. Finally, other intermediate phenotypes may be operating and hence could explain the relationship between the fibrinogen haplotypes and MI.

### **Effects of fibrinogen SNPs on intermediate phenotypes**

#### ***Fibrin clot structure***

The fibrin clot structure is a complex trait governed by an intricate interplay between genetic and environmental factors.<sup>145</sup> Formation of a rigid fibrin clot structure consisting of thin and tightly packed fibers *in vitro* has been associated *in vivo* with increased risk of precocious MI.<sup>150</sup> Therefore, factors having an unfavourable impact on the fibrin clot structure are also likely to confer an increased risk of MI. Accordingly, the observed relationship between the fibrinogen haplotypes, containing the FGG 9340T>C and FGA 2224G>A htSNPs, and MI could be mediated via haplotypic effects on the fibrin clot structure. The FGG 9340T>C SNP is located in close vicinity to the splicing site that generates the fibrinogen  $\gamma'$  chain variant, known to confer

resistance to fibrinolysis.<sup>32,230,289</sup> Moreover, the FGA 2224G>A promoter SNP may have regulatory functions and is in allelic association with the FGA Thr312Ala genotype known to influence the fibrin clot structure. Therefore, it is not unreasonable to presume that these SNPs may be associated with variation in fibrin clot structure and stability.

An inverse correlation between the plasma fibrinogen concentration and the fibrin clot porosity was observed, in agreement with other studies.<sup>148,165</sup> A low fibrin clot porosity, which can be considered a consequence of the clotting potential (i.e. fibrinogen and thrombin concentration),<sup>299</sup> reflects a tighter clot structure that is less permeable and therefore more resistant against fibrinolysis.<sup>229,300</sup> In addition, the relationship between the plasma fibrinogen concentration and the fibrin clot porosity seems to be influenced by other factors such as non-synonymous SNPs in the FGA and FXIII genes (i.e. the FGA Thr312Ala and FXIII Val34Leu polymorphisms)<sup>165,301</sup> and as noted in this study by the FGA 2224G>A promoter polymorphism. The rate of change in fibrin clot porosity at increasing plasma fibrinogen concentration varied significantly according to the FGA 2224G>A genotypes, being lowest in homozygotes for the minor FGA 2224A allele. A possible explanation for the latter finding could be that the FGA 2224G>A htSNP is a proxy for a functional SNP that has quantitative effects on the plasma fibrinogen concentration. Interestingly, evidence to suggest the presence of a quantitative trait locus in close vicinity to the FGA 2224G>A SNP has been reported,<sup>68</sup> implying that this polymorphism may be involved in the regulation of the plasma fibrinogen

concentration. Alternatively, our findings may reflect the LD between the FGA 2224G>A SNP and the FGA Thr312Ala SNP (paper II), which in the present study could not be evaluated in relation to fibrin clot structure due to its linkage with the FGG 9340T>C SNP according to which the selection of the individuals was performed.

The FGA Thr312Ala polymorphism is localized in a region implicated in the FXIII mediated cross-linking,<sup>155,156</sup> known to influence the fibrin clot stability and resistance to fibrinolysis.<sup>286,287</sup> Interestingly, the FGA\*3 haplotype, containing the FGA 2224A and FGA Thr312 variants appeared to confer protection against MI and these two alleles are also present together with the FGG 9340C allele in the extended and also protective FGG-FGA-FGB\*5b haplotype. Notably, an increased risk of post-stroke mortality in patients with atrial fibrillation<sup>38</sup> and of pulmonary embolism<sup>302</sup> has been reported in the presence of the FGA 312Ala allele as compared with the FGA Thr312 allele, which supports the observed protective effect of the FGA\*3, FGG-FGA\*4 and the FGG-FGA-FGB\*5b haplotypes. Additional support was provided by the observation that in the presence of the FGG-FGA\*4 haplotype, of which the FGA 2224A allele is a component, the rate of change in fibrin clot porosity is significantly lower at increasing plasma fibrinogen concentrations.

In conclusion, the fibrin clot porosity seems to be an intermediate phenotype between the fibrinogen haplotypes and the risk of MI. The clinical implication of these findings is that individuals in whom the phenotypic response to environmental or disease related stimuli

may be stronger as a consequence of genetic predisposition could be identified before clinical disease develops.

#### ***Epistatic effects on plasma fibrinogen $\gamma'$ concentration***

Nearly a century ago, William Bateson coined the term “epistasis” describing interactions between genes that may lead to novel phenotypes. Although it is a fundamental and ubiquitous biological phenomenon<sup>10</sup> epistasis has been overlooked by far too long.<sup>303</sup> A prerequisite for epistatic analyses is that the genetic variants in question are independent of each other, and, needless to say, biological plausibility is essential.

Both genetic variation in the fibrinogen gene cluster and the fibrinogen  $\gamma'$  concentration appeared to be related to the risk of MI. These findings posed the question of whether these genetic variants also influence the plasma fibrinogen  $\gamma'$  concentration. And as Bateson said in 1902: “That is where our *exact* science will begin”. Indeed, the FGG 9340T>C and FGA 2224G>A genotypes appeared to have both individual main effects and to interact on plasma fibrinogen  $\gamma'$  concentration. As a consequence of the latter the plasma fibrinogen  $\gamma'$  concentration was significantly higher in patients than in controls in carriers of the TT/GG genotypes. Carriers of the TT/GG haplotype might exhibit a more pronounced increase in plasma fibrinogen  $\gamma'$  concentration in response to disease related stimuli.

Thus, the increased risk of MI in carriers of the TT/GG haplotype may be partly mediated via effects on the plasma fibrinogen  $\gamma'$  concentration.

#### ***Pleiotropic effects on serum IL6 concentration***

Inflammation has been recognized as a key component in the etiology of atherosclerosis.<sup>3</sup> IL6 is a pro-inflammatory cytokine that has been demonstrated to be an active partaker in processes leading to endothelial dysfunction<sup>219</sup> and development of atherosclerotic plaques.<sup>304</sup> Local elevations of IL6 concentrations seem to reflect plaque instability<sup>305</sup> whereas systemic elevations have been observed in patients with unstable angina pectoris<sup>306</sup> and may confer an increased risk of future MI among seemingly healthy men.<sup>307</sup>

In this light, the regulation of the serum IL6 concentration seems to be of importance. Interestingly, one decade ago Smith et al<sup>308</sup> stated that there is convincing evidence that fibrin(ogen) “fragment E stimulates macrophages and connective tissue cells to produce interleukin-6”. Since then, even more experimental evidence has accumulated, implicating fibrinogen in the regulation of IL6 production.<sup>221,309</sup> It could be argued that such pleiotropic effects (i.e. effects of a gene on more than one phenotype) at sites of inflammation such as atherosclerotic plaques may amplify the response to injury, hence exacerbating the atherogenic processes that culminate in overt atherothrombotic disease.

Therefore, the possibility that fibrinogen gene variants may exert pleiotropic effects on serum IL6 concentrations was addressed in the SHEEP study. In male participants, the fibrinogen haplotypes FGG-FGA\*1 and FGG-FGA\*4 appeared to influence the serum IL6 concentration in a manner consistent with their impact on risk of MI: i.e. the FGG-FGA\*1 haplotype which appeared to confer an increased risk of MI, was associated with increased serum IL6 concentrations,

whereas the opposite relationship was observed for the protective FGG-FGA\*4 haplotype. The same associations were not found in women, which may be ascribed to differences in molecular and cellular (patho)physiology of the cardiovascular system between genders.<sup>310</sup> Notably, the serum IL6 concentration differs significantly between male cases and controls, while no such differences were noted amongst women. Therefore, the serum IL6 concentration may be a better risk indicator in men and than in women participating in the SHEEP study,<sup>311</sup> which is in agreement with recent findings from the prospective FINRISK study.<sup>312</sup>

In conclusion, these results strengthen the line of evidence implicating the FGG 9340T>C and FGA 2224G>A SNPs as contributors to the risk of MI. A dynamic crosstalk between the inflammation and coagulation pathways is likely to contribute to the complex mechanisms underlying MI. Therefore it is not unreasonable to conceive a bidirectional relationship between fibrinogen and IL6 that may be of importance in relation to MI. Nevertheless, these novel data need be confirmed and extended in independent epidemiological and experimental study settings.

### **Gene-gene and gene-environment interactions in relation to MI**

One of the objectives of this thesis was to study gene-gene and gene-environment interactions in relation to MI. Such studies are quite challenging to perform, given the multidimensional nature of MI, i.e. considering that an intricate interplay between genetic and environmental factors contribute to its etiology. The importance of the

environmental factors is well established. In contrast, despite the numerous genotype-phenotype association studies conducted so far, very few have provided consistent results (e.g. the ApoE studies). There are several reasons for these inconsistencies. Firstly, MI is a polygenic disease. Therefore, most genes involved are likely to have small effect sizes that are difficult to detect. Secondly, epistatic effects play an important role. However, these types of effects are often missed, partly because they are not considered at all in most genotype-phenotype association studies and also because the SNPs without individual main effects are usually excluded from further analyses. In addition, epistatic effects are easily overlooked when inadequate statistical tools are employed.

In the studies performed within the framework of this thesis, SNPs from different pathways (i.e. the coagulation and inflammation pathways) have been examined in relation to MI. None of these SNPs appeared to have any individual main effect on the risk of MI. These findings were in accordance with studies reporting lack of association between the IL6 1510G>C (known as the -174G>C SNP) and the F13A1 Val34Leu SNPs and risk of MI.<sup>313,314</sup> However, an array of data indicating that these two SNPs contribute to the variation in the risk of MI has also been reported.<sup>162,315</sup> This posed the question of whether these SNPs would behave differently when studied simultaneously in a broader genetic and environmental context. The MDR method, which seems to be better adapted for studies of multilocus associations, was employed in order to approach this question.

In the first analyses the FGG 9340T>C, FGA 2224G>A, FGB 1038G>A, IL6 1510G>C and F13A1 Val34Leu SNPs were included and the most parsimonious significant model of interaction consisted of the FGG 9340T>C and FGB 1038G>A SNPs. These results were confirmed by logistic regression analyses and also by haplotype analyses. Thus, the IL6 1510G>C and F13A1 Val34Leu SNPs did not appear to have a predictive value above the one offered by the fibrinogen SNPs. However, the gene-gene interaction model consisting of the FGG 9340T>C and the FGB 1038G>A SNPs had a rather low predictive accuracy. In the subsequent analyses, environmental factors, e.g. smoking, waist-to-hip ratio, alcohol consumption and risk factors such as hypertension, dyslipidemia and hyperglycemia, were added and as expected their effect was much stronger than the effects of these SNPs. A dyslipidemic phenotype together with increased waist-to-hip ratio appeared to offer a higher predictive accuracy, emphasizing the importance of these risk factors in relation to risk of MI.

Potential interactions on MI risk were then searched for between the plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations and the FGG 9340T>C and FGA 2224G>A SNPs which appeared to be independently related to MI. The FGB 1038G>A was also included in these analyses since it appeared to interact with the FGG 9340T>C SNP on the risk of MI. A high order interaction model, involving the plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations and the FGG 9340T>C and FGA 2224G>A SNPs was noted to confer a 3 fold increase in risk of MI. This constellation of factors appeared to confer a higher risk of MI than the one

involving the FGG 9340T>C and FGB 1038G>A SNPs ( $OR_{MDR}$  3.2 vs 1.8). Individuals having a plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations above the 75<sup>th</sup> percentile and who are carriers of the major FGG 9340T and FGA 2224G alleles run the highest risk of MI. Notably, these findings lend further support to the observation that fibrinogen haplotypes contribute to the variation in risk of MI, as the major FGG 9340T and FGA 2224G alleles are part of the haplotype that appeared to confer an increased risk (i.e. the FGG-FGA\*1 haplotype) and are involved in the high-order interaction model.

Thus, both the genetic and the environmental context in which genotype-phenotype association studies are performed play a major role. Notably, a gene may have a certain effect against a particular genetic background, and none/or the opposite effect against another genetic background. This may explain why it is so difficult to replicate studies in different genetic backgrounds.

In conclusion, epistasis involving fibrinogen gene polymorphisms and the plasma fibrinogen  $\gamma'$  and total fibrinogen concentrations contribute to the risk of MI. These results may pave the way for new gene tests, which could contribute to improved risk assessment strategies. Also, they might help identifying individuals in whom the fibrin clot structure may be more thrombogenic and display an impaired response to thrombolytic therapy due to an increased total plasma fibrinogen and fibrinogen  $\gamma'$  concentration in genetically predisposed individuals (i.e. carriers of the FGG 9340T and FGA 2224G alleles).

## Methodological considerations

The data presented in this thesis rest on findings from three population-based case-control studies, namely the HIFMECH, the SCARF and the SHEEP studies. A case-control study design is a powerful epidemiological tool. However, one must bear in mind that it confers several limitations, which in the worst-case scenario might undermine and invalidate the results.

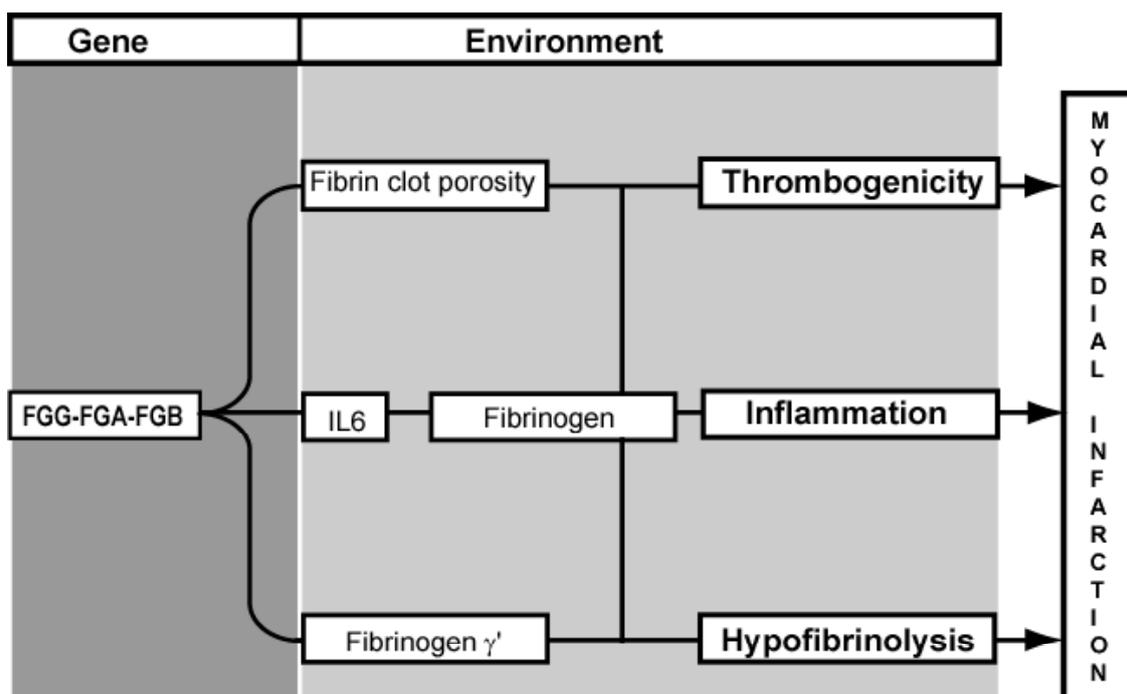
First, case-control studies are prone to confounding due to differential ascertainment of exposures between cases and controls. However, all three studies included in the present thesis have limited this possibility by including survivors of a first MI, thus reducing the risk that individuals with previously overt cardiovascular disease have changed their lifestyle. In this way, the possibility that inflammation, which is a hallmark of atherothrombotic disease, may have influenced the plasma fibrinogen concentration has also been reduced. On the other hand, it cannot be excluded that lifestyle modifications (e.g. smoking cessation, improved diet and increase physical activity) may have occurred in the early post-MI period. Moreover, since the analyses conducted in the present thesis have been restricted to postinfarction patients who survived the acute event, it cannot be excluded that subjects who died had a different genetic predisposition and/or different exposures to the risk factors studied, which may have been a source of selection bias.

Second, the selection of the controls is critical for the validity of the comparisons of the exposures. The controls included in the three study samples have been recruited from the

general population and from the same catchment areas as the patients. In addition, the controls were matched for age and sex, and were free from clinically overt cardiovascular disease. In the SHEEP study, five control candidates were sampled at the same time, in order to limit the risk of ending up with fewer controls due to non-responders. Due to a late response from some of the initial controls, occasionally both the initial and the alternative controls have been included. As a consequence, there are more controls than patients in the SHEEP study group.

Third, recall bias is a limitation inherent in the retrospective design of case-control studies. For instance, patients might be more prone to remember things potentially related to the disease but that may not always be completely accurate or relevant.

Fourth, the assessment of some of the exposures considered in the present thesis is based on biological assays, which even under optimal circumstances are not free of error. The method itself, the technical expertise and the biological variability of exposures like fibrinogen may influence the results. The plasma fibrinogen concentration, a key exposure considered in the present thesis, has been measured by the Clauss assay,<sup>265</sup> which seems to be quite robust.<sup>316</sup> However, there are various assays of fibrinogen that are known to yield different concentrations. For instance, the prothrombin time assay gives higher fibrinogen values than the Clauss assay.<sup>317</sup> On the other hand, the immunonephelometric method does not differ significantly from the syneresis method within a certain range of plasma fibrinogen concentration (2-12 g/L).<sup>318</sup> Nevertheless, despite the different



**Figure 10. Myocardial infarction is a multidimensional condition caused by a complex interplay between genetic and environmental factors through effects on intermediate phenotypes.**

fibrinogen assays employed, most studies have provided consistent evidence to suggest that plasma fibrinogen concentration is an independent predictor of CHD.<sup>180</sup>

Case-control studies, however, offer many advantages as well. They are attractive since a fairly rapid and cost-effective recruitment of study participants can be achieved. Moreover, case-control studies allow for the evaluation of a plethora of exposures. Given that, due to the rapid development of high-throughput SNP genotyping platforms, genome-wide association studies are becoming feasible,<sup>319</sup> well defined clinical study samples like SCARF, SHEEP and HIFMECH will be invaluable in the search for disease-causing genes.

### Future perspectives

While numerous epidemiological studies indicate that fibrinogen is an independent predictor of MI, the central question of

causality remains an area of future research. Evidence from *in vitro* studies, clinical studies and animal models implicates, indeed, fibrinogen as more than a simple bystander in the initiation, progression and exacerbation of atherothrombotic disease. Genetic variation in the fibrinogen gene cluster seems to predict the risk of MI, and this relation might entail effects on (1) fibrin clot structure, (2) serum IL6 concentration and (3) plasma fibrinogen  $\gamma'$  concentration (Figure 10). An altered fibrin clot structure with low porosity may be more thrombogenic, whereas a raised plasma fibrinogen  $\gamma'$  concentration may render such clots resistant to fibrinolysis, and elevated serum IL6 and plasma fibrinogen concentrations may signal the occurrence of progressive systemic inflammation, which at the plaque level reflects instability and propensity to rupture.

At first glance, it appears quite peculiar that the plasma fibrinogen concentration

seems not to be an intermediate phenotype linking fibrinogen SNPs to MI. First, this could be due to the multidimensionality of such complex traits. For instance, *trans*-acting loci (i.e. genes encoding transcription factors) may be quantitatively more important for the regulation of gene expression than *cis*-acting variation in the gene itself.<sup>320</sup> Moreover, an array of different genes and environmental factors may be operating in different individuals. Furthermore, the ubiquitous nature of epistasis and pleiotropy add yet another level of complexity in this context. Second, environmental and/or disease related stimuli could evoke a stronger increase in the plasma fibrinogen

concentration in genetically predisposed individuals that may be more important than the basal concentrations. Third, it cannot be excluded that the local enrichment of fibrin(ogen) in developing plaques is more detrimental than the systemic elevations in the plasma fibrinogen concentration may suggest. Accordingly, the role of fibrin(ogen) in atherothrombotic disease needs to be clarified at the plaque level, which requires a wide spectrum of *in vitro*, animal and clinical studies. Such studies may substantiate the relationship between fibrinogen SNPs and MI, which for the time being seems so fragile, subtle and difficult to seize.

## CONCLUSIONS

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**Plasma fibrinogen concentration is an independent risk factor for MI.**

**Elevated plasma fibrinogen  $\gamma'$  concentration is associated with increased risk of MI.**

**Genetic variants in the fibrinogen genes contribute to the variation in risk of MI and this association seems to be mediated through effects on plasma fibrinogen  $\gamma'$  concentration, fibrin clot structure and serum IL6 concentration.**

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