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Influence of fibrinogen and factor XIII genotypes on inflammatory processes

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Abbreviations and Units

°C	Degree Celcius
&	and
%	percentage
A	adenine
ACPA	anti-citrullinated protein antibody
Act	activity
Ag	antigen
Ala	alanine
Arg	arginine
AU/ml	absorbance unit per milliltre
BMI	body mass index
C	cytosine
CI	confidence interval
CD99	cluster of differentiation 99
CRP	C reactive protein
DAS28	disease activity score 28
DNA	deoxyribonucleic acid
DVT	deep vein thrombosis
EC	endothelial cell
e.g.	exempli gratia
ELISA	Enzyme-linked Immunosorbent Assay
ESR	erythrocyte sedimentation rate
et al.	et alia
etc.	et cetera
F13	factor 13
FDPs	fibrin degradation products
Fib	fibrinogen
FGA	fibrinogen alpha polypeptide
FGB	fibrinogen beta polypeptide

FGG	fibrinogen gamma polypeptide
FpA	fibrinopeptide A
FpB	fibrinopeptide B
G	guanine
g/l	gram per litre
Gln	glutamine
Glu	glutamic acid
Gly	glycine
H_2O	dihydrogen monoxide
HLA	human leucocyte antigen
i.e.	id est
IL6	Interleukin 6
IQR	interquartile range
IU/ml	international units per millilitre
kg/m^2	kilogram/square metre
Leu/L	leucine
Lys	lysine
$MgCl_2$	magnesium chloride
mg/l	milligram/litre
mL	millilitre
mM	millimolar
mm	millimetre
mm/h	millimetre/hour
MA	minor allele
min	minute
MNC	mononuclear cell
Mono	monocyte
n	number
ng	nanogram
OR	odds ratio
P_{adj}	p after the adjustment for non-independent observation

PAI-1	plasminogen activator inhibitor -1
PAI-2	plasminogen activator inhibitor-2
PCR	polymerase chain reaction
PE	pulmonary embolism
PECAM-1	platelet endothelial cell adhesion molecule-1
PMN/PMNC	polymorphonuclear cell
RA	rheumatoid arthritis
RF	rheumatoid factor
s	second
SE	shared epitope
SEM	standard error of the mean
SJC28	number of swollen joints
SNP	single nucleotide polymorphism
T	thymine
TAFI	thrombin activated fibrinolysis inhibitor
Thr	threonine
TJC28	number of tender joints (0-28)
Tm	primer melting temperature
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
Val/V	valine
VAS	visual analogue scale
VE-cadherin	vascular endothelial cadherin
vs	versus
wt	wildtype
y	year
µl	Microlitre
α	alpha
β	beta
γ	gamma

Abstract

Fibrinogen and factor XIII are primarily known for their functional role in the process of coagulation. They contribute to the formation of stable fibrin clots, which are essential for proper function of the haemostatic system. Fibrinogen synthesis and factor XIII functionality are influenced by genetic factors. For example, the β-fibrinogen (FGB) variant rs1800790 G>A is related to a stronger increase of fibrinogen levels during inflammation. The factor XIII A-subunit (F13A) variant Val34Leu influences the activation kinetic of factor XIII and results in altered fibrin clot structure in dependency of fibrinogen levels. Furthermore, it could be shown that FGB rs1800790 G>A and α-fibrinogen (FGA) Thr312Ala rs6050 influence inflammation interactively with F13A Val34Leu. It was hypothesized that those genotype constellations predisposing for dense fibrin clot structures (FGB rs1800790 A carriage & F13A 34Val/Val) are related to reduced, and those predisposing for loose fibrin clots (FGA 312Ala carriage & F13A 34Val/Val) to increased inflammatory activity.

In this study we tested in a cohort of patients suffering from rheumatoid arthritis (RA) if the variants FGA rs2070006 G>A and FGA rs2070016 T>C in combination with F13A Val34Leu influence inflammatory activity. This activity is measured by C-reactive protein (CRP) levels, clinical disease activity score (DAS) of 28 joints and morning stiffness, which is a feature typically found in RA patients. Both FGA variants are not in strong linkage dysequilibrium with FGA Thr312Ala and are known to influence fibrinogen levels.

In agreement with the hypothetical model, we found for FGA rs2070006 G>A and F13A Val34Leu an interplay with respect to CRP levels comparable to that found for FGA rs6050 Thr312Ala. Surprisingly, for FGA rs2070016 T>C associations with lower CRP levels were found that seemed to be independent from F13A Val34Leu genotype. However, this variant FGA rs2070016 T>C in F13A 34Val/Val patients was associated with reduced clinical disease activity, a finding, which would corroborate the hypothesis. Finally, FGA rs2070016 T>C exhibited a trend for shortened morning stiffness.

In conclusion, the findings of these analyses are in part in agreement with the hypothesis to be tested. The independency of the relation between FGA rs2070016 T>C on CRP levels from F13A Val34Leu genotype as well as the interesting connection with clinical disease activity should be addressed in further studies.

Zusammenfassung

Fibrinogen und Faktor XIII werden in erster Linie mit der Hämostase in Verbindung gebracht. Sie sind für die Generierung stabiler Fibringerinnsel und damit eine effektive Blutungsstillung verantwortlich. Sowohl die Fibrinogensynthese als auch die Funktionalität des Faktor XIII unterliegen genetischen Einflüssen. Beispielsweise ist die β-Fibrinogen (FGB) Variante rs1800790 G>A mit einem starken Anstieg der Fibrinogenkonzentration bei Entzündungsreaktionen verbunden. Die Variante Val34Leu der Faktor XIII A-Untereinheit (F13A) beeinflusst die Aktivierungskinetik vom Faktor XIII und damit in Abhängigkeit von der Fibrinogenkonzentration die Fibringerinnselstruktur. Außerdem konnte gezeigt werden das FGB rs1800790 G>A und α-Fibrinogen (FGA) Thr312Ala rs6050 gemeinsam mit F13A Val34Leu Entzündungsprozesse beeinflussen. In einem hypothetischen Modell wurde angenommen, dass diejenigen Genotypkonstellationen, die für dichte Fibringerinnsel prädisponieren (FGB rs1800790 A Träger & F13A 34Val/Val) mit einer verminderten und diejenigen, die für lockerere Fibringerinnsel prädisponieren (FGA 312Ala Träger & F13A 34Val/Val) mit einer gesteigerten Entzündungsaktivität verbunden sind.

In dieser Arbeit wurde in einer Kohorte von Patienten mit Rheumatoider Arthritis untersucht, ob die Varianten FGA rs2070006 G>A und FGA rs2070016 T>C in Kombination mit F13A Val34Leu die entzündliche Aktivität beeinflussen. Als Marker der entzündlichen beziehungsweise Krankheitsaktivität wurden die Konzentration des C-reaktiven Proteins (CRP), der klinische *Disease Activity Score* (DAS) aus 28 Gelenken und die Morgensteifigkeit verwendet. Beide FGA Varianten stehen nicht in einem starken Kopplungsungleichgewicht mit FGA Thr312Ala und wurden als Einflussfaktoren der Fibrinogenkonzentration beschrieben.

In Übereinstimmung mit der oben beschriebenen Hypothese, konnte für FGA rs2070006 G>A und F13A Val34Leu ein Effekt auf die CRP-Konzentration nachgewiesen werden, der mit dem früher für FGA Thr312Ala beschriebenen vergleichbar ist. Überraschenderweise zeigte sich für FGA rs2070016 T>C eine Beziehung zu niedrigeren CRP-Konzentrationen, die unabhängig vom F13A Val34Leu Genotyp zu sein scheint. Jedoch konnte für diese Variante FGA rs2070016 T>C bei F13A 34Val/Val Patienten zusätzlich eine Assoziation mit niedriger klinischer Krankheitsaktivität gefunden werden, was das hypothetische Modell zu unterstützen scheint. Außerdem zeigt sich für FGA rs2070016 T>C ein statistischer Trend für eine verkürzte Morgensteifigkeit.

Zusammengefasst bestätigen die Untersuchungen das hypothetische Modell zum Teil. Die Unabhängigkeit der Beziehung zwischen FGA rs2070016 T>C und CRP-Konzentration vom F13A Val34Leu Genotyp sowie die Beziehungen zu klinischen Endpunkten wie dem DAS28 und der Morgensteifigkeit könnten in weiteren Studien untersucht werden.

1. Introduction

In the common perception, fibrinogen, fibrin and the fibrin network are primarily involved in coagulation, i.e., the final result of a procoagulatory trigger is the formation of a fibrin clot, which helps the organism to stop bleeding.

However, over the last two decades evidence has shown that the haemostatic system in general and fibrinogen and its derivatives in particular are also involved in the inflammatory process. Therefore, various components of the haemostatic system are related to the propagation of inflammation, while others are known to suppress the intensity of the inflammatory process.

1.1 Fibrinogen in haemostasis

Fibrinogen or factor I is a polypeptide synthesized in the liver (Tennent et.al, 2007), playing a functional role in different processes such as blood coagulation, fibrinolysis, wound healing, inflammation and cell and matrix interactions. It belongs to the acute-phase-proteins and can be found in great quantities in the plasma and the α granules of platelets.

As a substrate for thrombin, the main function of fibrinogen is to contribute to coagulation. Under the influence of thrombin, soluble fibrinogen is converted to soluble fibrin, which polymerises. Covalent crosslinking by factor XIII transforms this product of polymerisation to the insoluble fibrin gel and as a result a stable blood clot is created. The formation of this clot prevents or at least diminishes blood loss.

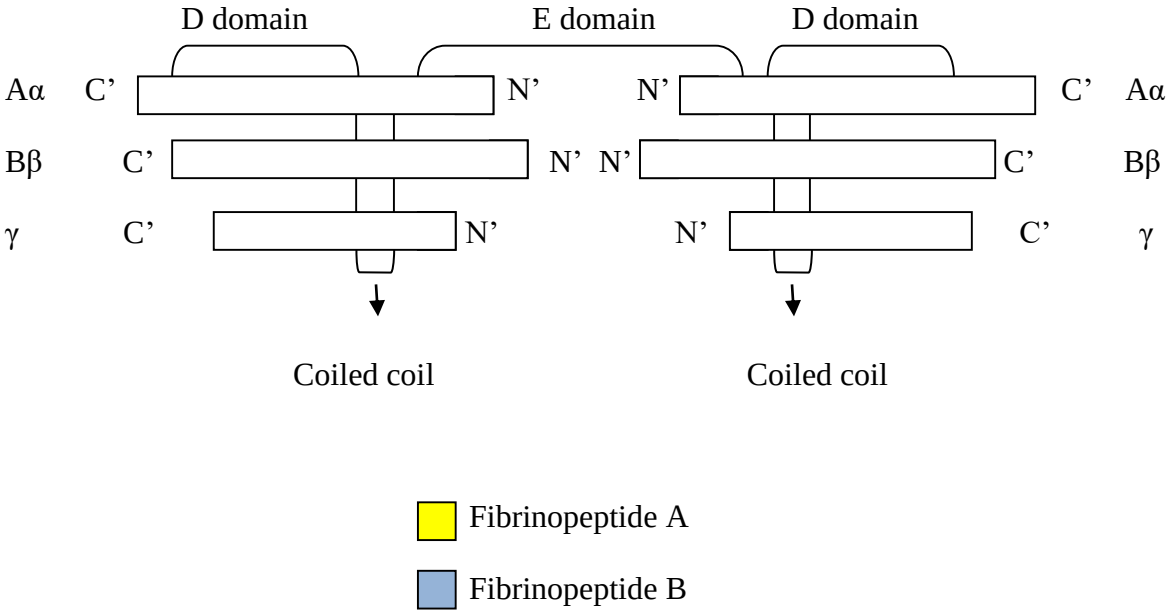
1.2 Fibrin polymerisation and fibrin network formation

Fibrinogen is a multidomain protein with a (hetero-) dimeric structure. It is composed of three sets of polypeptide chains (Aα, Bβ and γ/γ') interconnected by a multitude of disulfide bonds. Thus, fibrinogen contains predominantly 2 Aα-, 2 Bβ- and 2 γ-chains (AαBβγ/AαBβγ) and to a lesser extent 2 Aα- and 2 Bβ-chains are combined with 1 γ- and 1 γ'-chain (AαBβγ/AαBβγ').

Fibrinogen's structure (**Figure 1.1**) consists of a central E domain. The N-termini of the α-, β- and γ-chains are located here and exhibit amino acid sequences of great functional importance. The amino acid sequences of α- and β- chains are called fibrinopeptide A (FpA) and fibrinopeptide B (FpB), respectively, and after their removal by thrombin the so-called "knobs" are revealed. The E domain is connected by coiled structures with two outer D domains. The surface of the fibrinogen D

domains carries functionally relevant impressions, which are called “holes”. Knobs and holes combine with each other when the fibrin clot is formed.

(a)



(b)

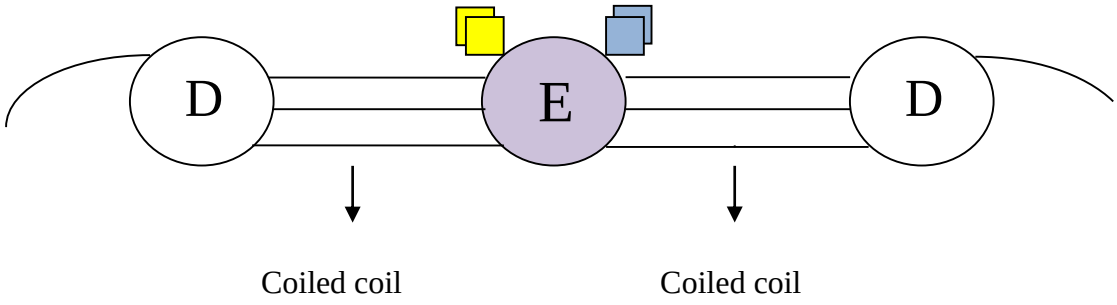


Figure 1.1: Structure of fibrinogen. Fibrinogen’s composition of 2 Aα-, 2 Bβ- and 2 γ-chains (AαBβγ/AαBβγ) and its domains (E and D) are given in (a) and (b) respectively.

The transformation of fibrinogen to fibrin takes place in three phases: (1) the phase of hydrolytic cleavage of FpA and FpB by thrombin, (2) the phase of polymerisation and (3) the phase of crosslinking (Georgoulis, 2010) (**Figure 1.2**).

In the first phase, FpA and FpB of the soluble fibrinogen monomer are cut under the influence of thrombin. This cleavage transforms fibrinogen to fibrin and exposes the so-called "knobs" of the E domain, which before this event were hidden by FpA and FpB. This demasking allows for the binding of "knobs" to so-called "holes" and the interaction between E and D domains, so that the E domain of one fibrin molecule becomes connected to the D domain of other fibrin molecules.

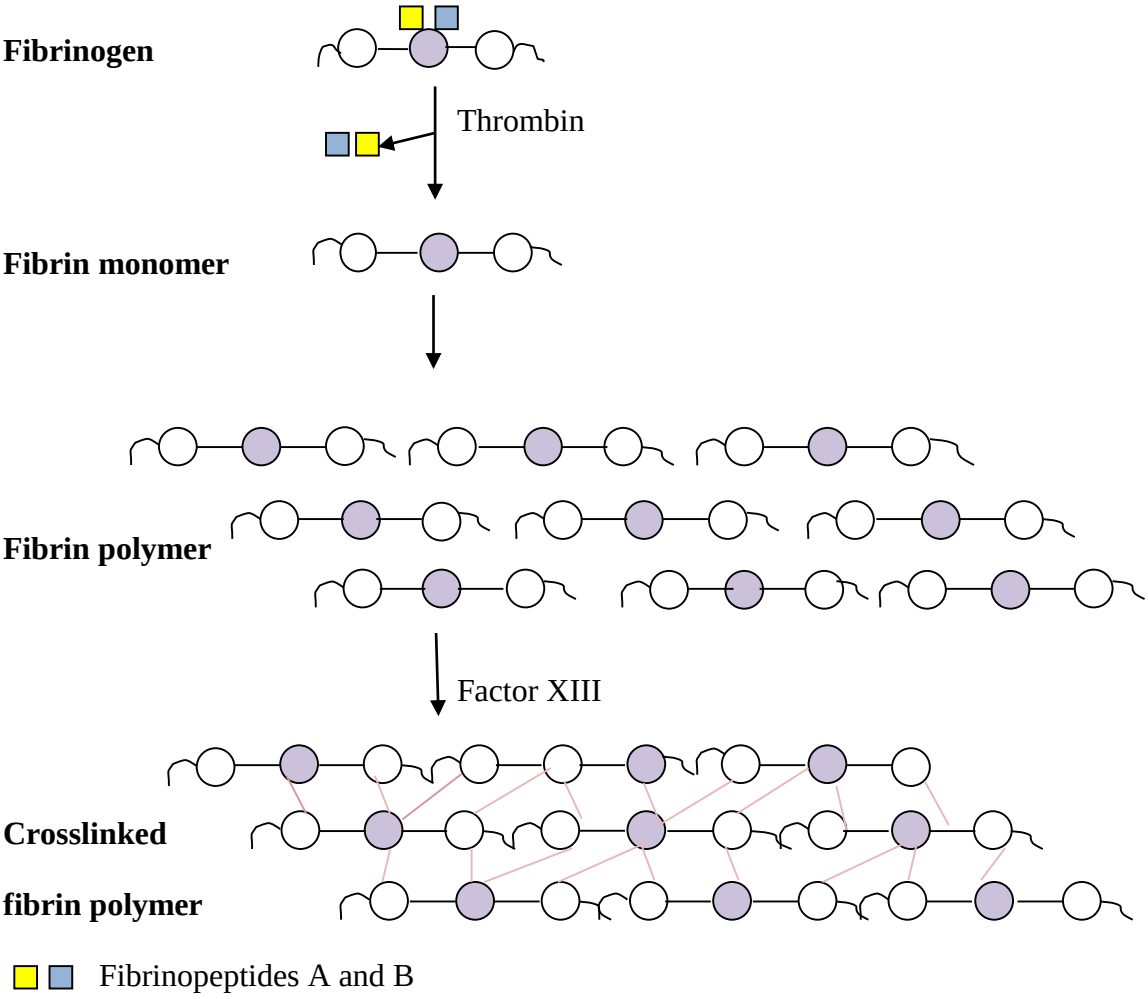


Figure 1.2: Activation of fibrinogen and crosslinking. The process of fibrinogen activation by thrombin as well as fibrin polymerisation and crosslinking are shown.

The fibrin monomers polymerise to fibrin fibrils and strands. At this stage, the fibrin gel is unstable. In the third phase, the fibrin gel is modified by factor XIII by covalent crosslinking. In this way, the unstable fibrin gel is transformed to a mechanically more stable fibrin network, which exhibits increased resistance to fibrinolysis. The importance of crosslinking and creating a stable clot correlates with the significance of withstanding against both fibrinolysis and mechanical stress (Weisel, 2007). Various studies have proved that there is a direct relation between the formation of a highly crosslinked fibrin network and reduced susceptibility to fibrinolysis. Under the influence of active plasmin, fibrin clots made of condensed, thin fibers with many crosslinks exhibit a slower fibrinolytic degradation than those with a loose fibrin structure with thicker fibers and a low grade of crosslinking (Weisel, 2007). The variables that influence the fibrin gel architecture are described in the following sections.

1.3 The role of factor XIII in fibrin crosslinking

Factor XIII is a transglutaminase and very important in haemostasis due to its role in the generation of a firm clot.

Two different forms of factor XIII can be distinguished: One is plasmatic, the other cellular (Komáromi et al., 2011). Plasmatic factor XIII is a tetramer composed of two catalytic A subunits and two B subunits (**Figure 1.3**). The main function of the B subunits is to carry the As and to mediate the binding to fibrinogen. The cellular form of factor XIII can be found in platelets and consists solely of two A subunits. The synthesis of the A units of factor XIII occurs in hepatocytes, monocytes and megakaryocytes, while the B units are synthesised solely in the liver (Ariëns et al., 2002).

Circulating factor XIII is enzymatically inactive. It becomes active after a thrombin-mediated cleavage of the activation peptides which block the active sites of the proenzymes (**Figure 1.3**). After the activation of factor XIII to factor XIIIa, the B subunits are released and the active dimer consisting of two factor XIII A subunits catalyzes fibrin crosslinking.

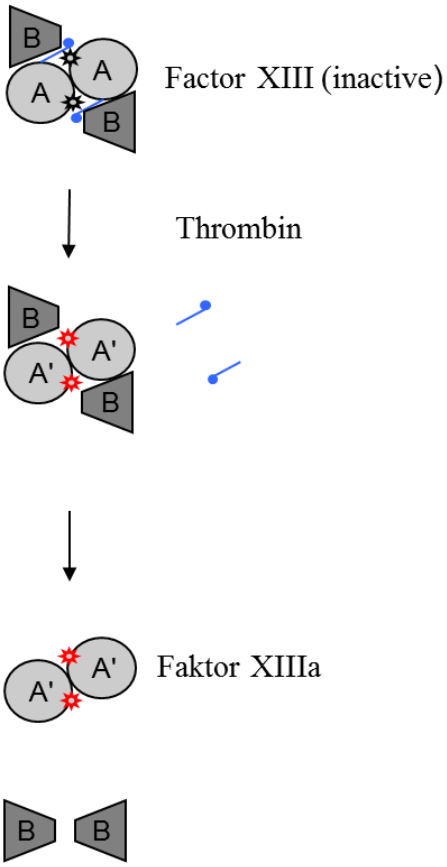


Figure 1.3: Structure and activation of plasmatic factor XIII. Plasmatic factor XIII consists of a tetramer formed by two A and two B subunits. The inactive factor XIII is converted to the active form (factor XIIIa) by the thrombin-mediated cleavage of the activation peptides.

The activated factor XIII crosslinks fibrin fibrils by creating covalent bonds between the C-terminal ends of the γ-chains of fibrin (Mosesson, 2005). The bonds are created between the Lys406 residues of the γ-chains of one fibrin molecule and the Gln 398/399 residues of the γ-chains of another fibrin unit. Additional crosslinks are also created at a slower rate between amine donors and lysine acceptors of two α-chains or one α- and one γ-chain.

The major importance of factor XIII becomes evident by the clinical symptoms related to a factor XIII deficiency. Patients with a lack of factor XIII have a severe haemorrhagic tendency. Factor XIII deficient children develop bleeding from the umbilical cord in the first days after birth (Karimi et al., 2009). Superficial bruising, bleeding in the oral cavity, and post-surgery bleeding are some of

the most common symptoms in factor XIII deficient patients. Moreover, congenital factor XIII deficiency in women is associated with a strong history of repeated spontaneous abortions. The mechanism of this phenomenon is not well understood, however, the causality seems to be proved by the clinical improvement of pregnancy outcome under substitution therapy with plasmatic factor XIII. Interestingly, the substitution of the cellular form of factor XIII is not sufficient for the prevention of abortions (Karimi et al., 2009). According to the literature ‘‘the level of plasma A-subunit of factor XIII antigen (XIII-Ag) or factor XIII activity (XIII-act) must be at least 2%-3%, and if possible, higher than 10% to prevent decidual bleeding and miscarriage during the pregnancy’’ (Asahina et al., 2007).

1.4 Fibrinolysis and factor XIII

The balance of the coagulation cascade is based on the formation of the fibrin clot as well as its degradation, which is called fibrinolysis. This process starts with the activation of plasminogen either by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (Chapin & Hajjar, 2015). The main activator is tPA while uPA seems to play a mainly extravascular role. After plasminogen is activated, it triggers the cleavage of fibrin into fibrin degradation products (FDPs). A special type of these fragments, which consist of two crosslinked D domains, are called D-dimers and they are of great diagnostic importance due to a high negative-predictive value of low D-dimer levels with regards to deep vein thrombosis (DVT) and pulmonary embolism (PE). An excessive activity of plasmin is circumvented by the existence of different inhibitors, e.g. plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2), α2-antiplasmin and thrombin activated fibrinolysis inhibitor (TAFI).

Hethersaw et al. proved that factor XIII not only contributes to the cross-linking of fibrin but also affects the architecture of the fibrin clot (Hethersaw et al., 2014). In factor XIII crosslinked fibrin gels, the fibers are thinner and the network is denser than in un-crosslinked fibrin gel. This more compact architecture as well as a higher content of α2-antiplasmin results in a significant delay of fibrinolysis and a decreased fibrinolysis rate.

1.5 Genetic variability of fibrinogen and factor XIII

As mentioned above, fibrinogen consists of three pairs of polypeptide chains (αα, ββ and γγ or γγ'). The synthesis of these polypeptides is encoded by three genes - FGA for the α-, FGB for the β- and FGG for the γ-/γ'-chain, which are located within 50 kilobases on chromosome 4 (4q31) (Neerman-Arbez & de Moerloose, 2007) (**Figure 1.4**).

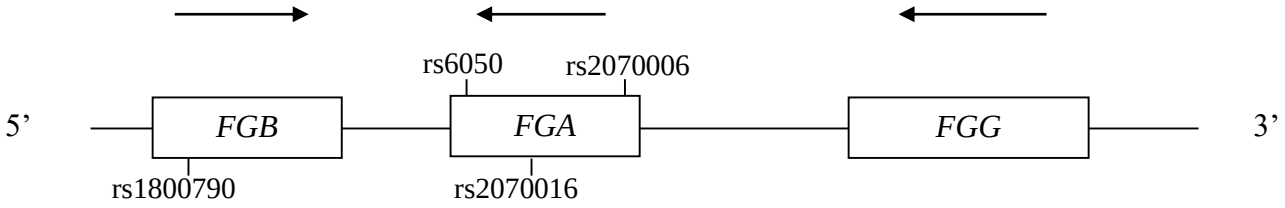


Figure 1.4: Genetic organisation of fibrinogen's loci. The genetic loci of the β-fibrinogen (FGB), α-fibrinogen (FGA) and γ-fibrinogen (FGG) chain are given. The single nucleotide polymorphisms (SNPs) related to this study are indicated.

The genetic loci of fibrinogen exhibit a considerable genetic variability. The best characterised genetic variants concern the α-chain (rs6050, FGA Thr312Ala) and the β-chain (rs1800790, FGB -455G>A). Functionally, both variants are associated with the fibrinogen synthesis rate during acute-phase-reactions. While the FGA rs6050 Thr312Ala variant carriage is assumed to result in lower increases in fibrinogen levels under inflammatory stimuli, the FGB rs1800790 G>A variant is associated with higher fibrinogen elevations under these conditions. Many other studies indicate a relevant genetic background of fibrinogen levels in response to inflammatory triggers (e.g. Jacquemin et al., 2008, Peters et al., 2009). Interestingly, the AIRGENE Study Group (Peters et al., 2009) described a significant influence of the fibrinogen loci on the fibrinogen response to ambient particulate matter in the air.

Furthermore, there are many publications describing possible associations between different fibrinogen genotypes and clinical phenotypes like venous thromboembolism or atherothrombosis. These disease associations are put in relation to different fibrinogen levels. Thus, Siegerink et al. described that in women of 18-50 years of age the FGA variant rs6050 Thr312Ala correlates with

lower plasma fibrinogen levels and is associated with a decreased risk of ischaemic stroke (Siegerink et al., 2009). Compared to FGA rs6050 Thr312Ala, the variant FGB rs1800790 G>A was characterised as having opposite effects on the biochemical as well as on the clinical endpoint. Not in complete agreement with these findings, when analysing the combined data of the Copenhagen General Population Study and the Copenhagen City Heart Study, Klovaite and colleagues (Klovaite et al., 2013) showed that fibrinogen genotypes like FGB rs1800790 G>A and the strongly linked FGB rs4220 Arg448Lys are associated with higher plasma fibrinogen levels, but that there is no correlation between increased plasma fibrinogen levels and the risk of deep vein thrombosis (DVT). Interestingly, when looking at the risk of pulmonary embolism (PE) in the same study a clear relation to plasma fibrinogen levels was found.

The genes of the factor XIII A- and B-subunit (F13A, F13B) are located on chromosome 6 (6p25.1) and 1 (1q31.3), respectively. The variant F13A rs5985 (factor XIII A-subunit Val34Leu), which is the subject of this research, has been studied in detail both functionally and epidemiologically. This polymorphism is quite frequent in our population (almost 25%) (Ariëns et al., 2000). The association between F13A rs5985 Val34Leu and the risk of DVT was the subject of a large control study, The Leiden Thrombophilia Study (Van Hylckama Vlieg et al., 2002). The results showed that men who were homozygous for the factor XIII A-subunit 34Leu allele exhibited a 30% lower risk of DVT. For heterozygous carriers the association was considerably weaker. However, it should be mentioned that this relation between F13A rs5985 Val34Leu and thrombosis is still subject to discussion as it could not be replicated in some other studies (e.g. Tiedje et al., 2011).

In a more functional view, F13A rs5985 Val34Leu is correlated with the rate of FXIII activation and the susceptibility of the resulting fibrin clot to fibrinolysis. The substitution of G with T at codon 34 results in a replacement of valine with leucine (Ariëns et al., 2002). The fact that this variant affects the amino acid sequence near the thrombin cleavage site has a functional consequence on factor XIII activation. Ariëns et al. were able to demonstrate that in individuals with factor XIIIA-subunit 34Leu the cleavage rate by thrombin is higher and occurs at lower thrombin activity compared to the activation process with factor XIII A-subunit 34Val (Ariëns et al., 2000). Thus, the factor XIIIA-subunit Val34Leu genotype influences the timing of the activation kinetics of factor XIII **(Figure 1.5).** This affects the composition of the fibrin clots which "in the presence of Leu34 factor XIII have thinner fibers, smaller pores, and altered permeation characteristics when compared with fibrin clots formed in the presence of the Val34 variant''(Ariëns et al., 2002).

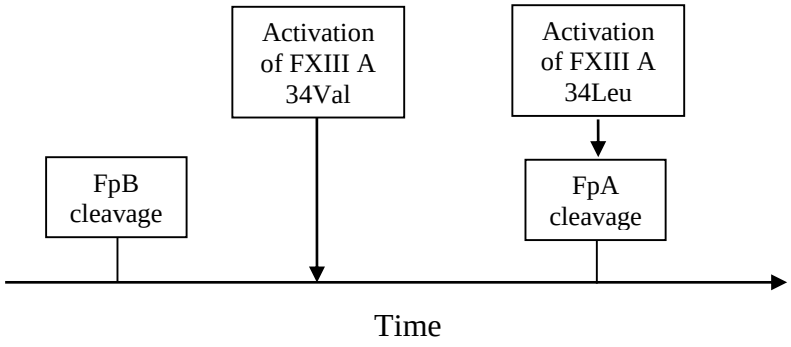


Figure 1.5: Functional consequences of factor XIII A-subunit Val34Leu. The factor 13 subunit A (FXIII A-subunit) Val34Leu genotype influences the kinetics of activation of factor XIII in relation to cleavage of fibrinopeptide A (FpA) and B (FpB).

The interrelation between the kinetics of FpA-/FpB-cleavage on the one hand and of the activation of factor XIII on the other has a crucial influence on the architecture of the resulting fibrin network (**Figure 1.5**). Thus, acquired and/or hereditary factors that influence this interrelation are of importance for the formation of the fibrin gel and its properties (e.g. density, mechanical stability, susceptibility to fibrinolysis) (Scott et. al., 2004).

1.6 Fibrin network formation in relation to factor XIIIA genotype

In 2003 Lim and colleagues published a study, investigating the association between different polymorphisms of factor XIII and fibrinogen and the structure of the fibrin clot. The results of these investigations were put in relation to the assumed association of these genotypes with vascular risk (Lim et al., 2003). As the results of this study are of special importance for my thesis, a more detailed description is given.

In this study (Lim et al., 2003), plasma samples of patients with a diagnosis of ischaemic stroke and with different genotype constellations at F13A, FGA and FGB were analysed (F13A Val34Leu (rs5985); FGA Thr312Ala (rs6050); FGB Arg448Lys (rs4220)). The experiments showed that in patients homozygous for the F13A 34Val allele, there was an inverse relation between the concentration of fibrinogen and the permeability of the resulting fibrin clot. Under this F13A genotype

constellation increasing fibrinogen levels resulted in decreasing permeability of the formed clot or in other words – as permeability and clot density exhibit an inverse relation – with increasing fibrinogen concentrations, the fibrin network became increasingly dense with thinner and highly crosslinked fibers. Conversely, in the case of homozygosity for F13A 34Leu the fibrinogen level and fibrin clot density were largely unrelated. That means that in the case of homozygosity for F13A 34Leu the fibrin gels exhibit an intermediate density, irrespective of the fibrinogen level used in the assay (**Figure 1.6**).

As it is known that patients with arterial or venous thrombosis exhibit a tendency to create highly crosslinked clots with high density and smaller pores, the studies of Lim et al. helped in the understanding of a possible relation of factor XIII and fibrinogen genetics with this clinical phenotype (Lim et al., 2003).

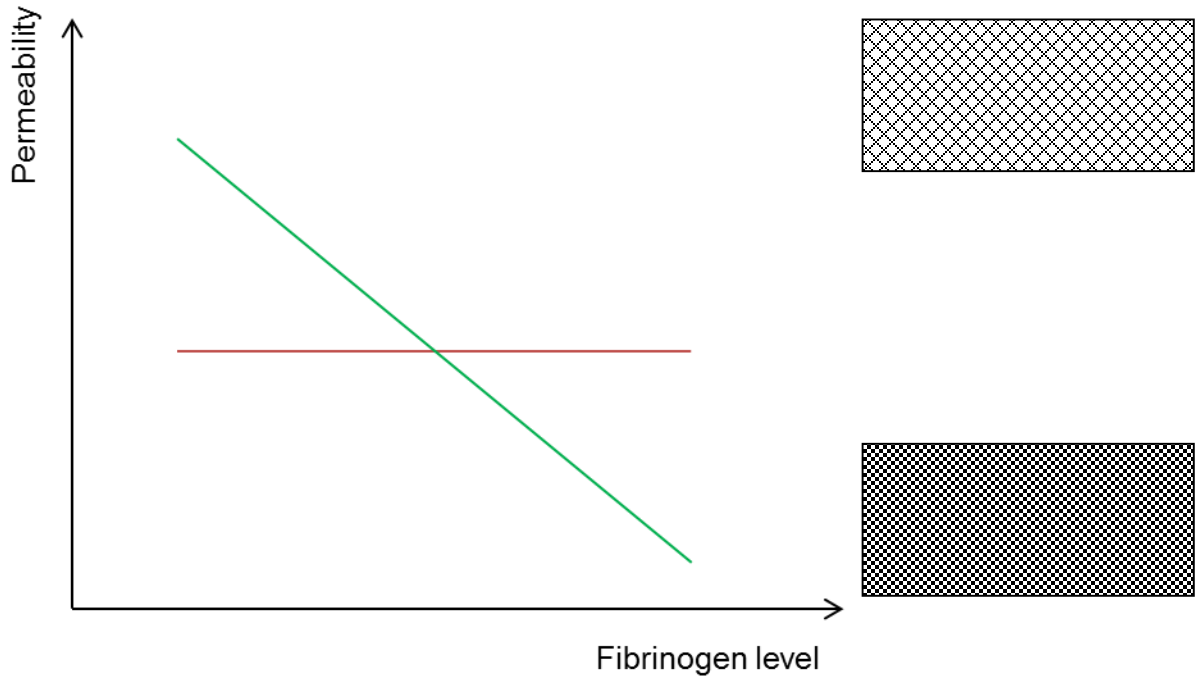


Figure 1.6: Influence of fibrinogen level on fibrin gel permeability in relation to F13A Val34Leu genotype. The lines represent schematically the fibrin gel permeability in relation to fibrinogen level in F13A 34Val/Val (green) and F13A 34 Leu/Leu individuals. (Figure adapted from Lim et al., 2003).

1.7 The relation of fibrin(ogen) derivatives and fibrin network to inflammation

Many studies have been conducted to investigate the influence of fibrin(ogen) and its derivatives on inflammation. In 1975 Richardson and his colleagues proved that FpB, after being cleaved by Contortrix venom, can induce monocyte and polymorphonuclear leukocyte (PMNC) migration (Richardson et al., 1976). Interestingly, this finding could not be detected for FpA. In the same study, the chemotactic properties of fibrinogen's D-domain and E-domain fragments towards monocytes were shown (**Figure 1.7**). On the other hand, the so-called X and Y fragments of fibrin did not show any corresponding activity. The role of FpB as a chemoattractant was further investigated by Senior and his colleagues (Senior et al., 1986). In this study the chemotactic activity of FpB on PMNC and fibroblasts but not on monocytes was described. The chemotactic response of PMNC to FpB was comparable with the one induced by other factors like the human complement C5a, leukotriene B4 and formyl-methionyl-leucyl-phenylalanine.

Apart from the effects of fibrin(ogen) derivatives on chemotaxis, the ability of mononuclear cells to migrate through fibrin gel matrices is influenced by different factors, as was shown in the study by Lanir et al. (Lanir et al., 1988). The researchers found that the presence of fibronectin and factor XIII in fibrin gels hindered migration of mononuclear cells (MNC), concerning both the number of migrating cells as well as the covered distance (**Figure 1.7**). There is evidence that this effect is not the result of small pore sizes, which could prevent MNC migration mechanically, but rather due to the influence of fibrin gel architecture on receptor mediated MNC-fibrin interactions.

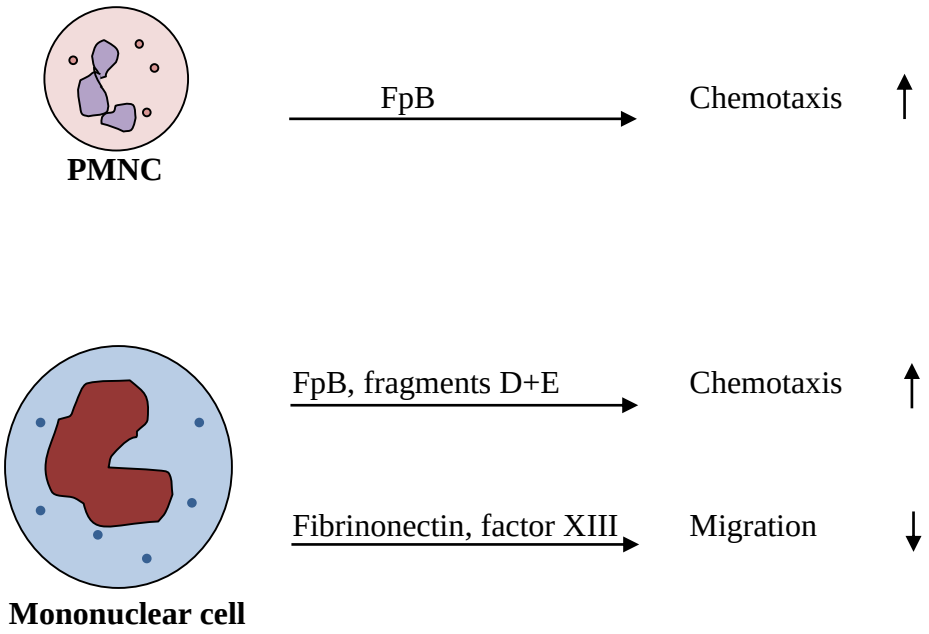


Figure 1.7: Schematic depiction of the influence of fibrin(ogen) derivatives and fibrin gel architecture on chemotaxis and migration. Fibrinopeptide B (FpB) as well as other fibrin fragments, fibronectin and factor XIII all exhibit differing effects on cellular migration, PMNC, polymorphonuclear leukocyte.

Finally, a short description of the data, which triggered the work of this thesis, should be given. In 2012 Hoppe et al. showed an interactive effect of fibrinogen and F13A genotypes on levels of C-reactive protein (CRP) (Hoppe et al., 2012a). It was demonstrated that in F13A rs5985 wildtype individuals the variant FGB rs1800790 G>A is associated with lower CRP levels in patients with rheumatoid arthritis as well as in those with non-autoimmune inflammatory diseases. The opposite relation existed with regards to F13A rs5985 wildtype individuals when looking at the FGA variant rs6050 Thr312Ala. Based on these results, a hypothetical model for these findings was established (**Figure 1.8**).

1.8 Aim of this thesis

The hypothetical model for the influence of fibrinogen and F13A genotypes on inflammation was based on FGB rs1800790 G>A, FGA rs6050 Thr312Ala and F13A rs5985 Val34Leu. To test the applicability of this model, other fibrinogen genotypes, which had previously been characterised for their effect on fibrinogen synthesis, and which are not in strong linkage dysequilibrium with the variants mentioned above, were tested. In our study, we investigated the association between the variants FGA rs2070006 G>A and FGA rs2070016 T>C and inflammation considering F13A Val/Val as a potential permissive factor (**Figure 1.8**). Our study was focused on patients suffering from rheumatoid arthritis. In addition to the effect on CRP, we explored the influence of these fibrinogen variants on the clinical features of this disease.

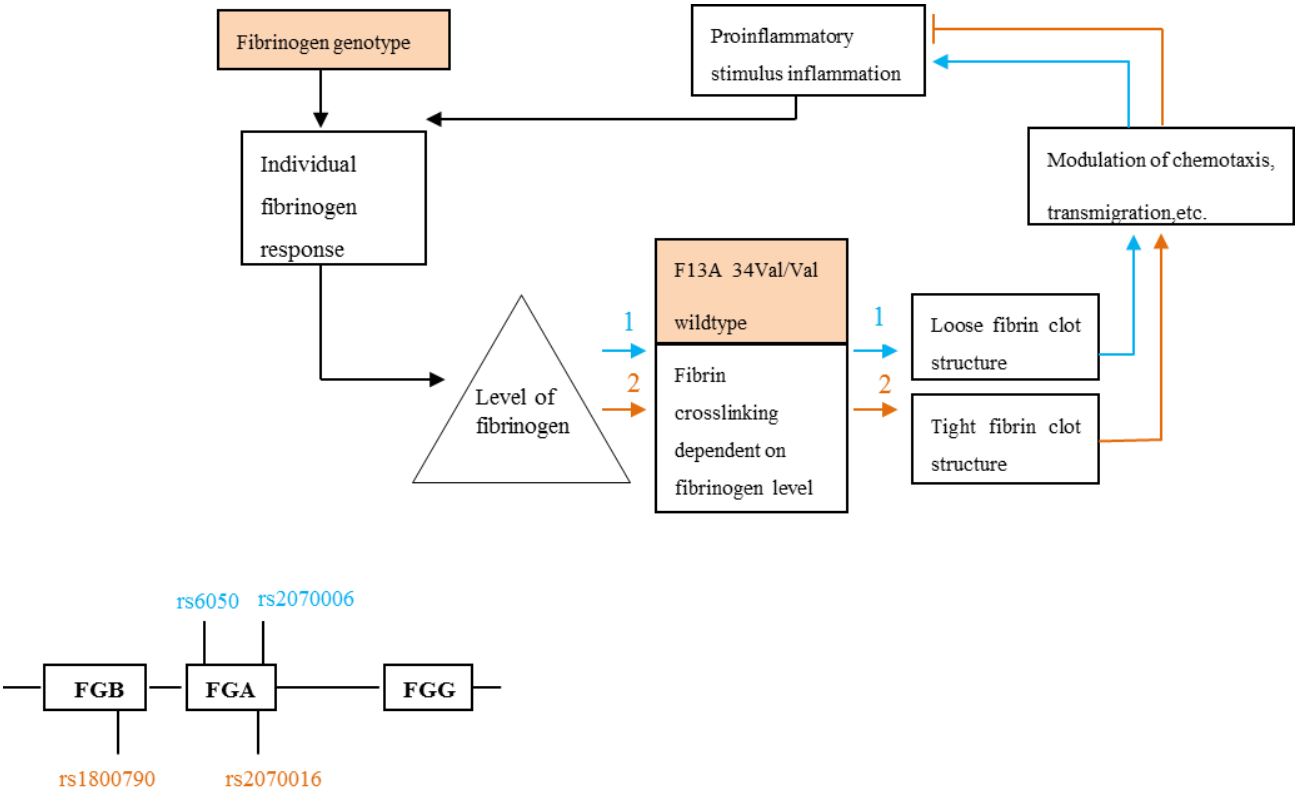


Figure 1.8: Hypothetical model for the influence of fibrinogen and F13A genotypes on the intensity of the inflammatory process. In addition to the variants FGB rs1800790 G>A and FGA rs6050 Thr312Ala, which were characterised in the initial studies, the FGA variant rs2070016 T>C and rs2070006 G>A of the present thesis are given. (adapted from Hoppe & Dörner, 2012b).

2. Materials and Methods

2.1 Patients

This investigation was based on a cohort of patients suffering from rheumatoid arthritis (RA) that was previously described (Hoppe et al., 2012a). All patients were under regular medical follow-up in the outpatient unit of the Department of Rheumatology and Clinical Immunology of the Charité–Universitätsmedizin Berlin. They fulfilled the 1987 criteria for classification of rheumatoid arthritis (RA) as published by the American College of Rheumatology (Arnett et al., 1988) and were included in the study after written informed consent was given. Work on this thesis as well as previous studies of this cohort are part of the BMBF-Forschungsverbund ArthroMark (01EC1009A) and were approved by the Charité ethics committee.

Originally, this cohort consisted of 373 consecutive RA patients (Hoppe et al., 2009) well characterised with regards to disease severity, medical history and pharmaceutical pretreatments. All patients presenting repeatedly (number of observations: $\geq$2) in the outpatient unit (n=288) were included in our initial study on a potential relation between fibrinogen and factor XIII genotypes and the inflammatory activity that was performed on β-fibrinogen (FGB) -455G>A (rs1800790), α-fibrinogen (FGA) Thr312Ala (rs6050) and factor XIII A- subunit (F13A) Val34Leu (rs5985) (Hoppe et al., 2012a).

In the context of this thesis, in 2016, this cohort (n=285 - in three cases no material was available for testing) was further characterised by testing for FGA rs2070016 T>C and FGA rs2070006 G>A, which are known to influence fibrinogen synthesis (Jacquemin et al., 2008) and not to be in strong linkage dysequilibrium with the variants described above.

2.2 Collection of clinical data

Clinical data necessary for the characterisation of disease activity as measured by the disease activity score 28 (DAS 28) included the erythrocyte sedimentation rate (ESR), number of tender joints (TJC28), number of swollen joints (SJC28), as well as the patient assessment of general health using a 100 mm visual analogue scale (VAS) **(Figure 2.1)**. Based on these data, individual DAS 28 were calculated as follows:

$$DAS28 = 0{,}56 \times \sqrt{TJC28} + 0{,}28 \times \sqrt{SJC28} + 0{,}014 \times VAS + 0{,}36 \times \ln(ESR + 1) + 0{,}96$$

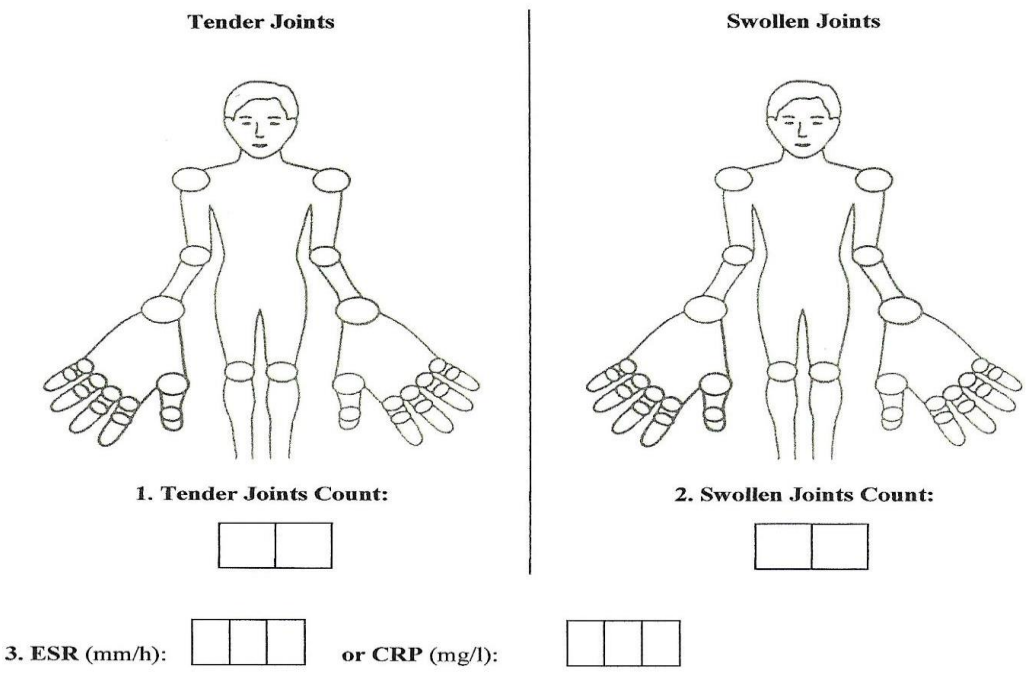


Figure 2.1: Parameters used in the calculation of DAS 28 score. The following parameters are used: number of joints with tenderness upon touching, number of swollen joints, erythrocyte sedimentation rate (ESR), subjective assessment of disease activity by the patient during the last week on a visual scale. (Approved schema, Department of Rheumatology and Clinical Immunology, Campus Charité Mitte, Charité–Universitätsmedizin Berlin.)

All these data as well as information on patient characteristics, individual clinical courses and treatment histories were collected by study nurses of the clinic's outpatient unit and deposited in a clinical database.

2.3 Laboratory testing

Measurements of anti-citrullinated protein antibodies (ACPA), rheumatoid factor (RF) and CRP levels as well as the characterisation for HLA-DRB1 shared epitope (SE), FGB rs1800790 G>A, FGA rs6050 Thr312Ala and F13A rs5985 Val34Leu were done previously (Hoppe et al., 2009 & Hoppe et al., 2012a) and are described briefly in the following sections.

2.4 Measurement of autoantibodies and C-reactive protein (CRP) levels

The ACPA levels of this cohort were quantified from serum usually taken at the time of first presentation in the outpatient unit using a second-generation Enzyme-linked Immunosorbent Assay (ELISA) (Euro-Diagnostica, Malmö, Sweden). For classification as ACPA negative or positive, a cut-off level of 25 units per mL was used.

For the quantification of rheumatoid factor (RF) levels serum was tested by an ELISA-based technique specific for IgM antibodies (DLD Diagnostika, Hamburg, Germany). A cut-off level of 24 IU per mL was used for classifying as RF negative or positive.

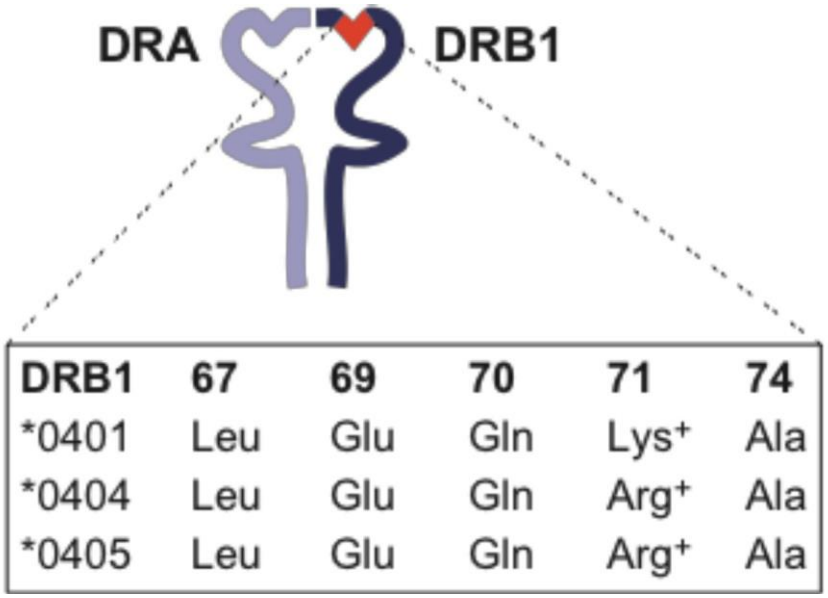
CRP levels were quantified by an immunoturbidimetric method on heparin plasma standardised according to the International Federation of Clinical Chemistry and Laboratory Medicine (Roche Diagnostics, Mannheim, Germany). ESR was determined locally in the rheumatological outpatients unit for the calculation of the DAS28 score.

2.5 Isolation of genomic DNA and molecular genetic characterisation

Genomic DNA was isolated from whole blood using a salting-out procedure (Super QUIK-Gene, Immucor, Dreieich, Germany). Aliquots were stored at < -20°C for further analyses.

Samples had been tested previously for FGB rs1800790 G>A, FGA rs6050 Thr312Ala, F13A rs5985 Val34Leu using allele specific primer pairs (Hoppe et al., 2012a) as well as for HLA-DRB1 SE using standard techniques (Dynal, Oslo, Norway; GenoVision, Vienna, Austria, Protrans, Hockenheim,

Germany). HLA-DRB1 SE was defined as the HLA-DRB1 alleles with the following DRβ1 chain residues: 67Leu – 69Glu – 71Lys or Arg – 74Ala – 86Gly or Val.



DRB1	67	69	70	71	74
*0401	Leu	Glu	Gln	Lys+	Ala
*0404	Leu	Glu	Gln	Arg+	Ala
*0405	Leu	Glu	Gln	Arg+	Ala

Figure 2.2: HLA-DRB1 shared epitope. Schematic diagram of the HLA-DRB1 shared epitope and its characteristic positions of amino acids.

The FGA variants rs2070006 G>A and rs2070016 T>C were characterised using LightSNiP assays (TIB MOLBIOL, Berlin, Germany) based on melting curve analysis on LightCycler 480 (Roche Diagnostics, Mannheim, Germany).

The PCR reaction mixtures (20 µl) were prepared as follows using FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany):

DNA (total: 50 ng):	1 µl
Reagent Mix:	1 µl
FastStart:	2 µl
$MgCl_2$ (25 mM):	1,6 µl
H_2O:	14,4 µl

Amplification and melting point analyses were performed on LightCycler 480 using the following programming:

Denaturation:

1 cycle, 95 °C (10 min)

Cycling:

45 cycles, 95 °C (10 s), 60 °C (10 s), 72 °C (15 s)

Melting: 1 cycle, 95 °C (30 s), 40 °C (2 min), 75 °C

The following Tm values were used to identify the respective genotypes:

rs2070016 C (Tm: 53,74 °C), T (Tm: 60,78 °C)

(TGGCCTCAATTTCCTGGCACCTAAT[C/T]TATGGCTAAGTGGACCCTCATTCCA)

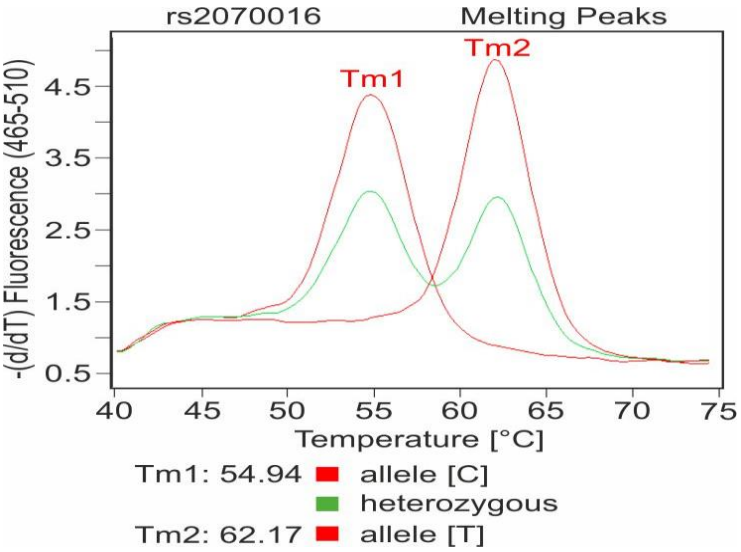


Figure 2.3: Genotyping of FGA rs2070016 T>C. Exemplary melting curves of the individual genotype constellations of FGA rs 2070016 T>C. The determined Tm values are indicated below the diagram.

rs2070006 G (Tm: 56, 33 °C), A (Tm: 61, 19 °C)

(GAACCCAAGACTTAGTAAACATTCA[A/G]TACACATTAGCTATTACTCTATTAG)

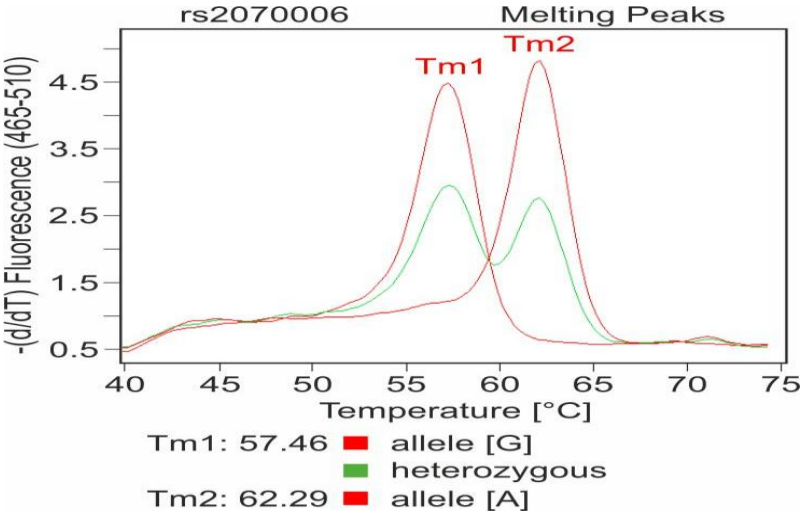


Figure 2.4: Genotyping of FGA rs2070006 G>A. Exemplary melting curves of the individual genotype constellations of FGA rs 2070006 G>A. The determined Tm values are indicated below the diagram.

2.6 Statistical analyses

The strategy for statistical evaluation of the data followed the one described previously (Hoppe et al., 2012a). For univariate analyses, odds ratios (OR) and exact 95% confidence intervals (95% CI) were calculated. For univariate analyses stratified by F13A 34Val/Val (wildtype) vs. 34Leu carriage, Mantel-Haenszel χ^2 testing for homogeneity was used to test whether an association differed significantly across the two strata. For the assessment of allele-dose dependency, the corresponding wildtype was used as reference and a test for linear trend of log odds (trend test) was performed. For multivariate analyses, OR and 95% CI were calculated by logistic regression analyses. Multivariate analyses were adjusted for potentially confounding factors (sex, erosive disease, ACPA, RF, HLA-DRB1 SE, BMI >25 kg/m^2). Non-parametric comparisons of CRP levels between different genotypes were performed by Mann-Whitney U test or in the case of testing for allele-dose dependency by a Cuzick non-parametric test for trend. As for each patient, CRP levels of different time points were included and p-values derived from analyses adjusted for non-independent observations within

clusters (p_{adj}) were calculated (Williams, 2000). All statistical analyses were performed using Stata Statistical Software for Macintosh (release 10.1, Texas, USA).

3. Results

3.1 Association of genetic fibrinogen variants with inflammation

As described in the introduction, the aim of this research was to corroborate the hypothesis that genetic variants, which influence the process of fibrinogen synthesis and/or fibrin crosslinking, also influence inflammatory processes. This initial hypothesis was derived from earlier work on variants FGB rs1800790 G>A, FGA rs6050 Thr312Ala and F13A rs5985 Val34Leu. In my thesis, we tested this hypothesis by analysing the single nucleotide polymorphisms (SNPs) FGA rs2070016 T>C and rs2070006 G>A, which are described to modulate fibrinogen synthesis and which in our study were not found to be in strong linkage dysequilibrium with FGA rs6050 Thr312Ala (rs2070016: r^2=0.07, rs2070006: r^2=0.51).

3.2 Characteristics of the study population

In our study we included patients suffering from rheumatoid arthritis (RA), who presented to the doctor at least twice. As a result, 285 patients were studied with a total of 905 observations, with 16, 203 and 66 of the patients presenting twice, 3-times and 4-times, respectively.

Our study population was representative for RA in respect of main demographic and diagnostic characteristics of this disease according to the literature (Klareskog et.al., 2008, Silman& Pearson, 2002). As shown in **Table 3.1** the majority of our patients were women, and about 2/3 to 3/4 of the patients exhibited serological markers of RA, i.e. anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF), which are typical for this disease.

Furthermore, about 2/3 of our patients were carriers of the HLA-DRB1 shared epitope (SE), which is known to be related to a susceptibility to RA and to represent the genetic background for ACPA generation. About 80% of the HLA-DRB1 SE carriers were heterozygotes.

Patients predominantly presented longstanding disease (median: 4.5 years) and – although different levels of disease activity were represented in our cohort – the main percentage of our study population (54.7%) suffered from disease with high activity (DAS28 $\geq$5.1). This is also reflected by a relatively high portion of patients on therapy with TNF blockers.

Table 3.1: Demographic, clinical and diagnostic characteristics of the study population

Characteristics	
Sex (female)	77.2%
Age, median (IQR) (y)	51 (39-61)
Duration of disease, median (IQR) (y)	4.5 (1.0-10.9)
Body mass index, median (IQR) (kg/m^2)	25.3 (22.1-28.6)
ACPA positivity (>25 AU/ml)	63.8%
Rheumatoid factor positivity ($\geq$25 IU/ml)	77%
HLA-DRB1 shared epitope	
- Carrier	62.5%
- Heterozygotes	49.4%
- Homozygotes	13.1%
Disease activity score 28 (DAS28)	
- Median (IQR)	5.35 (4.01-6.19)
- <2.6 (remission)	8.1%
- <3.2 (remission or mild disease activity)	13.3%
- $\geq$5.1 (high disease activity)	54.7%
C-reactive protein level (mg/l)	
- Median (IQR)	5.3 (2.5-17)
- Mean (SEM)	15.6 (1.00)
Patients receiving TNF blockers	16.1%
FGA rs2070016 T>C genotype	
- Carriers of the minor allele (C)	30.9%
- Heterozygotes (T/C)	25.6%
- Homozygotes (C/C)	5.3%
FGA rs2070006 G>A genotype	
- Carriers of the minor allele (A)	56.8%
- Heterozygotes (G/A)	40.7%
- Homozygotes (A/A)	16.1%

3.3 Inflammatory activity and body mass index (BMI)

Adiposity is known to influence inflammatory activity and to be related to increased levels of CRP (George et al., 2017). Thus, it was of importance for our study to characterise BMI as a possible confounding factor.

The median BMI of our cohort was 25.3 kg/m^2. In our cohort, patients with BMI >30 kg/m^2 had a significantly higher DAS 28 (P=0.0001) and significantly higher CRP levels (P<0.0001) than those with lower BMI. The mean CRP level for BMI ≤30 kg/m^2 was 15.2 mg/l (SEM: 1.15 mg/l) and for BMI >30 kg/m^2 it was 17.9 mg/l (SEM: 2.10 mg/l). Comparing the DAS 28 scores in patients above and below a BMI of 30 kg/m^2 resulted in a median of 4.9 (IQR: 3.9-6.2) for BMI ≤30 kg/m^2 and 5.9 (IQR: 5.0-6.7) for BMI >30 kg/m^2.

The influence of adiposity on inflammatory activity is impressively reflected by data given in **Table 3.2**. As one can see, the relative number of obese patients continuously increases with increasing inflammatory activity as measured by DAS28.

Table 3.2: Relation between BMI and inflammatory activity. Absolute numbers and percentages are given for each category of the disease activity score 28.

	Disease activity score 28				
BMI	**<2.6**	**2.6-3.2**	**3.2-4.0**	**4.0-5.1**	**≥5.1**
≤30 kg/m^2	22 (100%)	14 (93%)	30 (94%)	49 (84%)	119 (78%)
>30 kg/m^2	0 (0%)	1 (7%)	2 (6%)	9 (16%)	34 (22%)

3.4 FGA rs2070016 T>C genotype is associated with CRP levels

As presented in **Table 3.1**, the minor allele of the FGA rs2070016 T>C genotype was highly frequent in our study population with a carrier frequency of approximately 30.9%. As far as the allele dose is concerned, 25.6% of the patients carried one copy of the minor allele while the remaining 5.3% were homozygotes.

Our first analysis was focused on the association between FGA rs2070016 T>C genotype and CRP levels. As shown in **Table 3.3**, elevated CRP levels (>10 mg/l) were significantly less frequent in carriers of the minor allele of FGA rs2070016 T>C (29.6%) than in FGA rs2070016 wildtypes (40%) resulting in an odds ratio of 0.63 (P<0.003). These findings remained statistically significant after adjustment for non-independent observation (P_{adj}<0.03)

As can be seen in **Table 3.3**, the C allele dose of FGA rs2070016 T>C played an important role with respect to CRP, as well. The portion of observations with increased CRP levels (>10 mg/l) decreased significantly from wildtypes over carriers of one and two FGA rs2070016 C alleles respectively (P=0.002, P_{adj}=0.02).

Table 3.3: CRP levels in rheumatoid arthritis: Relation to FGA rs2070016 C allele carrier status and copy number. The percentages of observations in different CRP categories on the relation to carrier status or allele dose for FGA rs2070016 T>C are given.

	FGA rs2070016 C allele						
	Carrier status			**Copy number**			
	0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
CRP							
>10 mg/l	40.0	29.6	0.63 **(<0.003)**	40.0	30.7	24.5	**0.002**

With regard to the influence of the FGA rs2070016 T>C genotype on the CRP level categorised to $\leq$ 10 mg/l or >10 mg/l, our results were also corroborated in a non-parametric comparison of CRP levels in relation to this genotype by using the Mann-Whitney U test. Specifically, the FGA rs2070016 T/T wildtypes exhibited significantly higher CRP levels than carriers of one or two of the FGA rs2070016 C allele(s) (P=0.006). However, this finding lost its statistical significance after adjustment for non-independent observation (P_{adj}=0.061). When the FGA rs2070016 C allele dose was taken into consideration, the comparison between wildtypes and carriers of one or two FGA

rs2070016 C alleles again indicated significantly lower CRP levels with increasing C allele copy numbers (P=0.006), a finding that was of borderline significance after adjustment for non-independent observations (P_{adj}=0.057).

These results were clearly perceptible when comparing the mean values of the CRP levels of different FGA rs2070016 T>C genotypes. For the T/T wildtype patients the mean CRP level was 16.7 mg/l with a standard error of the mean (SEM) of 1.3 mg/l. The carriers of one or two FGA rs2070016 C allele(s) showed lower mean CRP levels (13.0 mg/l $\pm$ 1.3 mg/l). When looking at the C allele dose, the difference in CRP levels between T/T wildtypes and those patients with one copy of the C allele (13.4 mg/l $\pm$ 1.5 mg/l) and homozygous individuals (C/C) (11.3 mg/l $\pm$ 2.6 mg/l) indicated lower CRP levels with increasing numbers of the FGA rs2070016 minor allele **(Figure 3.1)**.

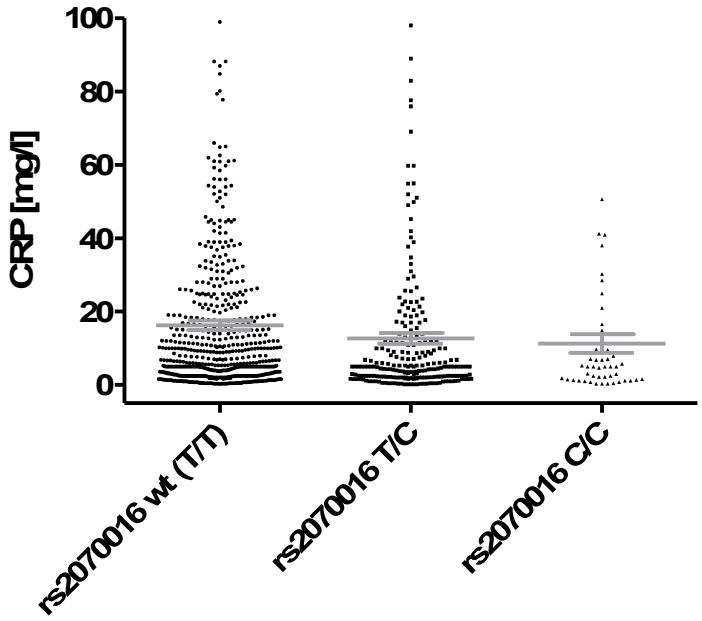


Figure 3.1: CRP levels in relation to FGA rs2070016 T>C genotype.

CRP levels are given as dots, mean and SEM are indicated.

As far as the association between FGA rs2070016 T>C and CRP is concerned, the findings described above were obviously the same when performing the analyses stratified by sex, ACPA status, RF status, HLA-DRB1 SE status, BMI >25 kg/m^2 or Steinbrocker Score $\geq$2, indicating that this association was not affected by these covariables.

Furthermore, the association between FGA rs2070016 T>C genotype and CRP elevations was tested in multivariate analyses, adjusted for the parameters mentioned above (sex, erosive disease, ACPA, RF, HLA-DRB1 SE carriage and BMI >25 kg/m^2). In these analyses the association between FGA rs2070016 C allele carrier status and CRP level could be confirmed (P=0.005) even after adjustment for non-independent observations (P_{adj}=0.044). The same conclusion could be drawn also after considering the rs2070016 C allele dose in our multivariate analyses (P_{adj}=0.016).

3.5 FGA rs2070016 T>C genotype is not related to disease activity score 28

In addition to the analyses on the relation between FGA rs2070016 T>C with CRP level, we studied the possible association between this genotype and the disease activity of RA, as measured by the DAS28. As shown in **Table 3.4,** the percentage of patients in remission (DAS28 <2.6) was almost the same between FGA rs2070016 T/T wildtype individuals (8.1%) and C allele carriers (8.0%). When considering the FGA rs2070016 C allele dose, the homozygous carriers (C/C) were slightly less frequently in remission (6.7%) compared to wildtypes (T/T) (8.1%) and heterozygous carriers (T/C) (8.2%). However, this finding clearly was not statistically significant (P=0.91).

Table 3.4: Disease activity score (DAS) 28 in rheumatoid arthritis: Relation to FGA rs2070016 C allele carrier status and copy number. The percentages of patients in different DAS28 categories in relation to carrier status or allele dose for FGA rs2070016 T>C are given.

	FGA rs2070016 C allele						
	Carrier status			**Copy number**			
	0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
DAS28							
<2.6	8.1	8.0	0.98 (0.96)	8.1	8.2	6.7	0.91
<3.2	11.7	17.1	1.6 (0.22)	11.7	16.4	20.0	0.20
≥5.1	56.9	50.0	0.76 (0.28)	56.9	49.3	53.3	0.37

Our data also showed no statistically significant relation between the FGA rs2070016 T>C genotype and low/moderate activity of the disease (DAS28 <3.2) (carrier status: P=0.22, allele dose: P=0.20) or patients with high activity (DAS28 ≥5.1) (carrier status: P=0.28, allele dose: P=0.37). However, when comparing the percentage of patients suffering from mild disease (DAS28 <3.2) in the different genotype constellation, a tendency for higher frequencies in patients with the FGA rs2070016 C allele became apparent.

The use of Mann-Whitney U test revealed that patients with the FGA rs2070016 C allele had a tendency for a lower DAS 28, although these results had no statistical significance when looking for carrier status (P=0.10) **(Figure 3.2)** or for allele dose dependency (P=0.14).

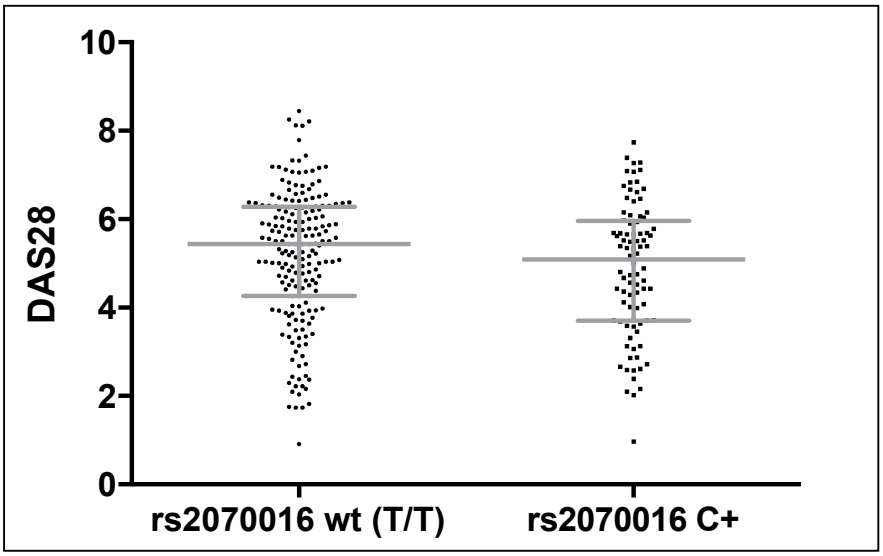


Figure 3.2: DAS28 in relation to FGA rs2070016 C allele carrier status.

DAS28 is given as dots in rs2070016 (T/T) wildtypes (wt) and C allele carriers (C+).

Median and IQR of DAS28 are indicated.

Using the same method of Mann-Whitney U test, we tested for the existence of an association between FGA rs2070016 T>C genotype and the duration of morning stiffness, a typical clinical feature of RA. It could be found that the duration of morning stiffness was shorter among the C allele carriers than in wildtypes (T/T). The wildtype individuals in median exhibited morning stiffness for 30 minutes (IQR: 10-90) while in carriers it lasted 15 minutes (IQR: 0-60). Statistical testing for this difference revealed a non-significant trend (P<0.07).

When categorising patients in those exhibiting morning stiffness $\leq$ or >60 minutes, statistical trends could be found for a possible negative association between FGA rs2070016 C allele carriage and the existence of morning stiffness longer than 60 minutes in Chi^2 testing (OR: 0.56, P<0.09) as well as when looking for a C allele dose dependency (P=0.11).

3.6 No association of FGA rs2070006 G>A genotype with CRP levels

As shown in **Table 3.1**, almost half of our population was carrier of the so-called minor A allele of the FGA rs2070006 G>A genotype (56.8%). The majority of patients were carrying one copy of the allele (G/A) (40.7%), while a smaller percentage were homozygotes (A/A) (16.1%).

With respect to FGA rs2070006 G>A genotype, again, the first constellation that was investigated in our patients was the possible association of this genotype with the level of CRP. Our data, as presented in **Table 3.5**, showed no association between carriage or allele dose of the minor A allele and increase of CRP to levels higher than 10 mg/l. The percentage of the FGA rs2070006 G>A wildtype patients (G/G) with CRP >10 mg/l (35.1%) was almost equal to this of the A allele carriers (38.1%) as well as patients with 1 (39.5%) or 2 A allele copies (34.7%) (carrier status: P=0.35, allele dose dependency: P=0.72).

Table 3.5: CRP-levels in rheumatoid arthritis: Relation to FGA rs2070006 A allele carrier status and copy number. The percentages of observations in different CRP categories in relation to carrier status or allele dose for FGA rs2070006 G>A are given.

	FGA rs2070006 A allele						
	Carrier status			**Copy number**			
	0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
CRP							
>10 mg/l	35.1	38.1	1.1 (0.35)	35.1	39.5	34.7	0.72

We further tested this relation in a non-parametric comparison using the Mann-Whitney U test. Carriers of the FGA rs2070006 A allele tended to present higher CRP levels (mean 17.1 mg/l $\pm$ 1.6 mg/l) than wildtypes (mean: 13.5 mg/l $\pm$ 1.0 mg/l) with no statistical significance (P=0.17).No allele dose dependency could be found for this tendency. The patients carrying one A allele had

slightly higher mean CRP levels (18 mg/l ± 2.0 mg/l) compared to those who carried two A allele copies (14.8 mg/l ± 2.0 mg/l).

3.7 No relation of FGA rs2070006 G>A genotype with disease activity score 28

As for the FGA rs2070016 T>C genotype, we also studied the possible influence of the FGA rs2070006 G>A genotype to the activity of the rheumatoid disease, as measured with the DAS28. As shown in the **Table 3.6**, no association could be found between the grade of the disease activity and the A allele carrier status of this variant. The same result was also found, when the A allele dose was taken into consideration.

Table 3.6: Disease activity score (DAS) 28 in rheumatoid arthritis: Relation to FGA rs2070006 A allele carrier status and copy number. The percentages of patients in different DAS28 categories in relation to carrier status or allele dose for FGA rs2070006 G>A are given.

	FGA rs2070006 A allele						
	Carrier status			**Copy number**			
	0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
DAS28							
<2.6	8.1	8.0	0.99 (0.97)	8.1	7.8	8.7	0.95
<3.2	13.8	13.0	0.93 (0.83)	13.8	13.8	10.9	0.68
≥5.1	52.9	56.2	1.1 (0.58)	52.9	57.8	52.2	0.85

The testing of the data by the Mann-Whitney U test showed that the DAS28 was essentially the same in the patients who were carriers of the FGA rs2070006 A allele (DAS28 median: 5.42, IQR: 4.26-6.26) compared to wildtypes (DAS28 median: 5.25, IQR: 3.86-6.15) (P=0.32). When considering the A allele dose, again no relevant relation to DAS28 could be found (1 A allele, DAS28 median: 5.47, IQR: 4.02-6.30, 2 A alleles, DAS28 median 5.15, IQR: 4.57-5.84) (P=0.54).

Additionally, the use of the Mann-Whitney U-test revealed that the duration of morning stiffness was quite similar in A allele carriers when compared to the FGA rs2070006 G>A wildtype patients (P=0.14). In the latter group the duration of this symptom was in median 30 min (IQR 0-90) while in the first group it was 37.5 min (IQR 10-90). Furthermore, no association could be shown between the FGA rs2070006 A allele carriage and the duration of morning stiffness longer than 60 minutes (OR: 1.46, P=0.22). The same conclusion could also be drawn when the A allele dose was considered (P=0.41).

3.8 Relation between FGA rs2070016 T>C genotype and CRP levels in F13A Val34Leu subgroups

As described in the introduction of this thesis, factor XIII plays a very important role in haemostasis by the crosslinking of fibrin, thereby, determining physical stability and fibrinolysis resistance (Weisel, 2007). The F13A Val34Leu genotype influences this effect more subtly with F13A 34Val/Val wildtype favoring thinner and denser fibrin network structures under high fibrinogen levels (Lim et al., 2003). Previous work showed that the F13A Val34Leu genotype, interactively with some fibrinogen genotypes, also influences inflammatory activity (Hoppe et al., 2012a). We therefore tested whether the relation between FGA rs2070016 T>C and FGA rs2070006 G>A with inflammatory activity would depend on the F13A Val34Leu genotype.

As shown in **Table 3.7,** the first subgroup that we studied were patients with F13A 34Val/Val wildtype and the association of CRP elevations >10 mg/l with FGA rs2070016 T>C genotype was assessed. In this subgroup, the patients who were carriers of the variant FGA rs2070016 C allele exhibited less frequently elevated CRP levels (27.6%) than the patients who were not carriers of this variant (36.8%). However, this result failed to be statistically significant (P=0.06).

When the FGA rs2070016 C allele dose was taken into consideration, it could be demonstrated that the highest frequency of patients with elevated CRP levels were FGA rs2070016 TT wildtypes (36.8%) while the percentage of patients with CRP >10 mg/l decreased with an increasing number of FGA rs2070016 C alleles (1 allele: 30.1%, 2 alleles: 14.3%). This finding was statistically significant (trend test, P=0.026), albeit lost its statistical significance after the adjustment for non-independent observation (P_{adj} =0.096).

The second subgroup that we studied with respect to a relation between FGA rs2070016 T>C genotype and CRP elevations were carriers of the F13A 34Leu variant. Our data showed that in this subgroup, again, the patients who were carriers of FGA rs2070016 C allele exhibited less frequently elevated CRP levels (31.5%) than those who were FGA rs2070016 TT wildtype (42.9%). Again, this result was statistically significant (P<0.02) and exhibited a statistical trend after the adjustment for non-independent observation (P_{adj}=0.07).

When considering the FGA rs2070016 C allele dose in this subgroup, CRP elevations were of comparable frequency in carriers of one (31.3%) or two C alleles (32.1%). Statistical testing with regards to allele dose dependency between FGA rs2070016 genotype and CRP >10 mg/l in F13A 34Leu carriers was significant (trend test, P=0.03) but failed to reach a significant level of P<0.05 after the adjustment for non-independent observations (P_{adj}=0.098).

Table 3.7: CRP levels in rheumatoid arthritis: Relation to FGA rs2070016 C allele carrier status and copy number in subgroups defined by F13A 34Leu carrier status. The percentages of observations in different CRP categories in relation to carrier status or allele dose for FGA rs2070016 T>C are given.

		FGA rs2070016 T>C genotype						
		Carrier status			Copy number			
		0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
F13A 34 Val/Val (wildtypes)	**CRP >10 mg/l**	36.8	27.6	0.66 (0.06)	36.8	30.1	14.3	**0.026**
F13A 34 Leu (carriers)	**CRP >10 mg/l**	42.9	31.5	0.61 **(<0.02)**	42.9	31.3	32.1	**0.03**

Our data were further assessed by a test of homogeneity. The purpose of implementing this test was to examine if there is a difference in the relation between FGA rs2070016 T>C and CRP elevations between the patients who were carriers of F13A 34Leu and F13A 34Val/Val wildtypes. The tests of homogeneity did not demonstrate any significant difference between the two categories (carrier status: P = 0.81, copy number: P = 0.82).

Finally, our data was tested using the Mann-Whitney U-Test. In our first subgroup (F13A 34Val/Val wildtypes) the test showed that the FGA rs2070016 TT wildtypes tended to present higher CRP levels compared to FGA rs2070016 C allele carriers (P=0.13). The patients who were FGA rs2070016 TT wildtype had a mean value of CRP of 17.4 mg/l (SEM $\pm$ 2.4) while in the carriers of the C allele the mean value of CRP was 12.6 mg/l (SEM $\pm$ 1.8). Regarding the allele dose, the FGA rs2070016 TC heterozygotes had a higher CRP of 13.5 mg/l (SEM $\pm$ 2.0) compared to C allele homozygotes with CRP of 7.4 (SEM $\pm$ 2.4) (P=0.07, P_{adj}=0.29). These results can be seen in **Figure 3.3**.

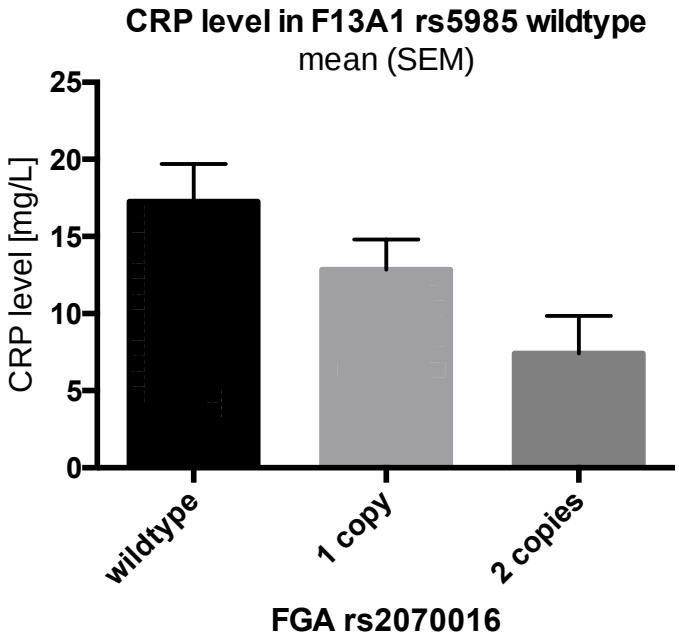


Figure 3.3: CRP levels in relation to FGA rs2070016 T>C genotype in F13A 34Val/Val wildtypes. CRP levels are given, mean and SEM are indicated.

As far as the second population (F13A 34Leu carriers) is concerned, the test showed that the patients who were carriers of FGA rs2070016 C allele exhibited lower values of CRP compared to FGA rs2070016 TT wildtypes (P=0.02, P_{adj}=0.095). Regarding the mean value of CRP in the first group (FGA rs2070016 TT wildtypes), it was 16.0 mg/l (SEM $\pm$ 1.17), while in the second group (FGA rs2070016 C carriers) it was 13.5 mg/l (SEM $\pm$ 2.0). The allele dose was also taken into consideration. The patients who were FGA rs2070016 TC heterozygous had a mean value of CRP of 13.3 mg/l (SEM $\pm$ 2.3). FGA rs2070016 CC homozygous patients exhibited a mean value of CRP of 14.3 mg/l (SEM $\pm$ 4.0). These results are given in **Figure 3.4**.

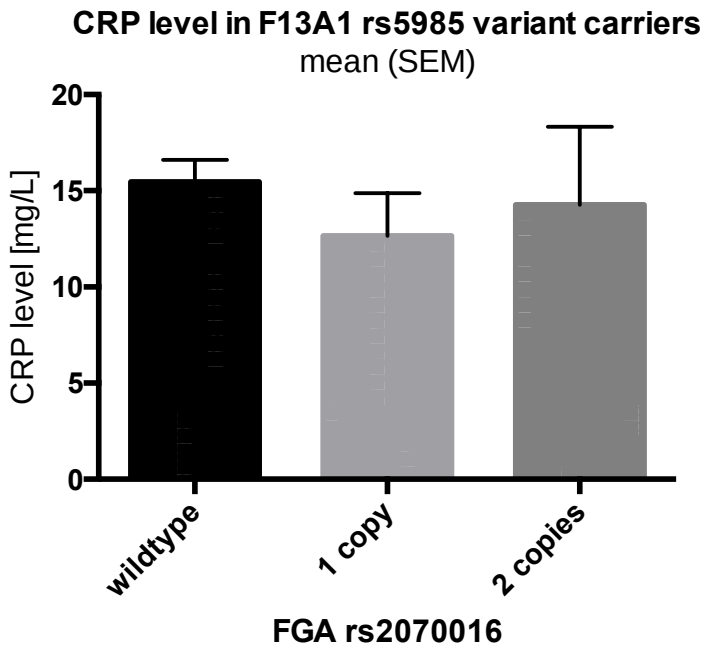


Figure 3.4: CRP levels in relation to FGA rs2070016 T>C genotype in F13A rs5985 34Leu carriers. CRP levels are given, mean and SEM are indicated.

3.9 Association of FGA rs2070016 T>C genotype with disease activity score 28 in relation to F13A Val34Leu genotype

The subgroups defined by F13A Val34Leu genotype, were further investigated regarding the disease activity of rheumatoid arthritis, as it is represented by DAS28.

As shown in **Table 3.8**, the first subgroup that we studied included patients who were wildtype for factor XIII A-subunit (F13A 34Val/Val). In the group of F13A 34Val/Val wildtype patients, low disease activity (DAS28 <2.6) seemed to be more often observed in FGA rs2070016 C carriers (9.3%, 4 of 43) than in FGA rs2070016 TT wildtypes (6.5%, 6 of 93) (P=0.55). When the FGA rs2070016 C copy number was considered, low disease activity was more frequent in homozygotes (14.3%, 1 of 7) than in carriers of one FGA rs2070016 C allele (8.3%, 3 of 36) (trend test, P=0.45). As indicated, it was obvious that both findings were of no statistical significance.

For moderate disease activity (DAS28 <3.2) similar findings were observed that were however statistically significant. A moderate disease activity (DAS28 <3.2) was less frequent in wildtypes (TT) of the FGA rs2070016 T>C genotype (10.8%, 10 of 93) when compared to C allele carriers (25.6%, 11 of 43) (P<0.03). In the last group of patients, the percentage of the C allele homozygotes (42.9%, 3 of 7) was almost double when compared to carriers of only one C allele (22.2%, 8 of 36). The findings with respect to C allele dose were also statistically significant (trend test, P= 0.01). After adjustment for potentially confounding variables (ACPA status, RF status, HLA-DRB1 SE, sex, BMI >25 kg/m^2), statistical analyses indicated an associated trend (carrier status: P<0.07, copy number: P=0.08).

In contrast, in the subgroup of patients with high disease activity (DAS28 $\geq$5.1), the majority of patients were FGA rs2070016 TT (wildtype) (59.1%, 55 of 93) compared to the C allele carriers (37.2%, 16 of 43). In C allele carriers, the clinical phenotype was more common in carriers of only one C allele (38.9%, 14 of 36) than in C allele homozygotes (28.6%, 2 of 7). Again, both of these outcomes were statistically significant (P<0.02, trend test, P=0.017, respectively) even after adjustment for potentially confounding variables (ACPA status, RF status, HLA-DRB1 SE, sex, BMI >25 kg/m^2) (carrier status: P<0.02, copy number: P=0.02).

The second subgroup that we studied, as seen in **Table 3.8**, consisted of patients who were carriers of the F13A 34Leu variant. When considering patients with low, moderate or high disease activity, the frequencies of FGA rs2070016 C allele carriers and non-carriers were distributed quite similarly. The same holds true for low and moderate DAS28 and FGA rs2070016 C allele copy numbers. When comparing patients with high disease activity to those not presenting a DAS28 of 5.1 or more, C allele homozygotes more frequently presented high disease activity (75%, 6 of 8) than those with one or no C allele (59.5%, 22 of 37 and 54.8%, 57 of 104, respectively). The latter relation was not statistically significant (trend test, P=0.28).

Table 3.8: Disease activity score (DAS) 28 in rheumatoid arthritis: Relation to FGA rs2070016 C allele carrier status and copy number in subgroups defined by F13A 34Leu carrier status. The percentages of patients in different DAS28 categories in relation to carrier status or allele dose for FGA rs2070016 T>C are given.

		FGA rs2070016 C allele						
		Carrier status			Copy number			
		0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
FXIII A 34Val/Val (wildtypes)	**DAS28**							
	<2.6	6.5	9.3	1.48 (0.55)	6.5	8.3	14.3	0.45
	<3.2	10.8	25.6	2.85 **(<0.03)**	10.8	22.2	42.9	**0.01**
	≥5.1	59.1	37.2	0.41 **(<0.02)**	59.1	38.9	28.6	**0.017**
FXIII A 34Leu (carriers)	**DAS28**							
	<2.6	9.6	6.7	0.67 (0.56)	9.6	8.1	0.0	0.42
	<3.2	12.5	8.9	0.68 (0.52)	12.5	10.8	0.0	0.36
	≥5.1	54.8	62.2	1.36 (0.40)	54.8	59.5	75.0	0.28

We further tested these associations for homogeneity between F13A 34Val/Val wildtypes and F13A 34Leu carriers. When looking at the relation of FGA rs2070016 T>C carrier status and DAS28 <3.2, there was a trend for heterogeneity between both F13A Val34Leu strata (F13A 34Val/Val, OR=2.86, F13A 34Leu, OR=0.68, test of homogeneity, P=0.063). When studying the relation between FGA rs2070016 C allele copy number and DAS28 <3.2 significant heterogeneity between both F13A Val34Leu strata was identified (P<0.02). The results were comparable, when looking for heterogeneity in DAS28 ≥5.1 (test of homogeneity, carrier status: P =0.02, copy number: P <0.02).

When looking for the distribution of DAS28 using the Mann Whitney U Test, in F13A 34Val/Val wildtypes **(Figure 3.5**), carriers of the FGA rs2070016 C allele tended to present a lower DAS28 than those without this allele (median DAS28, C carriers: 4.43 vs. TT wildtypes: 5.45, P<0.07). DAS28 decreased with C allele copy number (median DAS28, TT wildtypes: 5.45, one C allele: 4.52, two C alleles: 3.69, P= 0.054).

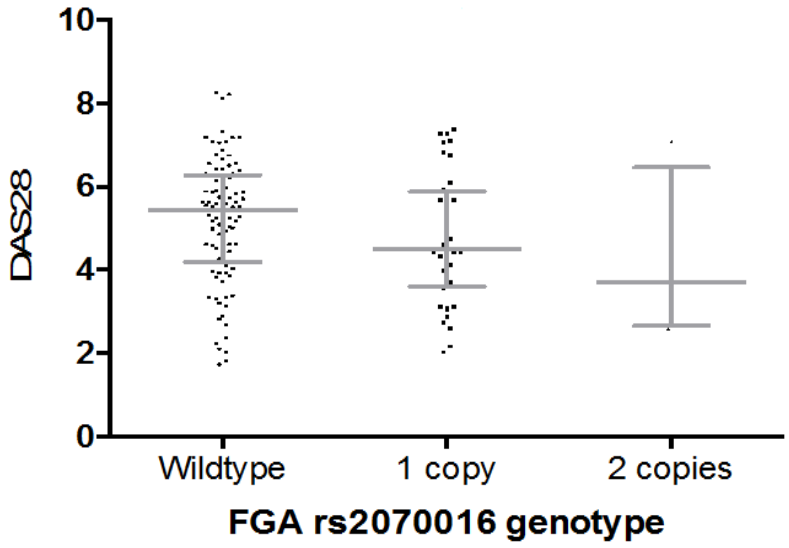


Figure 3.5: DAS28 in relation to FGA rs2070016 T>C genotype in F13A 34Val/Val wildtypes.
DAS28 is given as dots, median and IQR are indicated.

As can be seen in **Figure 3.6**, in F13A 34Leu carriers no relation was found between FGA rs2070016 T>C genotype and DAS28.

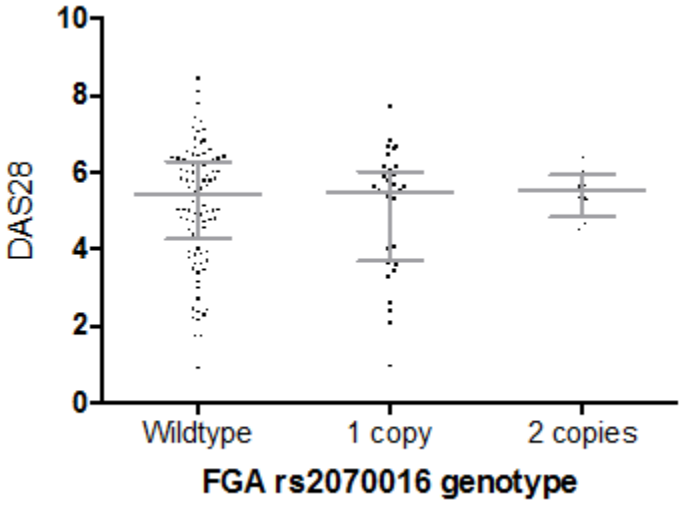


Figure 3.6: DAS28 in relation to FGA rs2070016 T>C genotype in F13A 34Leu carriers.
DAS28 is given as dots, median and IQR are indicated.

3.10 Association of FGA rs2070006 G>A genotype with CRP levels in F13A Val34Leu subgroups

We studied the association of FGA rs2070006 G>A genotype with CRP levels in strata defined by absence and presence of the F13A 34Leu allele, respectively. As shown in **Table 3.9** among the patients who were wildtype for F13A Val34Leu (34Val/Val), the patients who were carriers of the FGA rs2070006 A allele were more likely to have a CRP value higher than 10 mg/l (40.2%) compared to the FGA rs2070006 G>A wildtypes (27.9%). This association was statistically significant ($P<0.007$) even after adjustment for non-independent observations ($P_{adj}<0.05$). With increasing number of FGA rs2070006 A copies, the percentage of patients presenting increased CRP did not increase further ($P<0.08$, $P_{adj}=0.17$).

Table 3.9: CRP levels in rheumatoid arthritis: Relation to FGA rs2070006 A allele carrier status and copy number in subgroups defined by F13A 34Leu carrier status. The percentages of observations in different CRP categories in relation to carrier status or allele dose for FGA rs2070006 G>A are given.

		FGA rs2070006 A allele						
		Carrier status			Copy number			
		0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
FXIII A 34Val/Val (wildtypes)	**CRP >10 mg/l**	27.9	40.2	1.73 (<0.007)	27.9	43.5	33.3	<0.08
FXIII A 34Leu (carriers)	**CRP >10 mg/l**	44.6	36.6	0.72 (0.09)	44.6	36.9	35.9	0.13

On the other hand, in patients who were F13A 34Leu carriers, carriers of the FGA rs2070006 A allele showed a tendency of lower CRP values compared to the wildtypes (P=0.09). No significant difference could be shown between the homozygous and the heterozygous carriers (P=0.13).

The association between FGA rs2070006 G>A (carrier status) and CRP elevations exhibited a significant heterogeneity between strata defined by absence (OR: 1.73, P<0.007) or presence of F13A 34Leu (OR: 0.72, P=0.09) (test of homogeneity, P=0.0019, P_{adj}=0.02). For FGA rs2070006 A allele dosage, a similar relation between the different F13A Val34Leu strata was found (test for homogeneity, P=0.02, P_{adj}<0.08).

Mean CRP levels in F13A 34Val/Val wildtypes is given in **Figure 3.7**. For this subgroup, in FGA rs2070006 G>A wildtypes the mean value of CRP was 10.7 mg/l (SEM ± 1.2), while in carriers of the FGA rs2070006 A allele a mean CRP level of 21.3 mg/l (SEM ± 3.4) was found. This difference was statistically significant (P=0.0001, P_{adj}=0.008).

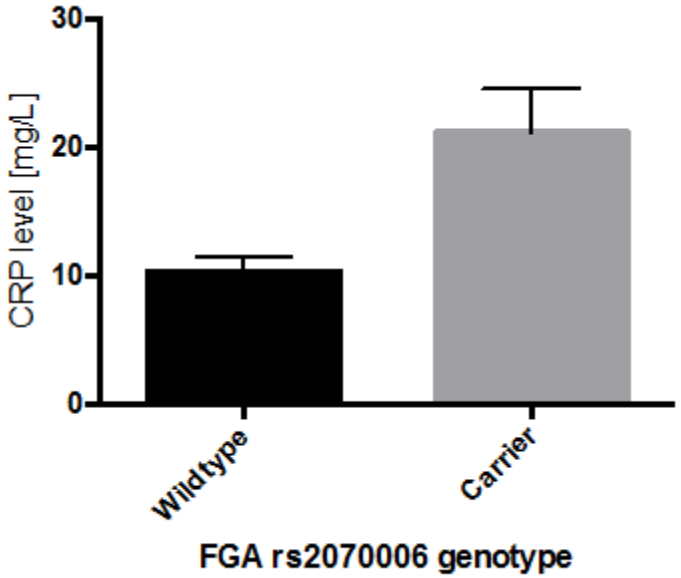


Figure 3.7: CRP levels in relation to FGA rs2070006 G>A genotype in F13A 34Val/Val wildtypes. CRP levels are given, mean and SEM are indicated.

On the other hand, the results for F13A 34Leu carriers are given in **Figure 3.8**. In this stratum mean CRP levels differed only slightly between FGA rs2070006 G>A wildtypes (17.3 mg/l, SEM ± 1.8) and A allele carriers (14.1 mg/l, SEM ± 1.2) (P=0.0538, P_{adj}=0.18).

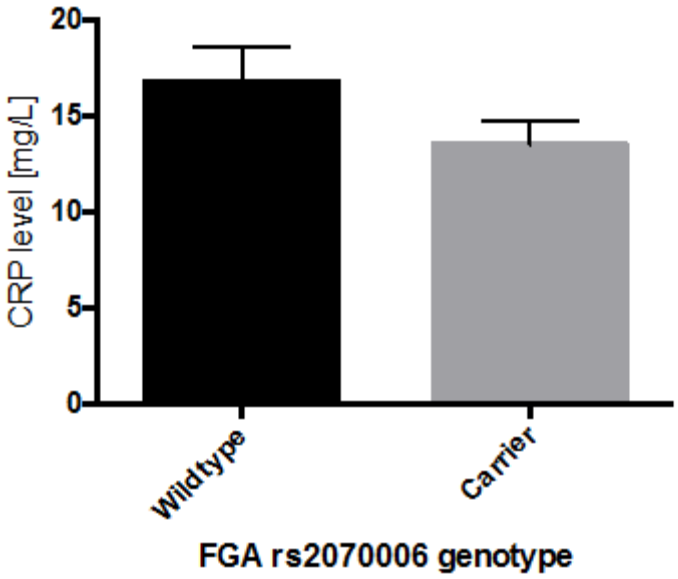


Figure 3.8: **CRP levels in relation to FGA rs2070006 G>A genotype in F13A 34Leu carriers.** CRP levels are given, mean and SEM are indicated.

3.11 No association of FGA rs2070006 G>A genotype with disease activity score 28 in F13A Val34Leu subgroups

Finally, we studied the relation between FGA rs2070006 G>A genotype and DAS28 in different F13A Val34Leu strata. As shown in **Table 3.10,** there was no statistical evidence for such a relation either when considering FGA rs2070006 A allele carrier status or A allele copy number. There was no heterogeneity between F13A 34Leu non-carriers and carriers.

Table 3.10: Disease activity score (DAS) 28 in rheumatoid arthritis: Relation to FGA rs2070006 A allele carrier status and copy number in subgroups defined by F13A 34Leu carrier status. The percentages of patients in different DAS28 categories in relation to carrier status or allele dose for FGA rs2070006 G>A are given.

		FGA rs2070006 A allele						
		Carrier status			Copy number			
		0 (wt) [%]	1 [%]	OR (P value)	0 (wt) [%]	1 [%]	2 [%]	Trend test P value
FXIII A 34Val/Val (wildtypes)	**DAS28**							
	<2.6	8.7	6.0	0.67 (0.54)	8.7	6.7	4.6	0.50
	<3.2	17.4	13.4	0.74 (0.52)	17.4	15.6	9.1	0.38
	≥5.1	47.8	56.7	1.43 (0.30)	47.8	60.0	50.0	0.56
FXIII A 34Leu (carriers)	**DAS28**							
	<2.6	7.4	9.5	1.31 (0.67)	7.4	8.5	12.5	0.50
	<3.2	5.0	12.0	1.42 (0.53)	9.3	12.7	12.5	0.60
	≥5.1	59.3	55.8	0.87 (0.68)	59.3	56.3	54.2	0.65

4. Discussion

Both the coagulation and fibrinolysis systems play an important role in the processes of inflammation control, wound healing, vascularisation, and maintenance, as well as endothelial function. These findings are based in part on animal experimental data, in which excessive inflammatory responses in impaired coagulation (e.g., fibrinogen knockout) were observed in various transgenic models (e.g. Flick et al., 2007). The importance for wound healing is also evident from clinical experience. For example, wound healing in patients with hereditary factor XIII deficiency is disturbed (Biswas et al., 2014, Weber et al., 2015). In this context substitution of factor XIII leads to significant improvement. Also, in the context of large area injuries (e.g., burn injuries) or pressure ulcers (e.g., in patients with spinal cord injury), a positive influence by the administration of factor XIII is described (Burkhardt et al., 1977, Erlebach & Hartung, 1999, Soendergaard et al, 2013). The influence of fibrin gel formation and cross-linking as well as fibrinolysis on vessel formation and endothelial function is well documented especially in experiments in cell cultures (e.g. Senior et al., 1986, Skogen et al., 1988, Petzelbauer et al., 2005). Epidemiological studies from our group support the above-mentioned relationship of coagulation and inflammation from a completely different perspective. As described in the introduction, an interplay of fibrinogen and factor XIII genetics in terms of the severity of inflammatory reactions indicates the importance of blood coagulation for inflammatory processes.

Before summarizing the findings for discussion, it clearly should be mentioned that due to the limited number of patients tested any interpretation should be considered carefully. Especially the subgroup analyses are at risk of false-positive and false-negative reporting because of the low sample size and statistical power. However, the findings can help to focus further research approaches on this topic.

The analyses presented in this thesis suggest lower inflammatory activity in relation to the FGA rs2070016 C-allele. This interpretation concerns CRP levels, DAS28 as well as morning stiffness in RA. When considering CRP levels, the trend for lower CRP levels in carriers of the FGA rs2070016 C-allele was homogenous between strata defined by F13A 34Leu carriage, in DAS28 a heterogeneity between F13A 34Val/Val wildtypes and F13A 34Leu carriers was found. The latter finding is reminiscent of the data published previously on FGB rs1800790 G>A and F13A Val34Leu (Hoppe et al., 2012a).

When considering FGA rs2070006 G>A genotype in the total study population, distributions of CRP levels as well as DAS28 were completely independent from this genotype. An analysis stratified for F13A 34Leu carriage revealed a strong heterogeneity between both subgroups, again, reminiscent of FGB rs1800790 G>A (Hoppe et al., 2012a).

A possible association of fibrinogen genotypes and inflammatory activity could be explained by considering the consequences of fibrinogen genetics on fibrinogen synthesis and the functionality as well as the effect of F13A Val34Leu genotype on fibrin gel formation.

4.1 Fibrinogen: genetics and synthesis

Fibrinogen is an acute phase protein whose synthesis is highly regulated (Fish & Neerman-Arbez, 2012). The gene loci of the β-, α- and γ- fibrinogen chains (FGB, FGA, FGG) contain regulatory gene segments which, on the one hand, influence the basal fibrinogen concentration, i.e. the fibrinogen concentration in inflammation free interval, which is interindividually different. On the other hand, there are regulatory gene segments that are indirectly activated, for example, by glucocorticoids or interleukin 6 (IL6). This activation results in an interindividual different level of increase of the fibrinogen concentration in inflammatory processes.

While in the infection-free interval, the fibrinogen concentration is usually in the range of 1.5-4.0 g/l, the concentration increases in inflammatory processes by 100% and even more. In addition, such increases can be seen, for example, in the context of uncomplicated pregnancy. Of central importance for the interindividual differences in the fibrinogen concentration is the variant FGB -455G>A (rs1800790) or a SNP highly coupled to this variant. Variant carriers have significantly higher fibrinogen concentrations than wildtype individuals. Work on the variant FGB -455G>A describes the increased binding of a repressor to the G allele and consequently a lower rate of synthesis of the downstream gene. (Fish & Neerman-Arbez, 2012).

Regardless of the haplotypes characterised by this SNP, there are several other variants in the three fibrinogen gen loci that appear to have an influence on the fibrinogen synthesis independent of variant FGB -455G>A. The knowledge of the importance of the genetic background on the fibrinogen concentration was substantially deepened by the AIRGENE study. In this study, as described in the introduction, the exposure of ultrafine particles was quantified as a measure of a proinflammatory trigger, thus investigating the relationship between proinflammatory trigger and fibrinogen

concentration with regard to the characterised genetic background (Jacquemin et al., 2008, Peters et al., 2009). According to this research, there is a strong dependence of fibrinogen concentration on the FGB -455G>A variant (rs1800790). As the number of variant alleles increases, so does the fibrinogen concentration. This influence is significantly increased in the context of acute phase reactions as measured by particulate matter exposure (Peters et al., 2009). Even under extreme physical stress, individuals with the FGB -455A allele show a particularly pronounced increase in fibrinogen synthesis (Brull et al., 2002).

For the uncoupled variants FGA rs2070006 G>A and FGA rs2070016 T>C, which are the subject of this thesis, an influence on the fibrinogen concentration is also described. In contrast to FGB -455G>A (rs1800790), the data situation in these two variants is not so clear and the effect significantly weaker. For FGA rs2070016 T>C, the minor C-allele is associated with higher fibrinogen concentrations. (Reiner et al., 2006). For the FGA rs2070006 G>A genotype, data are more diverse. The AIRGENE study showed a weak positive effect of the variant A-allele on the fibrinogen concentration (Jacquemin et al., 2008); in the CARDIA study, carriers of the variant FGA rs2070006 A-allele showed significantly lower fibrinogen concentrations than wildtype individuals (Reiner et.al, 2006).

In summary, it can be said that both the basal fibrinogen concentration and the extent of the inflammation-related increase in the fibrinogen concentration are interindividually different and that these differences are partly due to a genetic basis. Of greatest importance is the FGB -455G>A (rs1800790) variant or a haplotype coupled with this variant. Additional genetic factors, e.g. the variants FGA rs2070006 G>A and rs2070016 T>C, which have been investigated in this thesis, exert an independent influence on the fibrinogen concentration, affecting the subsequent processes.

4.2 Influence of fibrinogen concentration on terminal coagulation processes

The importance of the fibrinogen concentration for the last process of coagulation - fibrin gel formation - has already been described in detail in the introduction. It is, therefore, reminded at this point only in brief.

Considering the relation of fibrinogen concentration and fibrin gel density, it has been acknowledged that at higher fibrinogen concentrations, denser fibrin gels are formed, which are highly crosslinked (Lim et al.,2003). Highly crosslinked, dense fibrin gel structures are assumed to be

prothrombotic as they are associated with increased risk of thrombosis (Fatah et al., 1996, Undas et al., 2009). Some early findings on the influence of the F13A Val34Leu genotype on fibrin gel formation and risk of thrombosis appear to have generated some disagreement: Epidemiologically, the variant F13A 34Leu-allele is associated with a slightly lower risk of thrombotic events than the corresponding wildtype individuals (Kohler et al., 1998, Wartiovaara et al.,1999, Elbaz et al., 2000). Surprisingly, later on this F13A 34Leu allele was shown to be linked with an accelerated activation kinetic of factor XIII and an increased density of fibrin gel (Ariëns et al., 2000), i.e. ultimately a phenotype that is considered prothrombotic (Fatah et al.,1996, Undas et al., 2009).

Research conducted by the group of Lim et al. (2003) contributed to a better understanding of the relation between fibrinogen concentration and fibrin density in dependence of the F13A Val34Leu genotype: If one considers the fibrinogen concentration in investigations on the influence of the F13A Val34Leu genotype on fibrin gel formation, one can observe (see **Figure 1.6** of the introduction) that the fibrin gel density in F13A 34Leu carriers is significantly higher at relatively low fibrinogen concentrations than in 34Val/Val individuals. On the contrary, at elevated fibrinogen concentrations, e.g. induced by inflammation, this relation is reversed, as in 34Val/Val individuals the fibrin gel density is significantly higher than in carriers of the 34Leu allele (Lim et al., 2003). This set of findings helps us to understand why the carriage of the F13A 34Leu seems to be "protective" against thrombosis - the term "protective" used in the literature on the subject is very bold and should only be interpreted as a slight negative association.

When translating the topic of this thesis into this context, genotypes like the variant alleles of FGB rs1800790 G>A and FGA rs2070016 T>C or the wildtype of FGA rs2070006 G>A, which are associated with increased fibrinogen levels and/or fibrinogen synthesis under proinflammatory triggers (Jacquemin et al., 2008, Peters et al., 2009), should result in increased fibrin gel densities in F13A 34Val/Val individuals.

The impact of fibrinogen as well as of fibrinogen- and factor XIII-genetics on fibrin gel formation described above is related primarily to haemostasis and thrombosis. The relevance of these topics for inflammatory processes is intelligible only if one considers the influence of fibrin gel matrix architecture and fibrin (ogen) cleavage products on cellular migration processes, which is described in the following section.

4.3 The role of fibrinogen or fibrin(ogen)-derivatives in inflammatory processes

The influence of fibrinogen and fibrin(ogen) derivatives on inflammatory processes is manifold and can only be summarised selectively in this work. In part, for reasons of clarity, details that were already mentioned in the introduction are summarised here in brief.

Very early studies dealt with the chemotactic effect of various fibrinogen cleavage products generated during coagulation and fibrinolysis (Senior et al., 1986, Skogen et al., 1988, Richardson et al., 1976) **(Figure 4.1).** Thus, the fibrinopeptides B (FpB) act chemotactically on granulocytes and monocytes, the so-called β15-42 peptide, which is released after the action of plasmin on the fibrin clot, acts selectively on granulocytes and fragments of the fibrinogen D and E domains attract monocytes.

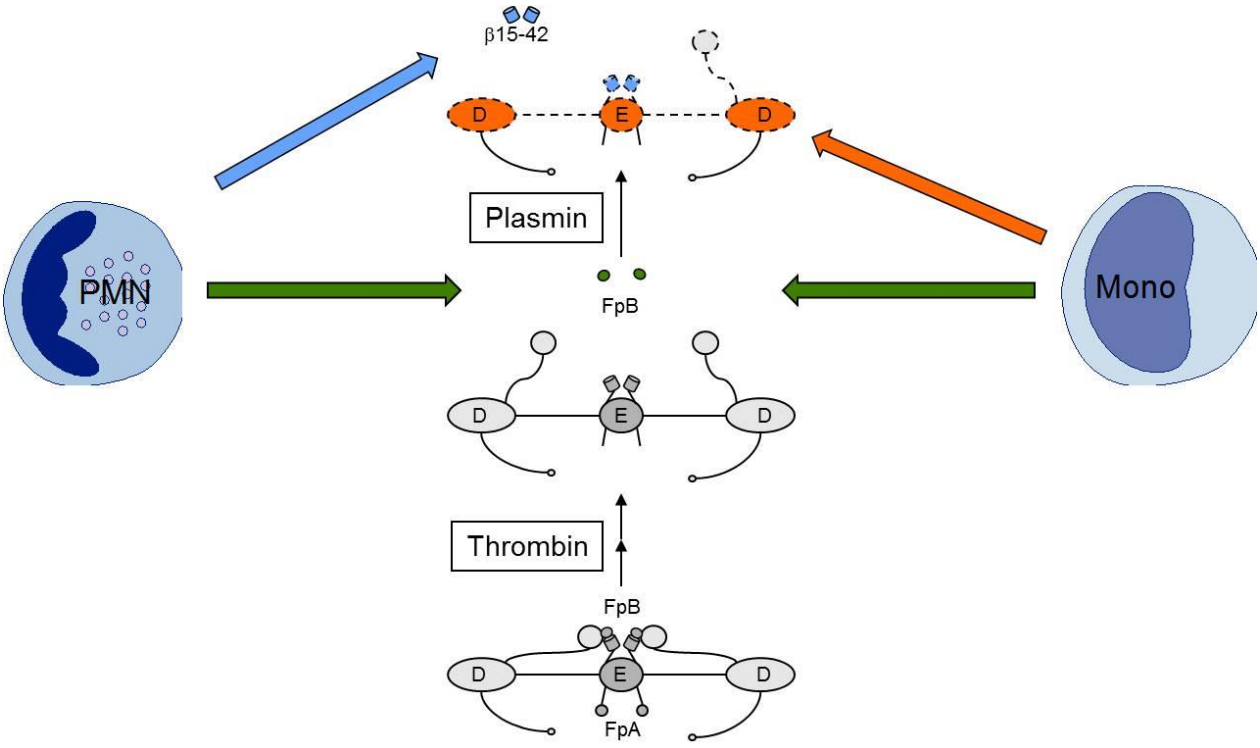


Figure 4.1: Chemotactic action of different fibrin(ogen) derivatives. Fibrinogen including the D- and E-domains, fibrinopeptides A (FpA) and B (FpB) as well as the β15-42 peptide are given. PMN: polymorphonuclear leukocyte, Mono: Monocyte.

Furthermore, in a complex process, fibrin(ogen) cleavage products, in this case the so-called fibrin E1 fragment, influence transendothelial migration processes. Leukocytes face an endothelial barrier if they must invade into inflammatory tissues **(Figure 4.2).** In part, this barrier is loosened by low-level fibrin activation and fibrinolysis at the inflammatory focus by the generation of fibrin E1 fragment **(Figure 4.3)**.

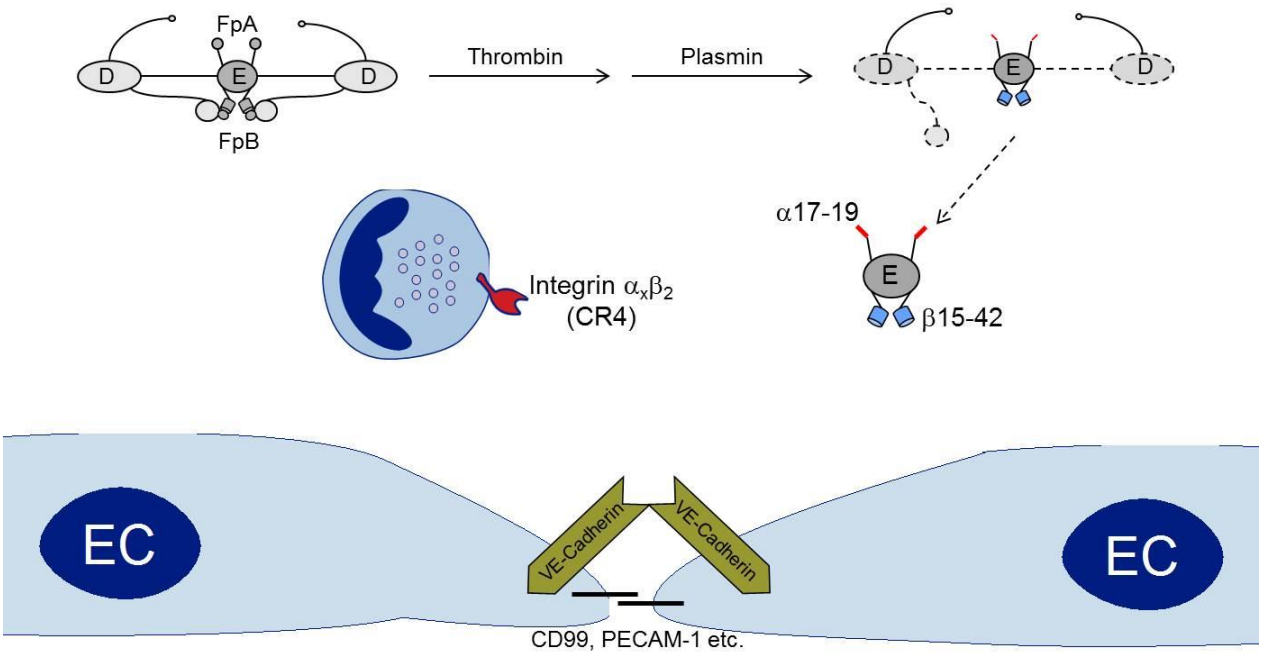


Figure 4.2: **Cell-cell interaction between two endothelial cells (EC).** Different molecules, which mediate this cellular interaction (VE-cadherin, CD99, PECAM-1), are depicted. After activation of fibrinogen by thrombin the fibrin gel is digested by plasmin. The fibrin E1-fragment, which carries two binding motifs (α17-19, β15-42), is released.

This fibrin cleavage product has binding sites for the endothelial VE-cadherins (vascular endothelial cadherin) and a leukocytic receptor. Hence, on the one hand it dissolves endothelial-endothelial bonds and, on the other, it supports the leukocyte to penetrate this barrier (Petzelbauer et al., 2005, Zacharowski et al., 2006).

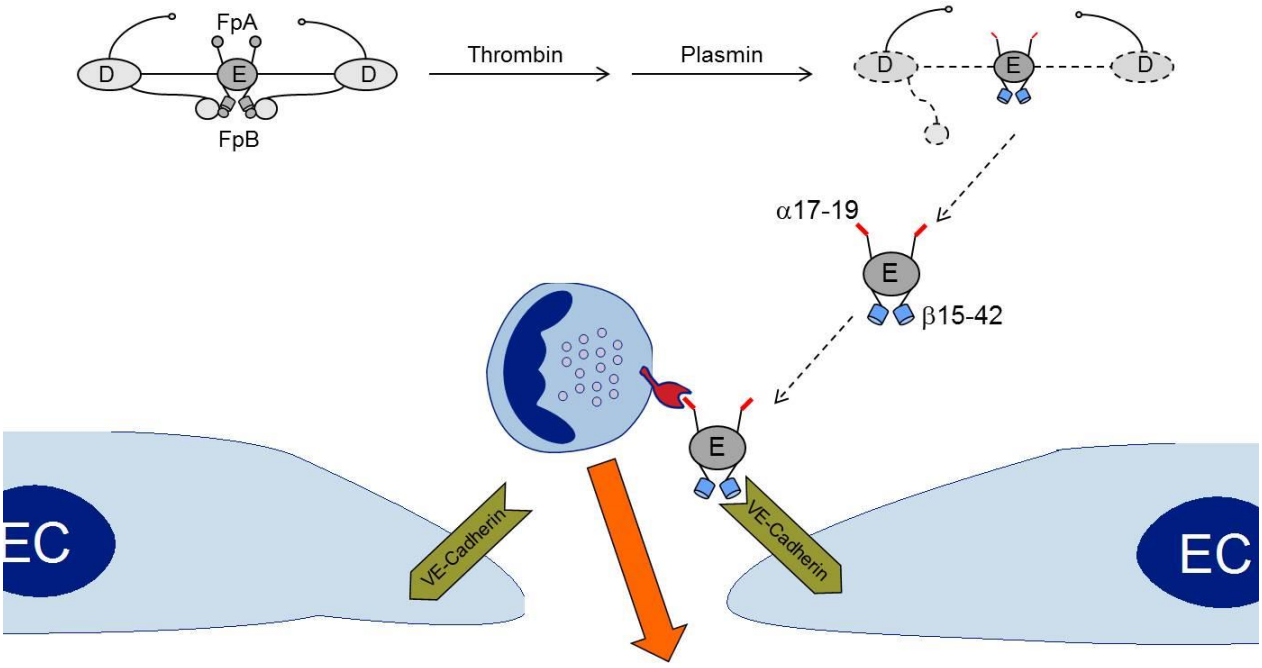


Figure 4.3: Opening of the cell-cell-interaction between two endothelial cells (EC). The fibrin E1-fragment interacts via α17-19 with a leukocytic integrin, the β15-42 motif mediates the interaction with VE-cadherins.

This pathway is not only important in "conventional" inflammatory processes, it also plays a role in the so-called myocardial reperfusion injury. After a myocardial infarction and the reopening of the occluded vessel, a leukocytic invasion into the infarcted area induces additional tissue damage increasing the ischemia induced defect. By administering the β15-42 peptide, which is part of the fibrin E1 fragment, but which is unable to dissolve VE-cadherin interactions or to bind to leukocytes, the reperfusion damage can be significantly reduced in the animal model because the leukocyte infiltration into the infarct area is suppressed. (Petzelbauer et al., 2005) **(Figure 4.4).**

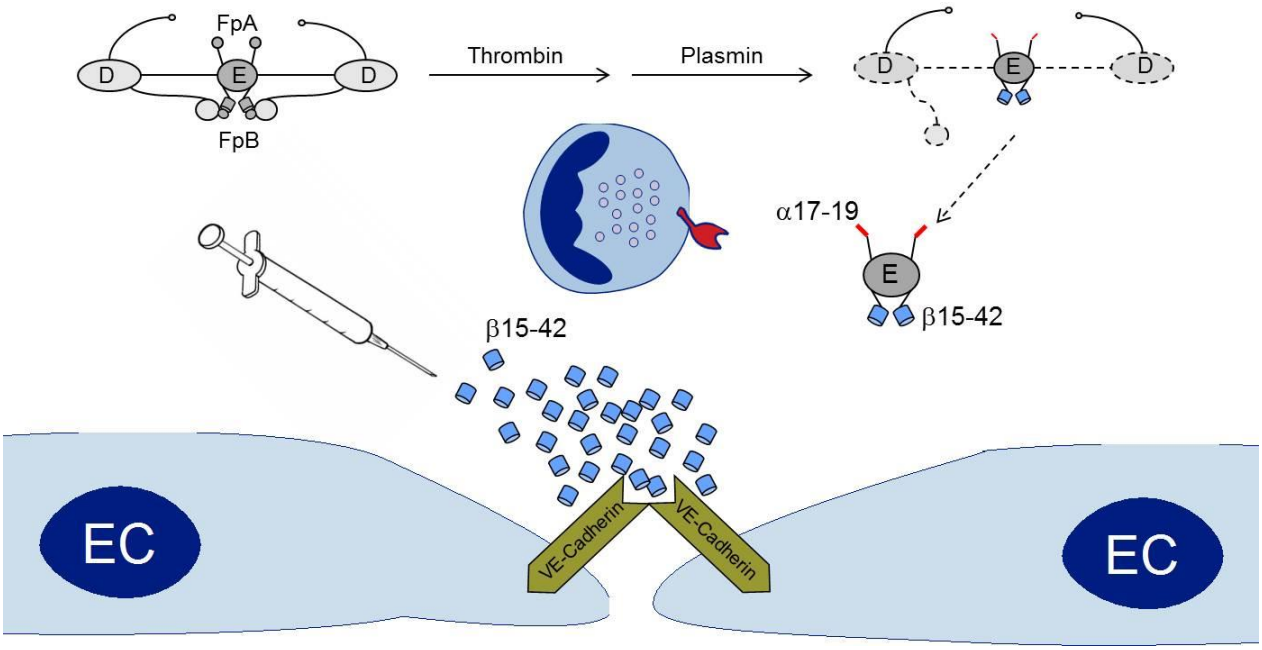


Figure 4.4: Reduction of postischaemic reperfusion injury by β15-42 peptides. The application of β15-42 peptides in an animal model of postischaemic reperfusion injury blocks fibrin E1-fragment induced opening of cell-cell interactions between two endothelial cells, thereby ameliorating postischaemic reperfusion injury.

As the genetic variant FGA rs2070016 T>C presumably influences the process of fibrin gel formation, while potentially modulating fibrinolytic processes, it could be argued that the tendency for reduced inflammatory activity in carriers of the combination "FGA rs2070016 C-allele plus F13A 34Val/Val" could be related to a predisposition for dense fibrin gels under proinflammatory triggers, reduced fibrinolysis and – consequently – reduced leukocytic invasion. This could be translated in reduced CRP-levels as well as in a tendency for lower inflammatory activity in RA. The reverse argumentation could be used to explain the finding for "FGA rs2070006 A-allele plus F13A 34Val/Val".

4.4 Fibrinogen in the collagen-induced arthritis model

Finally, an example of a study on transgenic mice should be mentioned, which illustrates how fibrinogen is involved in the inflammatory process of collagen-induced arthritis (Flick et al., 2007). In this model, application of collagen II and other supplements induces chronic arthritis in mice that is similar in micro-pathological features to rheumatoid arthritis in humans **(Figure 4.5).**

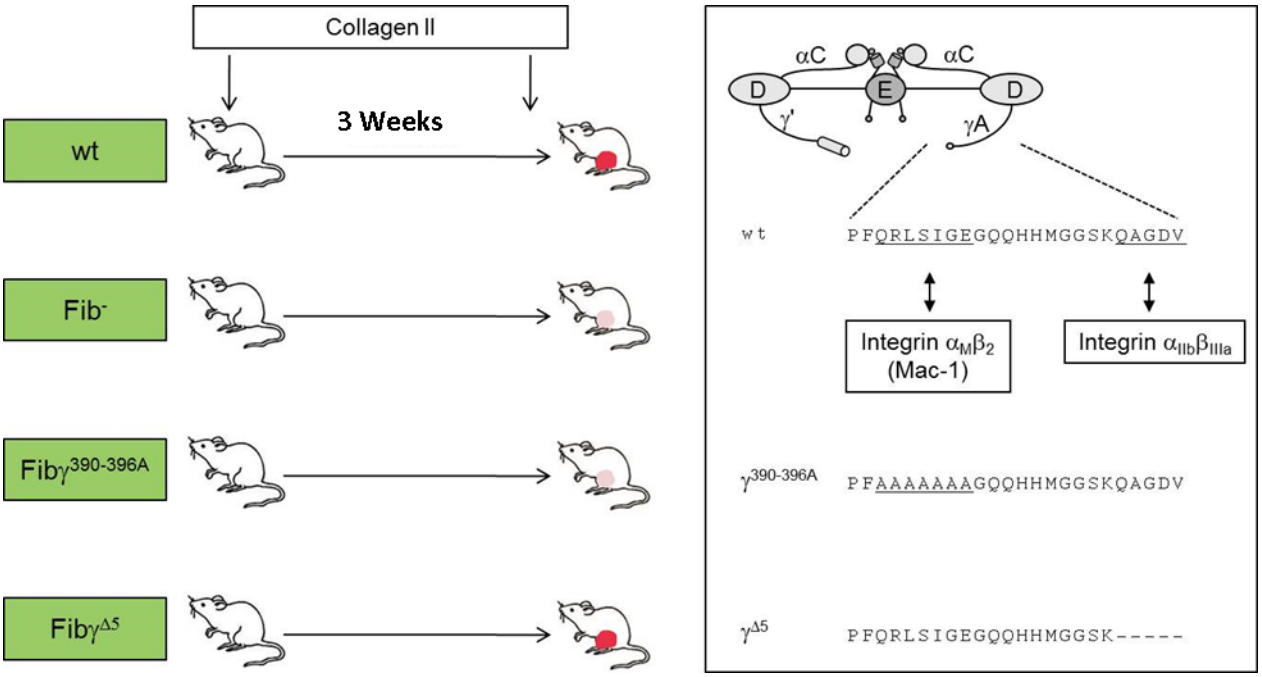


Figure 4.5: Impact of fibrinogen on inflammatory activity in collagen-induced arthritis. The inflammatory activity of collagen-induced arthritis in wildtype (wt) as well as in transgene mice (Fib^- : fibrinogen knock-out, $Fib\gamma^{390-396A}$, $Fib\gamma^{\Delta 5}$) is indicated by red (high inflammatory activity) and pink (low inflammatory activity) dots.

In wildtype mice, arthritis develops about 3 weeks after the first immunisation. If fibrinogen is completely lacking in these mice (Fib^-), the resulting arthritis severity is considerably reduced. The same is true for transgenic mice, that produce haemostatically active fibrinogen, lacking a specific motif on the γ-fibrinogen chain ($Fib\gamma^{390-396A}$), that functions as binding partner for leukocytic integrins. However, if the five terminal amino acids at the γ-fibrinogen chain are deleted ($\gamma^{\Delta 5}$), the full arthritic

phenotype can be detected. This study underscores that the fibrinogen molecule serves as an essential interaction partner for inflammatory mediators.

4.5 Influence of fibrinogen- and factor XIII-genetics on inflammatory activity in rheumatoid arthritis

The aim of this research was to test a hypothesis derived in 2012 from studies on variants FGB -455G>A (rs1800790), FGA Thr312Ala (rs6050) and F13A Val34Leu (rs5985) with respect to inflammatory reactions (Hoppe et al., 2012a).

According to this model, a genetic background that predisposes to a strong fibrinogen response in the case of acute phase reaction (e.g. carriage of FGB rs1800790 G>A minor allele), predisposes to lower CRP levels in individuals with the F13A 34Val/Val genotype, which allows for an extended fibrin crosslinking under high fibrinogen levels. The F13A 34Val/Val genotype is considered as a permissive factor in this model **(Figure 4.6).**

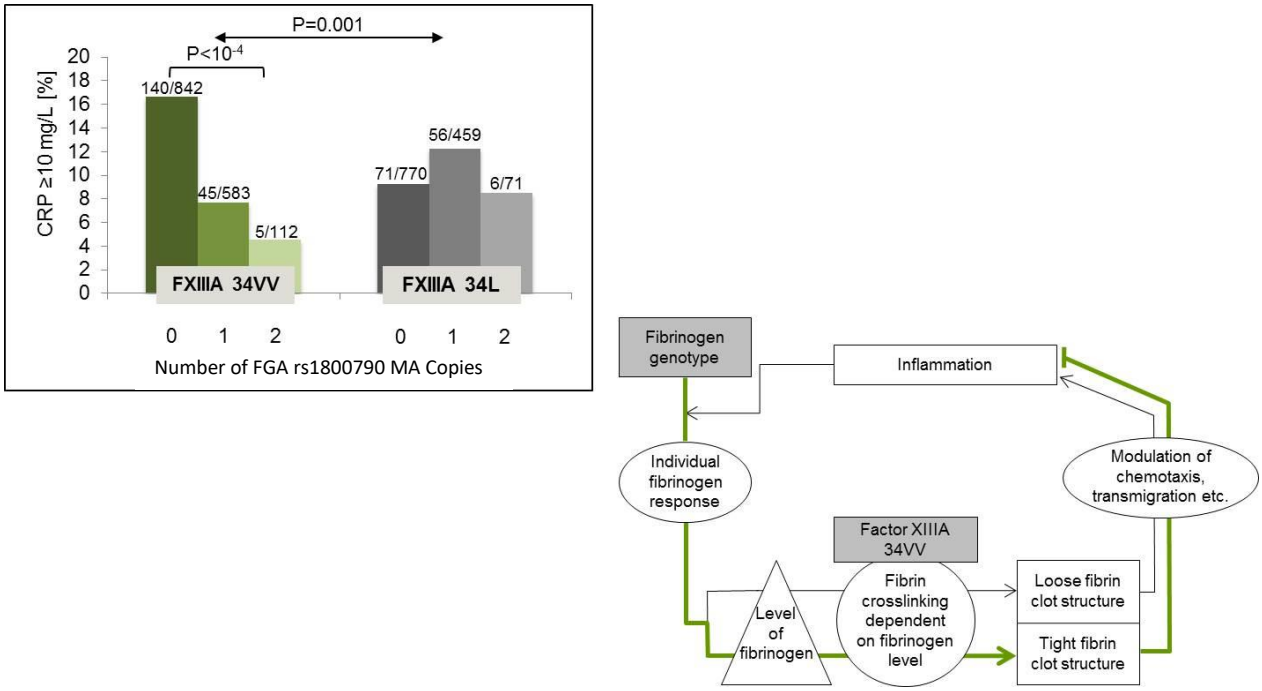


Figure 4.6: **Influence of FGB rs1800790 G>A and F13A rs5985 Val34Leu genotypes on inflammatory activity.** Data from previous works as well as a hypothetical model are given. (Figure adapted from Hoppe ,2014).

Conversely, the proportion of elevated levels of CRP increases when the focus is on a fibrinogen variant, where the minor allele is associated with lower levels of fibrinogen in inflammation (e.g., FGA rs6050 Thr312Ala) **(Figure 4.7).**

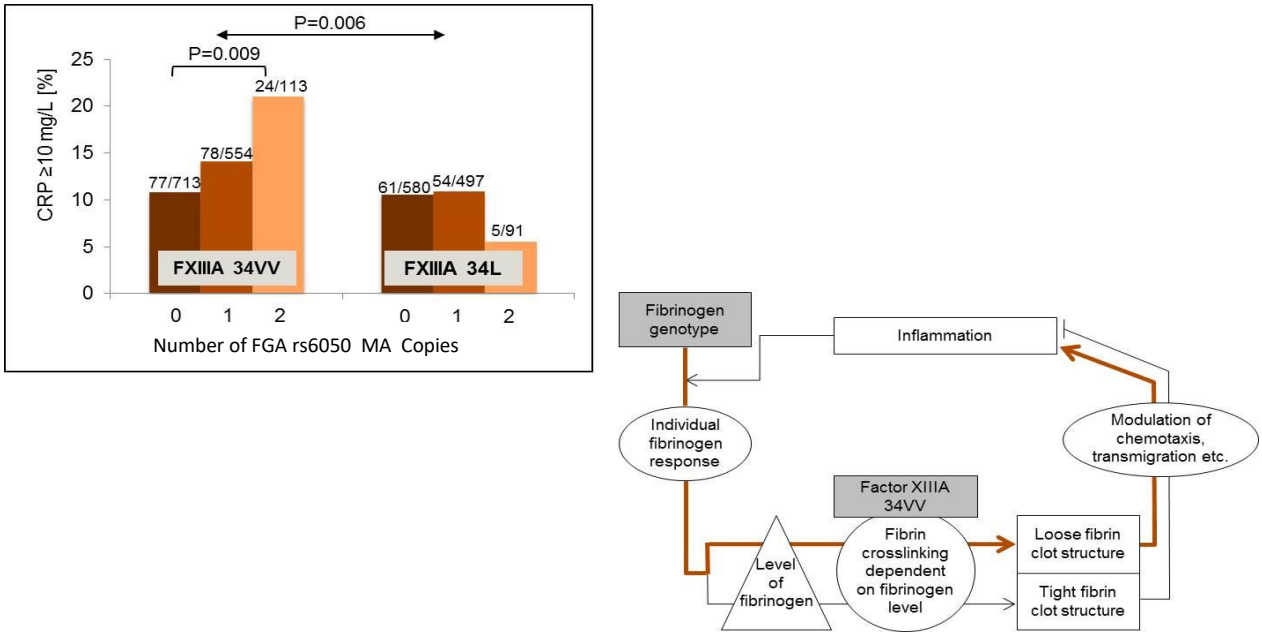


Figure 4.7: **Influence of FGA rs6050 Thr312Ala and F13A rs5985 Val34Leu genotypes on inflammatory activity.** Data from previous works as well as a hypothetical model are given. (Figure adapted from Hoppe, 2014).

To test this hypothesis, two other genetic variants have now been investigated at the α-fibrinogen locus, FGA rs2070006 G>A and rs2070016 T>C, which in our study population are not in strong linkage dysequilibrium with the FGB rs1800790 G>A and FGA rs6050 Thr312Ala variants.

Analyses were based on the RA cohort, which was used for the initial study (Hoppe et al, 2012a). As described in the section of characteristics in the results, the group of patients we examined consisted of 285 patients with rheumatoid arthritis. About 30% of them were carriers of the FGA rs2070016 minor allele (C) and about 57% had the FGA rs2070006 minor allele (A). In this respect,

they are high-frequency genetic variants, which is advantageous for a statistic assessment of effects **(Table 3.1).**

The patient population was typical for RA in terms of gender distribution with 77% of it being women. Also, the laboratory diagnostic characteristics corresponded to the expected distributions for this disease (reviewed in Klareskog et.al, 2008). Nearly 2/3 of the patients were ACPA positive. HLA-DRB1 SE, which is very strongly associated with ACPA formation, was found at approximately equal frequency. The rheumatoid factor was detectable in almost 3/4 of patients.

Consistent with previous work (Visser et al., 1999, Timpson et al., 2011), BMI was strongly correlated with the level of inflammatory activity in our patient population. A BMI> 30 kg/m^2 was significantly associated with higher CRP concentrations.

As shown in **Table 3.3**, the relation between FGA rs2070016 T>C and CRP was first regarded without considering the factor XIII genotype. The proportion of elevated CRP concentrations (CRP >10 mg/L) was shown to be higher in the wildtype patients (40%) and it decreased by increasing numbers of FGA rs2070016 C alleles to 30.7% (1 C allele) and 24.5% (2 C alleles) (P = 0.002). As the FGA rs2070016 C-allele was described to be related to increased fibrinogen levels (Jacquemine et al., 2008) – comparable to FGB rs1800790 G>A but to a smaller degree – the finding that FGA rs2070016 T>C behaves similar to FGB rs1800790 G>A with respect to inflammation is fully consistent with the hypothesis to be tested.

The situation is different with additional consideration of the F13A Val34Leu genotype **(Table 3.7)**. In the presence of the "permissive" genotype F13A 34Val/Val described in the hypothetical model, the relation between FGA rs2070016 C-carrier status and elevated CRP levels was detectable only as a statistical trend (WT: 36.8%, C-allele carrier: 27.6%, P = 0.06). When considering the allele dose, elevated CRP levels were less frequently observed with increasing C-allele copy numbers (wild type: 36.8%, 1 C allele: 30.1%, 2 C alleles: 14.3%, P = 0.026). The lack of a statistically significant relation between FGA rs2070016 C allele carrier status and CRP elevations in the F13A 34Val/Val (wt) stratum may be due to the small number of cases in this subgroup and thus the low statistical power (136 patients, 436 observations). In contrast, the relation was statistically significant in individuals with F13A 34Leu genotype (WT: 42.9%, Carrier: 31.5%, P <0.02 and 1C allele: 31.3%, 2C alleles: 32.1%, P = 0.03). In summary, in both F13A Val34Leu-strata the proportion of CRP elevations was lower in FGA rs2070016 C allele carriers compared to non-carriers. Surprisingly, this

finding appears to be independent of the F13A Val34Leu genotype. As described above, in the initial study the association between FGB rs1800790 G>A and CRP was limited to the F13A 34Val/Val genotype (Hoppe et al., 2012a), so the latter genotype was considered as a necessary and permissive condition.

For the variant FGA rs2070006 G>A (**Table 3.5)**, no relation could be shown (WT: 35.1%, A-carrier: 38.1%, 1 A-allele: 39.5%, 2 A-alleles: 34.7%). with CRP elevations (>10 mg/l) for A-allele carrier status or for A-allele copy number. However, with additional consideration of the F13A 34Val/Leu genotype **(Table 3.9)**, this picture changed. In the presence of the permissive F13A 34Val/Val genotype, the frequency of CRP elevations was significantly higher in A-allele carriers (40.2%) than in FGA rs2070006 G/G wildtypes (27.9%) (OR:1.73, P<0.007). For F13A 34Leu carriers **(Table 3.9)**, the frequency of CRP elevations in FGA rs2070006 G/G wildtypes (44.6%) tended to be higher than in the FGA rs2070006 A allele carriers (36.6%) (OR: 0.72, P=0.09). The relation between FGA rs2070006 G>A genotype and CRP elevations was significantly different between the F13A 34Val/Val and F13A 34Leu subgroups (test for homogeneity, P =0.0019). Since the FGA rs2070006 A-allele is associated with lower fibrinogen concentrations when compared to the wildtype – similar to the variant FGA rs6050 Thr312Ala from the initial study (Hoppe et al, 2012a) – the findings for FGA rs2070006 G>A are consistent with the hypothetical model.

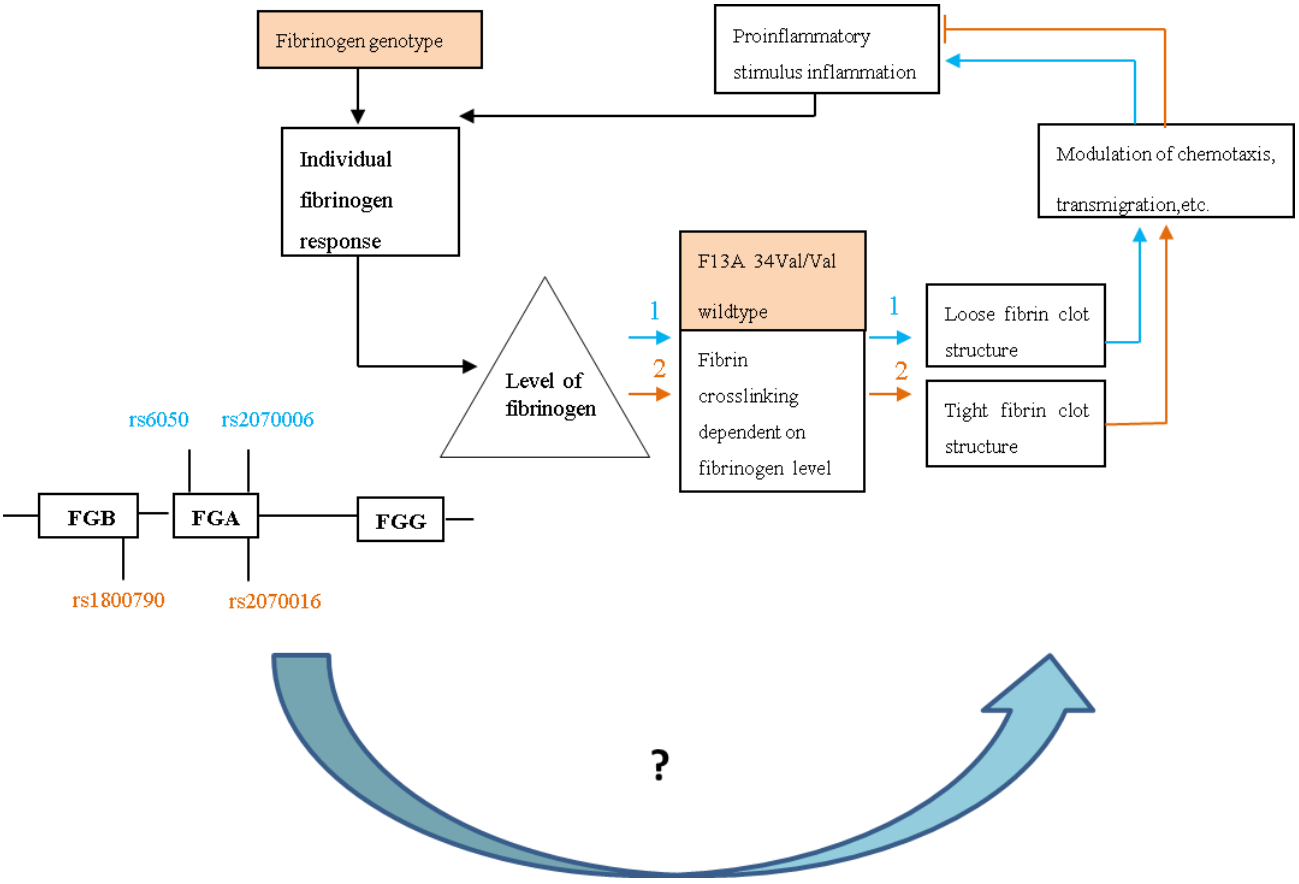


Figure 4.8: Hypothetical model for the influence of fibrinogen and F13A genotypes on the intensity of the inflammatory process. In addition to the variants FGB rs1800790 G>A and FGArs6050 Thr312Ala, which were characterised in the initial studies, the FGA variants rs2070016 T>C and rs2070006 G>A of the present thesis are given. A possible direct relation between FGA rs2070016 T>C genotype and formation of tight fibrin clot structures is indicated (Figure adapted from Hoppe & Dörner, 2012b).

The initial hypothesis on the relation between fibrinogen/factor XIII genetics and inflammatory activity basically focused on the interplay between fibrinogen concentration and factor XIII genotype with respect to the quality of the fibrin gel (Lim et al., 2003). In addition, it included the underlying genetic background of the fibrinogen gene loci (especially FGB rs1800790 G>A),

which affects basal and reactive fibrinogen levels (Jacquemine et al., 2008, Peters et.al., 2009). For all genetic fibrinogen variants other than FGB rs1800790 G>A - also for FGA rs2070006 G>A and rs2070016 T>C - the effect is clearly less pronounced and not completely or consistently documented (Jacquemine et al., 2008, Peters et al., 2009). In this respect, the findings for FGA rs2070006 G>A confirm the hypothesis. The fact that FGA rs2070016 T>C is associated with CRP elevations independent of the factor XIII genotype could be explained by a possible direct influence of this variant on the structure of the fibrin gel.

With the exception of dysfibrinogenemias (Tiscia & Margaglione, 2018), direct influences of fibrinogen genetics on the structure of the fibrin clot are quite poorly documented. One example of a variant influencing fibrin gel architecture directly, is given by a splice variant of fibrinogen, the so-called γ'-chain. In contrast to the dominant, 411 amino acid residues long γA chain, the γ'-chain has an extended C-terminus (a total of 427 amino acid residues) (Uitte de Willige et al., 2005, Uitte de Willige et al., 2007, Uitte de Willige et al., 2009). Usually almost 8-15% of the γ-fibrinogen chains belong to the γ'-chain. This means that in these cases, the fibrinogen molecule is not formed from a homodimer (αβγA/αβγA) but rather from a heterodimer (αβγA/αβγ'). Through the extended C-terminus, the fibrinogen interacts with the B subunit of factor XIII. The extent of the γ'-chain is subject to genetic control. Thus, a haplotype in the FGG gene has been described (FGG rs2066861, FGG rs2066864 and FGG rs2066865, the latter probably playing the most important role), which reduces the proportion of γ'-chain (Uitte de Willige et al., 2005 , Uitte de Willige et al., 2007, Uitte de Willige et al., 2009). Interestingly, both a reduced proportion of the γ'-chain and the corresponding genetic variants are associated with an increased risk of thrombosis (Uitte de Willige et al., 2005, Uitte de Willige et al., 2007, Uitte de Willige et al., 2009). Considering that a dense, highly cross-linked fibrin gel is generally considered to be prothrombotic (Lim et.al, 2003), it can be assumed that a reduced concentration of the γ'-chain or the corresponding genetic FGG variants are also associated with this fibrin gel architecture. Because of a strong linkage between FGG rs2066861 G>A and FGA rs6050 Thr312Ala (Uitte de Willige et al., 2005) it can be assumed that the effect of FGA rs6050 Thr312Ala on fibrin gel formation is in part related to the amount of γ'-chain. Thus, it would be conceivable that FGA rs2070016 T>C influences fibrin gel formation and inflammatory processes indirectly by influencing the amount of γ'-chain. In order to verify this assumption, further characterisation of the

cohort with regard to relevant FGG genotypes and possibly also the concentration of the γ' chain would be necessary.

4.6 Influence of fibrinogen and factor XIII genetics on disease activity in rheumatoid arthritis

For the assessment of disease activity in rheumatoid arthritis, the DAS 28 is used in clinical practice. This score integrates information on the status of the joint (pain, swelling) as well as a self-assessment of the patient on the general feeling of health. In addition, the laboratory diagnostic characterisation of the inflammatory activity in the form of the erythrocyte sedimentation rate is part of DAS 28.

The studied patient group showed a relatively high disease activity (DAS 28 median: 5.35), 54.7% had a high disease activity (DAS 28 $\geq$ 5.1) **(Table 3.1)**. Among other reasons, this is due to the fact that patients with a previously unfavorable course of the disease are frequently referred to the highly specialised rheumatological out-patient unit of the Charité.

The BMI of the patients was very likely to have a significant influence on the DAS28 due to the fact that it represents a proinflammatory trigger and is known to influence inflammation related markers like CRP **(Table 3.2)**. Thus, at a BMI >30 kg/m^2 0 (0%) and 1 (2,2%) of the patients showed a remission (DAS 28 <2.6) or mild disease activity (DAS28 2.6-3.2), respectively, whereas 22 (9.4%) and 14 (6.0%) patients with a BMI $\leq$30 kg/m^2, respectively, belonged to these activity groups. In the groups of high disease activity (DAS 28$\geq$ 5.1), 34 patients (73.9%) with a BMI> 30 kg/m^2 were found while at a BMI $\leq$30 kg/m^2 119 patients (50.9%).

We also investigated the influence of fibrinogen/factor XIII genetics on the DAS 28 score. In the isolated consideration of the variant FGA rs2070016 T>C **(Table 3.4)** C-allele carriers and wildtypes were almost equally distributed in the different DAS 28 groups (<2.6, <3.2, $\geq$ 5.1). The result did not change when considering the number of C-alleles. Only in the category of patients suffering from mild disease (DAS28<3.2) was a tendency shown for higher frequency in the carriers. When looking at the distribution of the DAS 28 values in different FGA rs2070016 T>C genotypes in the rank sum test, a non-significant trend towards lower DAS 28 values was found in C allele carriers (P=0.10). Interestingly, in this genotype group, the duration of morning stiffness also tended to be shorter with a median of 15 min than in the wildtype group (median: 30 min, P <0.07). This finding calls to mind a very recent publication describing increased morning stiffness in those patients

with intrasynovial fibrin deposits (Orange et al., 2019). For the variant FGA rs2070006 G>A there was no trend detectable, not in DAS 28 groups (<2.6, <3.2, ≥5.1) or in the distribution of individual DAS 28 values, or in the duration of morning stiffness **(Table 3.6)**.

After stratification for the F13A Val34Leu genotype, the effects described in the previous paragraph for the variant FGA rs2070016 T>C were accentuated in the group F13A 34Val/Val genotype (wildtype) (DAS 28 <3.2: OR: 2.85, P <0.03, DAS 28≥ 5.1: OR: 0.41, P <0.02) **(Table 3.8).** That means that in this subgroup, the C allele was over-represented in patients with mild disease activity, whereas it was less abundant in the high disease activity group. In the F13A 34Leu subgroup, no relation between the FGA rs2070016 T>C genotype and the disease activity could be detected. This finding is surprising since with respect to CRP elevation for FGA rs2070016 T>C, no heterogeneity could be found between F13A 34Val/Val (wildtype) and 34Leu carriers **(Table 3.7)**.

In contrast, for FGA rs2070006 G>A no interplay with F13A Val34Leu genotype with respect to DAS28 could be found **(Table 3.10)**, even though there was significant interaction when considering CRP elevations **(Table 3.9)**.

5. Final Conclusion

The aim of this study was to examine whether a model for the relation between fibrinogen/factor XIII genetics and inflammation, which was developed from investigations of the variants FGB rs1800790 G>A, FGA rs6050 Thr312Ala and F13A rs5985 Val34Leu, can also be applied to the variants FGA rs2070016 T>C and rs2070006 G>A.

For FGA rs2070006 G>A the results were fully consistent with the 2012 model. For FGA rs2070016 T>C, a relation with CRP could be found that was in agreement with previous work, however, when considering the interactive effect with the F13A Val34Leu genotype, there were some discordances that should be addressed in further studies. Further investigations of the FGG genotypes, the γ'-fibrinogen, the genetic variability of the B-subunit of factor XIII (F13B) and also the genetic variants in the α2-antiplasmin gene would be of interest in order to further develop the fibrin-based model of inflammatory control.

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Statutory Declaration

“I, Anneta Pistioli, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Influence of fibrinogen and factor XIII genotypes on inflammatory processes, Einfluss von Fibrinogen und Faktor XIII Genotypen auf Entzündungsprozesse], independently and without the support of third parties, and that I used no other sources and aids than those stated.

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My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

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