REGULATION AND MODULATION OF THE PLASMA FIBRINOGEN LEVEL

(REGULATIE EN MODULATIE VAN DE FIBRINOGEEN-WAARDEN IN PLASMA)

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aan mijn ouders
voor Dick

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ABBREVIATIONS

AMI	acute myocardial infarction
CABG	coronary artery bypass graft
CHD	coronary heart disease
CM	conditioned medium
CRP	C-reactive protein
DVT	deep venous thrombosis
EIA	enzyme immuno assay
FDP (TDP)	(total) fibrin(ogen) degradation products
HMW-fibrinogen	high molecular weight fibrinogen
HRG	histidine rich glycoprotein
IL1ß	interleukin 1ß
IL6	interleukin 6
LMW-fibrinogen	low molecular weight fibrinogen
LMW'-fibrinogen	low' molecular weight fibrinogen
PAI	plasminogen activator inhibitor
PPP	platelet poor plasma
PRP	platelet rich plasma
PTCA	percutaneous transluminal coronary angiography
RFLP	restriction fragment length polymorphism
TNFα	tumour necrosis factor α

INTRODUCTION

FIBRINOGEN

Fibrinogen is a soluble plasma glycoprotein that, under normal circumstances, is present in human plasma at a concentration of 2-4 mg/ml (6-12 μM). The plasma half-life is 3-4 days in humans and about 10-25% of the total body fibrinogen is extravascular[1]. Fibrinogen is composed of two sets of three polypeptide chains (Aα, Bβ and γ) that are interconnected by disulphide bridges (figure 1). The aminoterminal segments of all six chains form a central domain from which the Aα and Bβ chains protrude[2]. These aminotermini are the target of thrombin and small peptides (FPA and FPB) can be cleaved off.

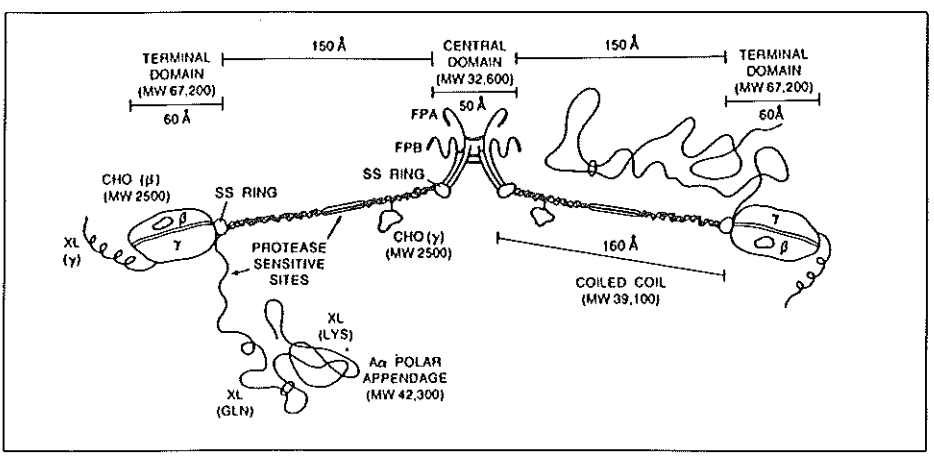

Figure 1. Model of the human fibrinogen molecule showing structural characteristics[2]

Three main molecular forms of fibrinogen have been identified in plasma[3-9]. Fibrinogen is assumed to be synthesized in the high molecular weight form (HMW)[10], with two intact carboxyl ends of the α-chain. In plasma two groups of degraded forms of fibrinogen can be distinguished. One group has one degraded Aα-chain carboxyl end and is called the low molecular weight (LMW) form, in the other form both Aα-chains are degraded and this is called the LMW' form. In a normal, average plasma 70% of fibrinogen is HMW, 26% is LMW and only 4% is LMW'. The LMW and LMW' forms are heterogenous due to different lengths of their residual Aα-chain. The enzymes responsible for the degradation have not yet been identified[11,12]. Recently, it has also been described that $\pm$ 3% of the natural occurring fibrinogen molecules have a globular C-terminal extension on both carboxyterminal ends which shares C-terminal homology with the ß and γ chains. The molecular weight of the fibrinogen molecules with the extended α chains is

420.000[13]. The extended Aα-chains of this fibrinogen are more resistant to degradation than the normal forms.

The main function of fibrinogen is in the clotting process. After thrombin has cleaved off the fibrinopeptides A from the N-terminal end of the Aα-chain the molecule changes its conformation and, via intermediate stages, polymerizes into an insoluble fibrin network. Somewhat delayed, the fibrinopeptides B are also cleaved off the Bß-chain, initiating the lateral polymerization of the fibrin fibres. This fibrin network is normally constructed of fibres that are about one μm in diameter, which means a width of several hundred molecules. Approximately 80% of the blood clot volume consists of solvent and the relative mass of fibrin in the clot is not more than 0.3%. The remainder includes blood elements like platelets and red and white blood cells.

A HIGH PLASMA FIBRINOGEN LEVEL - AN INDEPENDENT RISK INDICATOR FOR CARDIOVASCULAR EVENTS

Only about 30% of all cardiovascular events can be predicted by the established risk factors like hypercholesterolemia, smoking, overweight, diabetes, age, gender and hypertension[14]. This suggests the existence of more risk factors. In recent years the relation between plasma fibrinogen levels and cardiovascular risk has been evaluated in a number of epidemiological studies.

The Northwick Park Heart Study was the first large-scale prospective longitudinal study that described the association between plasma fibrinogen levels and the risk for cardiac events[15]. An increase of the plasma fibrinogen with one standard deviation of the mean (about 15%) gave almost a doubling of the risk for cardiac events in the next five years. After 10 years follow-up, approximately half of the coronary events had occurred in the upper tertile of the plasma fibrinogen levels. The prognostic value of fibrinogen is at least equal and probably greater than that of cholesterol (figure 2).

The association between the plasma level and cardiac events has since then been confirmed in a number of other studies, including the Gothenburg[16], the Framingham[17], Leigh[18], PROCAM[19], Scottish Heart Health[20], Caerphilly-Speedwell[21,22], the GRIPS[23] and the ECAT-Angina pectoris[24] studies (table 1). Elevated plasma fibrinogen levels have also been associated with an increased risk of myocardial infarction in patients with unstable angina[25] and in patients that had experienced a stroke[26] or a previous infarction[27,28]. Even though these studies differed in design and procedures they all identified fibrinogen as an independent risk indicator. The predictive value of elevated plasma fibrinogen levels for the incidence of fatal and nonfatal myocardial infarctions and cerebrovascular events were independent of the prevalence and distribution of other, established

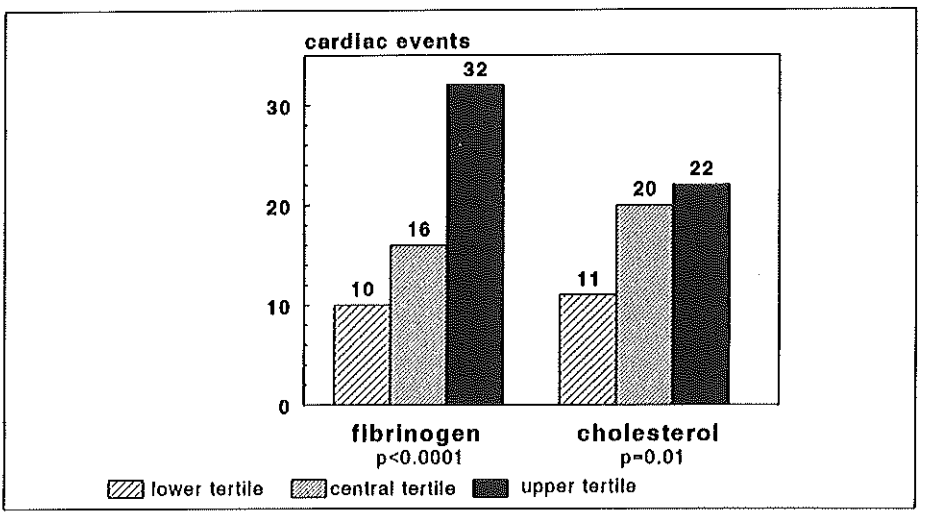

Figure 2. Relation between plasma fibrinogen and cholesterol levels and the incidence of cardiac events (in the 5 year follow up period) in the Northwick Park Heart Study[15]

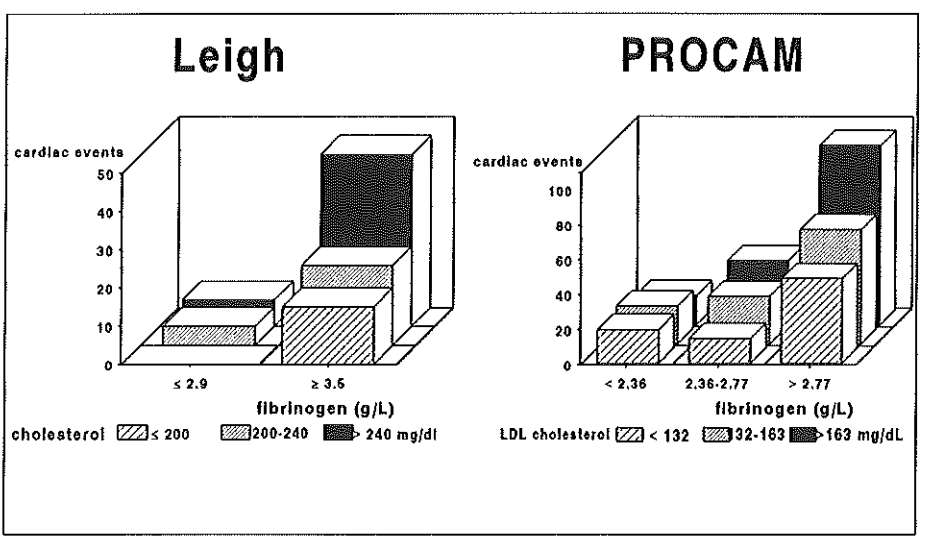

Figure 3. Relation between incidence of coronary events (events per 1000 patient years in Leigh Study and events in 6 years/1000 in the PROCAM Study) and plasma levels of fibrinogen and cholesterol in the Leigh Study[18] and the PROCAM Study[19]

cardiovascular risk factors.

Interactions between high plasma fibrinogen levels and other risk factors are repeatedly observed. In the PROCAM and the Leigh study it was shown that individuals with both high fibrinogen and high cholesterol had the highest cardiac risk, which was more than additive (figure 3), similar to individuals with high fibrinogen and high blood pressure (figure 4). Also, several gender differences were observed in the Framingham and the ECAT-angina pectoris studies which examined both men and women. For example, in the Framingham Study, it was shown that the magnitude of the fibrinogen-mediated risk in women declined with age. Also, in women elevated fibrinogen levels were not associated with risk of stroke[17], figure 5).

EFFECTS OF INCREASED PLASMA FIBRINOGEN LEVELS THAT MAKE A DIRECT RELATION WITH CORONARY ARTERY DISEASE PLAUSIBLE

It has been established in several epidemiological studies that elevated plasma fibrinogen levels represent an increased risk for the development of cardiovascular incidents (table 1). Even though the background of this relation has not yet been elucidated it is plausible that increased fibrinogen levels can give a causal contribution to cardiovascular disease: a) fibrinogen influences the formation and growth of the arteriosclerotic lesion[29], b) fibrinogen is the principal component of gelatinous and fibrous plaques[30], c) there is interaction between many cell types and fibrinogen: the proliferation[30] and migration[31] of smooth muscle cells is stimulated by fragments of fibrinogen, d) fibrinogen is an important promoter of the aggregation of activated platelets[review: 32], e) it also interacts with endothelial cells[33,34], monocytes[35] and macrophages[36,37], f) as part of the extracellular matrix fibrinogen is also involved in tumor growth, although not for metastases[1].

A direct contribution of fibrinogen to risk might also be through the plasma viscosity, which is mainly determined by the plasma fibrinogen level[38]. An increased plasma viscosity can induce microcirculatory disorders, like reduced oxygen release, in poststenotic vascular segments[39].

Relation between plasma fibrinogen levels and the severity of vascular disease

The relation between plasma fibrinogen levels and vascular thrombotic events is further strengthened by the correlations between plasma fibrinogen levels and the degree of coronary artery disease[29,40-44] and peripheral arterial disease[45].

Table 1. Prospective epidemiological investigations.

Study	sample size	mean age (range)	length of follow up (years)	method	endpoint	number of events
NPHS[15]	1511 white men	52 (40-64)	10.0	gravimetric	(1) IHD death (2) non-fatal	(1) 68 (2) 60
Gothenburg[16]	792 men	all 54	13.5	spectro-photometric	(1) MI (2) stroke	(1) 92 (2) 38
Leigh[18]	297 men	52 (40-69)	7.3	nephelometric	MI	40
Framingham[17]	554 men 761 women	>55 47-79	12	spectro-photometric	(1) IHD (2) stroke	(1) 312 (2) 92
Caerphilly/Speedwell[22]	4860 men	(45-49)	5.1 / 3.2 (2 cohorts)	nephelometric Clauss	IHD	251
PROCAM[19]	2116 men	48 (40-65)	6	Clauss	MI	82
GRIPS[23]	5239 men	(40-60)	5	Clauss	MI	107
ECAT-angina pectoris[24]	2587 men 456 women	56	2	Clauss	coronary events	106

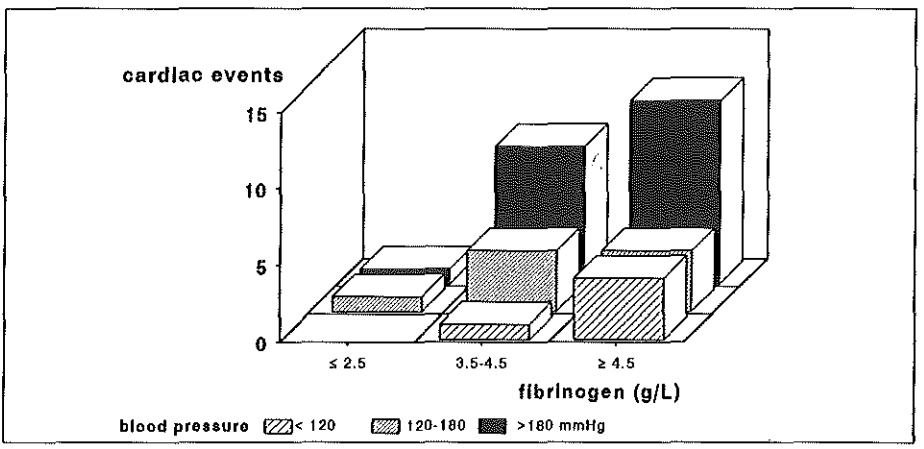

Figure 4. Relation between incidence of coronary events (incidence/1000 patient years), plasma fibrinogen levels and systolic blood pressure in the Göteborg Study[16]

MEASUREMENT OF PLASMA FIBRINOGEN LEVELS

The measurement of fibrinogen levels can be based on several different principles[46,47]. The first group is formed by the clotting rate assays, that give a functional fibrinogen level[48-50]. The second group of assays measures the amount of protein that can be clotted with thrombin[50-54]. The third group are the methods based on heat- and salt precipitation of fibrinogen[55]. Finally, there are the immunological methods[56,57], in which the interest has grown since the introduction of specific monoclonal antibodies that will make it possible to assay specific fractions of fibrinogen[58].

In studies where the different fibrinogen assays are compared, the agreements between the methods were acceptable[59,60]. However, because of the different characteristics of the assays it is expected that the different assays are not equally valuable for the use of fibrinogen as a risk indicator. This needs further attention in the future and has not been addressed systematically.

VARIATIONS IN PLASMA FIBRINOGEN LEVELS

physiological variation

The plasma levels of fibrinogen are related to several environmental, life-style and physiological factors (table 2). Smoking[61-66], age[67,68], gender[69,70], body weight[71], race[72], stress[71,72,75], social class[73,74,76], alcohol intake, season[77,78], exercise[79],

pregnancy[80], contraceptives[70,81,82], and menopause[70] have repeatedly been shown to affect the fibrinogen levels[overview: 69,83,84].

acute phase reaction

A part of the human defence system against tissue injury or infection is a change in the synthesis rate and possibly also the clearing rate of a group of proteins, named the acute phase proteins. Plasma fibrinogen levels can increase two to threefold as the consequence of an acute phase reaction, C-reactive protein can increase more than thousandfold[85]. Albumin and transferrin are two negative acute phase proteins, whose synthesis is decreased by the acute phase reaction. These acute phase induced changes in the synthesis rate are mainly the result of increased concentrations of circulating interleukin-6, interleukin 1ß and tumour necrosis factor α[86,87].

Atherosclerosis has been suggested to be an inflammatory process of the vascular wall[88]. Because fibrinogen is an acute phase protein the high plasma fibrinogen levels are suggested to be a reflection of vascular damage. This theory of inflammation as a mechanism in coronary heart disease is also indicated by the results of the ECAT Angina Pectoris study where both fibrinogen and C-reactive protein were risk indicators for a cardiac event within two years in patients with angina pectoris. Fibrinogen and C-reactive protein were strongly correlated to each other in this study, which strengthens the role of the acute phase reaction. In the Framingham Study an association has been described between cardiovascular risk and white blood cell count which also suggests a role of the inflammatory process[89].

genetic predisposition

The genetic contribution to the plasma level of fibrinogen is calculated to be between 20%[90,91] and 50%[92]. It is now becoming clear that an individual's response to environmental changes is also partly determined by genetic variation[90,93,94]; it has already been indicated that the increases in fibrinogen levels by smoking is associated with the genetic genotype[95,96].

chronic disease related increase

It has already been mentioned above that plasma fibrinogen levels are increased in patients with vascular disease. Also in hyperlipidaemic patients increased plasma fibrinogen levels are observed[97,98]. After surgery or as a result of other traumatic events the fibrinogen levels increase, probably as a result of an acute phase reaction.

FIBRINOGEN DECREASE BY DRUGS

Several drugs have been reported to reduce plasma fibrinogen levels, in addition to their main activity[review: 100]. Fibric acid derivatives and ticlopidine are two examples of such drugs which have been studied.

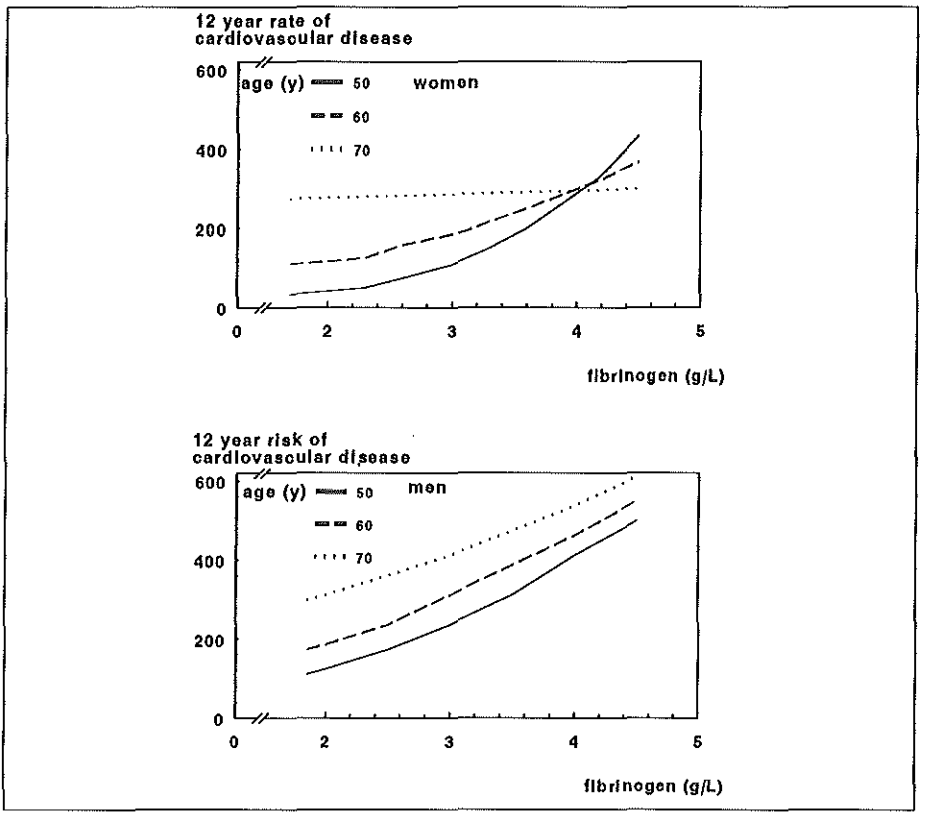

Figure 5. Risk of cardiovascular disease by fibrinogen level and age, 12-year follow up, Framingham Study[17]

Fibrates

In patients with high blood lipid levels an increased plasma fibrinogen level has been repeatedly observed[97,98]. When these patients were treated with the lipid lowering fibrates[91,99-108], the fibrinogen levels decreased to normal levels. Fibrates are a class of hypolipidemic compounds that effectively reduce plasma triglyceride and cholesterol levels and in many instances increase plasma high density lipoprotein levels[overview: 109]. There are several mechanisms responsible for the lowering of cholesterol and triglycerides, among which an inhibition of liver acetyl CoA carboxylase, the rate limiting enzyme in the synthesis of fatty acids, and an increase of the activity of lipoprotein lipase, which promotes the catabolism of plasma triglycerides[110]. Other lipid lowering drugs, the vastatins, have not been shown to be effective in lowering the fibrinogen level[104]. This suggests that the fibrinogen lowering is not directly coupled to the lipid lowering effect. By what mechanism fibrinogen is lowered by the fibrates needs to be further studied and the role of the acute phase mechanism or genetic factors need to be investigated.

Table 2. Variations in fibrinogen synthesis

physiological factor	effect on the plasma fibrinogen level
age	+ 3 to + 10% / 10 years[18,24,64,65,67-70,135,136] + 3 to + 5% / 10 years in non-smokers[70,135] + 8 to + 15% / 10 years in smokers[70,135]
smoking	no effect to + 33% in smokers[64,68-70,135,137,139] no effect in age < 35 years[70,135] 8% to + 14% in smoking men[64,69,137] 3% to + 7% in smoking women[64,69,137] dose dependent relation with sigarettes/day[44,64,70]
alcohol	- 0.8% with 10g/day alcohol[135] - 6% in men with <20 g/day alcohol[137] - 3% in women with <20g/day alcohol[137]
body mass	+ 14% in highest tertile[69] + 0.17% / (kg/m²)[135] + 12% in highest tertile in men[139] + 20% in highest tertile in women[139]
gender	+ 3% to +20% in women[68,135,137] no effect in non-smoking women[61,135] + 20% in smoking women[135]
race	- 16% in Japanese men compared to Caucasian[72] + 5% in black men, compared to white[44,91] + 7% and +9% in black women, compared to white[42,91]
stress	+ 1.3%[135] and +15%[74] with higher working stress + 8% after stress test[75]
season	+ 5% to + 38% in the cold months compared to the warm[77,78]
contraceptives	no effect[68,138], -2%[70] and + 7%[69] in pill users
physical exercise	no effect to -13%[79,139] no effect in persons <30 years
menopause	no effect of menopause[69] + 5%[96] and + 10%[70] in postmenopausal women without hormone replacement therapy[70]

Ticlopidine

Another drug that has been shown to decrease the fibrinogen levels is the platelet aggregation inhibitor ticlopidine. This drug strongly inhibits the ADP-induced aggregation of blood platelets[111-114]. The plasma fibrinogen levels were increased in patients with vascular diseases, and they could be normalized with ticlopidine[115-120, overview 121]. This effect might be associated with recovery of the disease since in patients with intermittent claudication the walking distance improved.

No mechanism has yet been identified, but it might be possible that recovery of the disease is accompanied by a reduction of the acute phase reaction. Another possible mechanism is a reduction of the level of fibrin(ogen) degradation products as the result of the inhibition of platelet aggregation. Since fibrin(ogen) degradation products are known as stimulants of the fibrinogen synthesis in the liver[121], a reduction of their levels may result in a decrease of the fibrinogen synthesis. Until now, it has only been recognized that there is an effect of ticlopidine on fibrinogen levels in patients with vascular diseases, but there have not been studies investigating the mechanism.

GENETIC CONTRIBUTION TO FIBRINOGEN LEVELS

The three chains of fibrinogen are encoded by different genes, which are located adjacent to each other on the long arm of chromosome 4[122,123]. The genes for the α and γ chain are directed in the same direction and are transcribed toward the ß gene. The ß gene is transcribed in the opposite direction (figure 6). The complete genomic DNA sequence for all three genes has been elucidated[124-126].

Several restriction fragment length polymorphisms (RFLP) in the genes for the three chains of fibrinogen have been described (figure 6)[90,127-130]. Fowkes et al reported a higher frequency of the rare allele of the *Bcl*1 RFLP of the Bß chain in patients with peripheral arterial disease[131]. The RFLP of the ß-fibrinogen gene are associated with the plasma fibrinogen levels in a number of studies[90,95,130,132], but not in all[96,131,133]. No significant associations were found between plasma fibrinogen levels and RFLP of the α and γ chains. In the Caucasian populations that have been studied until now, a strong linkage disequilibrium between the polymorphisms of the ß and α chain has been observed[127,129,132].

Interaction between environmental factors and the effect of genetic variation is observed in determining differences in biochemical and physical characteristics in healthy individuals[90,95]. DNA polymorphisms might also predispose to clinical disorders, like cardiovascular disease since the genetic-environmental interactions may cause some individuals to show large fluctuations while others in the same environment show small fluctuations. The identification of these genetic-environmental interactions might be important in identifying individuals that are prone to develop high plasma levels, and therefore a higher thrombotic risk, after a relatively small stimulus.

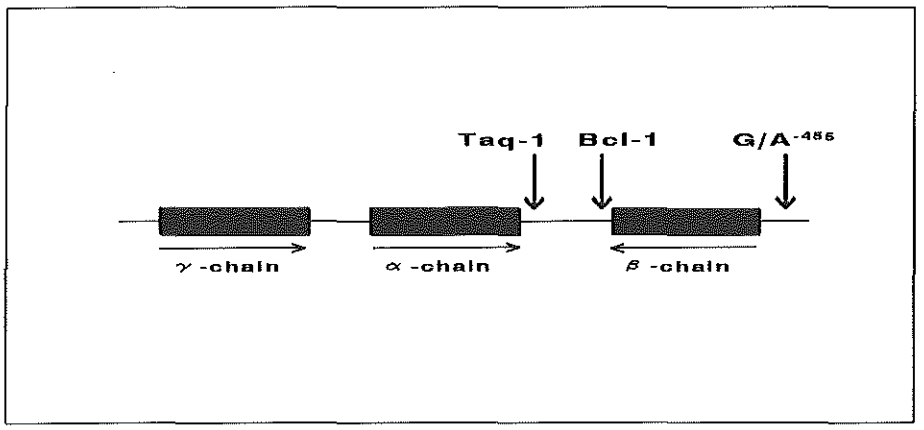

Figure 6. Structure of the genes for the Aα, Bß and γ-chains of fibrinogen with the locations of the restriction fragment length polymorphisms.

AIM OF THIS THESIS

Several epidemiological studies have identified elevated plasma fibrinogen levels as a risk indicator for cardiac events and this thesis concerns studies on the background of this relation.

Measurement of fibrinogen (Chapters 1-3)

Of primary importance when studying risk indicators in epidemiological or clinical studies are the characterization and standardization of the assays that are used. For fibrinogen, there now is a plasma standard available, but standardization of the assay method has not yet had much attention. One factor that will influence the fibrinogen assays is the large number of different molecular forms of fibrinogen normally found in the circulation. Our aim in the first part of this thesis was to evaluate the effect of variation in fibrinogen forms on the different assays.

The acute phase reaction and fibrinogen (Chapters 5-10)

The acute phase reaction is known to be a main mechanism for elevation of fibrinogen levels in blood. Major injury can result in 2-3 fold increases of fibrinogen levels. A low to moderate grade of an acute phase reaction is a candidate mechanism of elevating fibrinogen also for prolonged periods, and may for instance be a reflection of the inflammatory state of the vascular wall. The acute phase reaction may also be (part of) the mechanism by which fibrinogen is influenced by smoking or by medication (e.g. vitamin E, fibrates and ticlopidine).

The aim of this part of the study was to estimate the role of the low grade acute phase reaction in the regulation of fibrinogen levels by evaluation in cross-sectional

and intervention studies the association of fibrinogen with the well-known acute phase protein, C-reactive protein.

Modification of plasma fibrinogen levels by medication (Chapters 7-10)
Plasma fibrinogen levels are known to be modified by medication, for instance by fish oil plus vitamin E, fibrates and ticlopidine. However, the mechanism of it has not yet had much attention. Therefore, the aim of this part of the study was to elucidate the mechanism of fibrinogen lowering by medication:
1) to elucidate the contribution of the low grade acute phase reaction to fibrinogen lowering by medication
2) to study the association between the effects of medication on fibrinogen and fibrin(ogen) degradation products known to be involved in regulation of fibrinogen synthesis.
3) to study the contribution of genetic heterogeneity of fibrinogen to fibrinogen lowering by medication in order to determine whether it is possible to identify a group of patients that will benefit more from fibrinogen lowering therapy.

Genetic polymorphisms and fibrinogen (Chapters 6,8,11,12)
There is a difference in risk for cardiac events among individuals. This difference is partly genetic and might involve polymorphisms in the fibrinogen genes. These polymorphisms are associated with differences in the regulation of the fibrinogen levels. In this part of the thesis we studied whether the relation between polymorphisms and plasma levels of fibrinogen is different in groups of individuals with a different risk for an acute myocardial infarction, e.g. men and women, Inuit and Caucasians, patients with symptomatic coronary heart disease and healthy volunteers.

There is also a difference in the allelic frequencies of these polymorphisms in patients with peripheral arterial disease with an increase in the frequency of the rare allele that codes for higher fibrinogen levels in healthy individuals. We hypothesized that this might more generally be the case in vascular disease. This hypothesis was studied in groups with different risk of cardiac events, namely in Caucasians and Inuit, and in patients with symptomatic coronary heart disease and healthy volunteers.

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CHAPTER 1

CRITICAL EVALUATION OF FIBRINOGEN ASSAYS

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INTRODUCTION

Fibrinogen is a soluble plasma glycoprotein that is present in normal human plasma at a concentration of 2-4 g/L. In pathological states it can fluctuate from approximately 0 to above 10 g/L. It is composed of two pairs of three polypeptide chains (Aα, Bβ and γ) that are interconnected by disulphide bridges. The aminoterminal segments of the six chains form a central domain from which the fibrinopeptides A and B protrude[1]. During clotting thrombin cleaves off the fibrinopeptides A from the Aα chains which leads to a conformational change of the molecule and, via intermediate stages, eventually leads to polymerisation into the insoluble fibrin fibres. Somewhat delayed also the fibrinopeptides B from the Bβ-chain are cleaved off, which initiates the lateral polymerization of the fibrin fibres. The fibrin network is normally constructed of fibres that are about one μm in diameter, corresponding to a width of several hundred molecules. Only 0.3% of the mass of a blood clot is made up of fibrin, about 80% of the volume is liquid and the remainder includes blood elements like platelets and red and white blood cells.

Fibrinogen concentrations are subject to considerable biological variation. Fibrinogen is one of the major acute phase reactant proteins, and increased hepatic synthesis occurs as a physiological response to inflammation and tissue necrosis[2]. Altered protein catabolism due to intravascular consumption may also influence circulating plasma concentrations. The plasma fibrinogen concentration rises 5-7% per ten years of age[3-6], and a gender difference is described in both young and old groups[3,5]. Fibrinogen levels are also correlated with smoking[overview: 7], body weight[4,6] and degree of coronary stenosis[6,8-12]. In women, the fibrinogen levels rise after the menopause. The effects of hormone replacement therapy are inconsistent[13]. The plasma fibrinogen level is increased in users of combined oral contraceptives[14] and during pregnancy[15].

At least three molecular forms of fibrinogen have been identified in plasma[16-23]. Fibrinogen is synthesized in the high molecular weight form (HMW), with two intact carboxyl ends of the α-chain. In plasma two groups of degraded forms of fibrinogen can be distinguished. One group has one degraded Aα-chain carboxyl end and is called the low molecular weight (LMW) form, in the other form both Aα-chains are degraded and this is called the LMW' form. In a normal, average plasma 70% of fibrinogen is HMW, 26% is LMW and only 4% is LMW'. The LMW and LMW' forms are heterogenous due to the different length of their constituent Aα-chain. The enzymes responsible for the degradation have not yet been identified[24].

There are indications of a pathological significance of the occurrence of relatively high levels of HMW fibrinogen. Treatment of patients with acute myocardial infarction (AMI) with streptokinase results in a radical consumption of their fibrinogen. Fibrinogen is then newly synthesized in the HMW form[25] which has an increased clotting capacity. This procoagulant tendency might explain the

observation that in patients with the highest HMW-fibrinogen levels more reinfarctions are observed. For the management of AMI patients it is therefore advisable to measure fibrinogen levels with a method that is more sensitive to HMW fibrinogen than to the other fibrinogen forms, because such an assay may give the best estimation of the thrombotic risk. Promising methods for this purpose are in development and will be discussed. A prethrombotic state due to increased HMW-fibrinogen also rapidly develops after hip surgery; after MI not treated with streptokinase; or after any major trauma, where much HMW-fibrinogen is synthesized while the LMW only increases much later[26].

Usually plasma fibrinogen levels are measured in the management of consumptive coagulopathies. During the last decade fibrinogen levels have been studied in other diseases. An association between fibrinogen levels and the severity of atherosclerosis has been repeatedly observed[8-12,27]. Also, the results of the Northwick Park Heart Study[28] and several other studies in healthy populations[6,29-35] have shown that elevated plasma fibrinogen levels are associated with an increased risk for ischaemic heart disease (IHD). Although methods for fibrinogen quantification differ from study to study, a significant association between plasma fibrinogen levels and risk for cardiac events was found in all of them.

There are several different principles on which the measurement of fibrinogen can be based[36,37]. First, there are the clotting rate assays, that give a functional fibrinogen level. The second group of assays measure the amount of protein that can be clotted with thrombin. Furthermore, there are the methods based on heat- and salt precipitation of fibrinogen. Finally there are the immunological methods for which there is growing interest since the introduction of specific monoclonal antibodies.

Each of these methods will be susceptible in its own way to sources of variation. The expression of the three forms of fibrinogen mentioned above is likely to be different in clotting assays, because the thrombin clotting times of the three fibrinogen forms differ[18,21,22,38]. The different fibrinogen forms also produce clots with a different fibre structure[38], which might influence methods where the end point depends on the clot structure (Ellis and Stransky, Prothrombin Time-derived (discussed later)). In this paper, we will focus on the currently used methods to determine fibrinogen, and on the methodological aspects including specificity, sensitivity, interfering substances and calibration.

CLOTTING RATE BASED ASSAYS

The assays that are most often used in a routine laboratory are based on measuring the time it takes to clot plasma after the addition of excess thrombin[39]. The concentration of fibrinogen is expressed as a function of the clotting time. By applying different dilutions of the plasma, it is possible to measure fibrinogen levels between 0.5 and 10 g/L. It is also possible to use snake venom (Arvin) or reptilase

in clotting rate assays instead of thrombin. The latter enzymes function more specifically than thrombin because they will only split off the fibrinopeptide A[40].

It has to be remembered that this method yields a functional fibrinogen level, and not an absolute concentration of fibrinogen. As will be discussed below, the fibrinogen levels found with this method are influenced by forms of fibrinogen that have an altered fibrinogen clotting rate.

An additional point of importance is that the effect of certain factors (such as the degradation products of fibrin and fibrinogen (FDP); heparin) on the clotting rate assays may be different from the effect they have on the clotting rate in vivo.

Factors Influencing the Clotting Time

Fibrin degradation products (FDP) have a strong anticoagulant effect and affect the polymerisation times of fibrinogen solutions. This effect is most pronounced for fragments X and Y[41], but fragment D also has anticlotting properties[42]. Increased FDP levels are therefore expected to delay clotting in clotting rate based assays and as a consequence yield spuriously low fibrinogen levels. However, no effect of FDP ($< 190\ \mu g/mL$) on the Clauss assay was found in samples containing normal fibrinogen levels. Only in samples with fibrinogen levels below 1 g/L did the high FDP levels interfere[43,44]. This might be explained by a difference in the type of FDP in that situation[45,46]. Increased fibrin monomer levels in the patient are expected to cause spuriously high levels through reduction of the clotting time.

Also, an alteration in the distribution of the three fibrinogen forms (HMW, LMW and LMW') in plasma may affect the Clauss assay. The LMW and LMW' forms have a prolonged thrombin clotting time[16,18,22] and therefore changes in the ratio of the three fibrinogen forms will affect the thrombin clotting time and result in different values without differences in molar concentrations. The time required for the transformation from HMW- and LMW-fibrinogen to fibrin by thrombin is similar, but the rate of polymerization is reduced for LMW compared to HMW and is even lower for LMW'. Also, the different heights of the polymerization curves indicate different fibril length and thickness. Clots from HMW fibrin have thicker and shorter fibrils than those from LMW fibrin[38]. A more compact clot has a higher turbidity and firmness than a looser clot and this influences the turbidimetric and mechanical end point measurements.

Newly synthesized fibrinogen is more phosphorylated, particularly in fibrinopeptide A at serine 3, than normal fibrinogen[25,47,48]. The fibrinogen found after streptokinase treatment has 66% of the possible sites phosphorylated whereas normal fibrinogen has only 30%[25]. Reports on the effect of phosphorylation on clotting are conflicting. Witt et al[47] find no effect, but both Hanna et al[48] and Reganon et al[23] describe that phosphorylation increases the release of FPA, which they ascribe to an enhanced binding of thrombin to the phosphorylated fibrinogen. If indeed phosphorylated fibrinogen clots faster, this further emphasizes that not all

forms of fibrinogen show the same clotting behaviour.

Another component that influences the clotting rate 'assay is heparin, which in patients' plasma (concentrations 0.03 - 3 IU/mL) gives apparent increases up to 20% of the estimated plasma fibrinogen level in the Clauss assay[44]. This artefact might hinder the use of the fibrinogen level to predict the thrombotic risk. Polybrene (1,5-dimethyl-1,5-diazaundeca-methylene polymethobromide) can be used to neutralize heparin in the sample[49].

Little is known about the disturbance by lipids in the clotting rate assay. No effect was found when plasma from healthy volunteers was supplemented with plasma with increasing levels of β-lipoprotein[50]. In assays based on a turbidimetric end point, lipemic and haemolytic plasmas may disturb the measurements.

In patients with cirrhosis of the liver, the fibrinogen molecules have an increased sialic acid content. This causes prolonged clotting times with thrombin[51,52]. After removing the sialic acid from the fibrinogen molecules with neuraminidase, the clotting times normalize. It has been reported that during inflammation the glycosylation of the acute phase proteins is altered[53,54]. As fibrinogen is an acute phase protein, this may result in defective functional fibrinogen levels[55] in patients with an increased inflammatory status.

One freezing-thawing step does not give levels different from those found in fresh plasma[56].

Accuracy

In a quality control study performed in 18 different laboratories, the Clauss method was the most accurate haemostatic assay[57], reporting a coefficient of variation (CV) of 3.3% between duplicate measurements in the same series, a between day CV of 4.7% and a between centres CV of 5.3%.

Use as Routine Assay

The clotting rate based methods are the most frequently used in routine laboratories, because they measure the functional fibrinogen levels and also because they are fast, cheap and have little variation[58]. A major problem, especially in the assessment of thrombotic risk, is the discrepancy between the different laboratories. This will be discussed below.

Comments

In clotting rate assays the velocity of the fibrinogen to fibrin conversion can be enhanced by the presence of dextran and calcium chloride. Dextran increases the turbidity of the fibrin polymer at subnormal plasma concentrations of fibrinogen[49].

Calcium ions are not essential for fibrinopeptide release nor for polymerisation of fibrin monomers, but they greatly accelerate the aggregation of soluble fibrin

monomer[59,60]. Calcium also makes fibrinogen more resistant to thermal and acid denaturation, and to digestion by plasmin[61].

CLOTTABLE PROTEIN BASED ASSAYS

A. ASSAYS BASED ON CLOT MASS MEASUREMENT

Methods that determine the amount of clottable protein use the unique property of fibrinogen that it becomes a clot after the addition of thrombin. After *complete* clotting of the fibrinogen, the non-clottable proteins trapped in the fibrin network must be washed out before the weight or protein content of the clot is determined.

The methods are time-consuming, because both complete clotting and thorough washing out of the non-clottable proteins take time.

Compared to the clotting rate method, measurement of clottable protein is more representative of the molar concentration of fibrinogen, because fibrinogen forms with prolonged clotting times are also measured.

Interfering Factors

Arnesen et al[62] found only a minor increase of up to 1 g/L early FDP in the measured amount of total clottable protein. This is confusing, because the fragment X is an early FDP that still has clotting capacity. Hoffmann et al[46] describe a marked increase of the apparent fibrinogen levels when $\pm$ 2.5 g/L fragment X is added to pooled plasma, a small increase when fragment Y is added and no effect from fragments D and E. These concentrations are only obtained after thrombolytic therapy. Interference by heparin, haemolytic plasma or increased sialic acid content of fibrinogen has never been described and is unlikely.

Fat is trapped in the fibrin clot formed from blood collected during alimentary lipemia. The error in the determination of fibrinogen, due to trapped fat, is small provided correction is made for "extraneous absorption"[63,64]. Lipid containing plasmas that have been lyophilized cannot be used in a gravimetric assay, because clotting is disturbed by the lipids. We observed this repeatedly with this method, but not with other fibrinogen assays. One freezing-thawing step of the plasma yields the same levels as in fresh plasma[57].

Accuracy

The total clottable protein methods have a 4.6% variation in duplicate measurements[65]. The method is less accurate with low fibrinogen levels, because weak clots are formed that are difficult to handle.

Use in Routine Assays

This assay is hardly ever used in a routine laboratory, because it is very labour-intensive. However, two epidemiological studies, the Northwick Park Heart Study[28]

and the Framingham Study[29] employed this method.

Comments

Originally, the final step was drying the clot and weighing it (gravimetric assay)[66], as in the Northwick Park Heart Study[28]. Alternatively, the fibrin clot can be dissolved in urea and the protein content is then calculated from the absorbance at 279 nm[63]. To correct for non-protein contamination, such as lipids, the absorbance at 315 nm was subtracted from the absorbance at 279 nm[64]. Astrup et al[67] dissolved the clot in 2.5 N NaOH in a boiling water bath and then measured the protein content with sodium carbonate and phenol reagent, obtained thereby a variance of less than 1%. Ratnoff and Menzie[68] dissolved the clot in 1 N NaOH and measured the protein with the Biuret test.

B. ASSAYS BASED ON CHANGE IN TURBIDITY

The Ellis and Stransky method[69] is also based on complete clotting of fibrinogen by thrombin, but the quantitation is based on the increase in opacity (turbidity) at 470 nm, due to the formation of fibrin. This method gives additional information on kinetics of fibrin formation and fibrinolysis which can be obtained from the continuous monitoring of the absorbance.

Nowadays, many laboratories use automatic coagulometers. These machines often derive the fibrinogen levels from the prothrombin time (PT)[70,71]. This method is based on the Ellis and Stransky method because it measures increased optical density after clotting. It yields good results when the prothrombin time is relatively normal[49,70,71].

Accuracy

The Ellis and Stransky method has a 5.6% variation between duplicate measurements[65]. The PT derived fibrinogen assay gives a same day CV of 5.6% and a different day CV of 6.5%[49].

Use as Routine Assay

Measurement of PT-derived fibrinogen levels is used frequently nowadays, because this assay can be performed on automatic coagulatometers. Because this test can be performed in combination with the PT, it is relatively cheap.

Comments

Changes in the fibrin clot structure will affect the turbidity of the clot. Because the different molecular weight forms of fibrinogen and the degree of phosphorylation of fibrinogen affect clot structure, these factors influence the measurement of fibrinogen with this method. The presence of calcium during clot formation also modifies the clot structure[72].

The effects of lipids on turbidity and thereby on the PT-derived measurement can be corrected for with the newest apparatus. In patients on oral anticoagulants, results do not significantly differ from those in the Clauss assay[71]. Bilirubin (< 15 mg/dL) and haemoglobin (< 150 mg/dL) have no disturbing effects[70,72]. An effect of the clottable, early FDP has not yet been described, but is theoretically expected.

Unless clot formation is complete, spuriously low levels are obtained. This might be a problem in patients receiving heparin; therefore it is usually neutralized with polybrene[69]. One freezing-thawing step does not alter the measured fibrinogen levels[57].

PRECIPITABLE PROTEIN BASED ASSAYS

Fibrinogen can be precipitated by heating[65,73] or with salt[74-76]. These methods determine both the clottable and the non-clottable fibrinogen.

The heat precipitation method measures the amount of protein that precipitates at 56°C. It is very sensitive to assay conditions. An increase in the temperature of one °C leads to a 12% error due to co-precipitation of other proteins. The assay is also sensitive to the pH in the system.

Sulphite[74,76] and ammonium sulphate[75] have been used in quantitative assays because they specifically precipitate fibrinogen. Other salts have the disadvantage that they co-precipitate many other proteins. The best results are obtained with sodium sulphite because it cleaves the disulphide bonds of fibrinogen specifically and accurately[76].

Accuracy

If the assay conditions are well controlled the heat precipitation methods gives coefficients of variation of 3.9%, precipitation with sodium sulphite 7.1% and precipitation with ammonium sulphate 5.4%[65]. Because of the many possible technical errors and interferences, these methods are generally considered unreliable[65].

Use in Routine Assays

As the precipitation methods are prone to errors they are not routinely used.

Comments

Low levels of fibrin monomers (< 0.1 g/L) do not influence the precipitation of fibrinogen by heat or by salt. Lipids in the sample do not influence the turbidimetric measurement when sufficient care is taken of the cooling procedure in the heat precipitation method[73].

If the assay conditions are well controlled the heat precipitation method is not influenced by moderate haemolysis, heparin, fibrin monomers, hyperlipemia and bilirubin[73]. There is however an effect of early fibrin(ogen) degradation products (X

and Y), but not of D and E[36], if they are present in large amounts (>15% of the fibrinogen level).

IMMUNOLOGICAL ASSAYS

Immunological methods measure the fibrinogen related antigens, rather than the amount of fibrin that can be formed. They include immuno-precipitation methods like radial immunodiffusion (RID)[77] or rocket immuno electrophoresis[78] where polyclonal antibodies are applied. These precipitation assays need polyclonal antibodies, which unfortunately crossreact with fibrin degradation products, fibrinogen degradation products and soluble fibrin. The enzyme immune assays (EIA) can use either polyclonal or specific monoclonal antibodies (e.g. specific to HMW+LMW fibrinogen[79]). The use of fibrinogen-specific monoclonal antibodies specifically determines fibrinogen and gives an assay system that has a lower detection limit than clotting rate or total clottable protein methods. The disadvantage of EIAs is that they are more time-consuming than clotting rate assays, although for instance the recently developed HMW+LMW fibrinogen EIA[79] takes less than 1 hour (apart from sample preparation time).

The specificity of the monoclonal antibodies used in the HMW+LMW fibrinogen assay has the advantage of measuring specifically those molecular weight forms of fibrinogen that have the highest clottability and therefore are possibly associated with the highest thrombotic risk.

Interfering Factors

It depends on the specificity of the antibody[80] whether FDP are measured in immunological assays. Usually, polyclonal antibodies crossreact with FDP, but the FDP levels have to be very high to noticeably influence the fibrinogen levels. In patients treated with streptokinase, apparently normal fibrinogen levels are found in radial immuno diffusion assays due to the high level of FDP. The clotting rate and total clottable protein assays would suggest a marked reduction of fibrinogen in these patients[46].

The influence of the different forms of fibrinogen also depends on the specificity of the antibody. The recently developed HMW+LMW fibrinogen EIA[79] uses antibodies that do not detect the LMW' form of fibrinogen. Most polyclonal antibodies are not expected to react differently to the three fibrinogen forms. However, as stated above they will cross-react with FDP.

Effects of lipids, heparin, haemolysis, phosphorylation and sialic acid on the measurement have not been reported, but are unlikely. One step of freezing-thawing does not influence the RID assay[56]. In the rocket immuno-electrophoresis the addition of EDTA is required.

Accuracy

When RID is performed on blood plasma that has been collected in heparin, the fibrinogen levels will be 10.7% higher than in sodium citrate plasma, whereas the concentrations in EDTA plasma are 10.3% higher than the heparin plasma[81]. This difference may partly be due to the dilution effect of plasma in citrate. Heparin is known to inhibit the formation of antigen-antibody complexes in immunoassays.

The same day CV of the HMW+LMW EIA was between 3% and 7%, depending on the plasma dilution used, the different day CV was between 2.8% and 7.7%[79].

Use as Routine Assay

The traditional immunological methods were very time consuming, a radial immunodiffusion assay takes three days. In most routine laboratories the immunological methods are mainly used when the presence of dysfibrinogenaemia is suspected.

The earlier EIAs took one day because they used relatively long incubation periods. The new HMW+LMW fibrinogen EIA only takes one hour, from the first incubation to the end of the staining reaction[79], which makes it suitable for routine use.

COMPARISON BETWEEN METHODS AND BETWEEN LABORATORIES

Standards

To identify an increased fibrinogen level as a potential cardiovascular risk factor, it is essential that a correct standard is used. Furlan[82] reported that there were giant differences between the indicated levels of commercial fibrinogen standards and the actual levels. To overcome the problem of differences between standards, it is desirable to establish the potency of a standard after a collaborative study, by agreement with the participants. For a long term reference standard the stability has to be checked. An international standard for fibrinogen is now available[64].

Comparison Between the Different Methods

In various studies the available fibrinogen assay methods have been compared. In general, there is good correlation between different methods when rank order is considered (i.e. high levels with one method yield high levels in another and, similarly, for intermediate or low levels[73,83]. There is also a high degree of consistency in different studies of the association between fibrinogen levels on the one hand and physiological factors (e.g. smoking, age, body mass index (BMI)) and risk of arterial disease on the other.

One study compared the clotting rate[39], clottable protein (PT-derived) and immunological (RID) methods in plasma samples from healthy individuals and patients with acute pneumonia and postoperative patients with increased fibrinogen

levels[57]. Palareti et al[57] found large discrepancies between the results obtained with the same samples applying different methods when they used the standard included in the different test kits. When they calibrated all methods to the same reference, there was satisfactory agreement, i.e. in the relation between different methods and the average of the methods. The most accurate is the Clauss method, but all methods had an inaccuracy below 8%. Although agreement in the group as a whole was acceptable, differences in agreement between the healthy individuals and the patients was not studied. Less agreement might be observed in patients with increased levels of HMW- and phosphorylated fibrinogen.

In another study Exner et al[65] compared the Clauss method, total clottable fibrinogen (protein and clot opacity), salt ($(NH_4)_2SO_4$ and Na_2SO_3) and heat precipitation methods to measure the plasma fibrinogen levels in patients with various diseases, e.g. consumptive coagulopathy and hepatic cirrhosis. The correlation of the total clottable protein level with the other methods was satisfactory (>0.91), but the results of salt precipitation methods were inaccurate at low levels (below 1 g/L) and gave spuriously high values in jaundiced samples. The Clauss method had the best reproducibility.

Comparison Between Laboratories

The differences between laboratories are much larger than those between different methods in one laboratory. In 1993, the results of the College of American Pathologists (CAP) Proficiency Testing Program in 2250 laboratories from 1988 to 1991 were published[84]. Poor interlaboratory reproducibility was described. The authors ascribe this to both instrument and reagent variables. The absence of an international standard for plasma fibrinogen was believed to be the major reason for the reagent variation.

In the European Concerted Action on Thrombosis (ECAT) project the patients' plasma samples were not analyzed centrally and therefore the study included a quality control programme. The CV between the 16 participating European laboratories was assessed as 5.3% for the Clauss assay, which is only slightly higher than the mean different day CV of 4.7%[57]. These variation levels remained constant after five quality assessment exercises[85].

PHYSIOLOGICAL VARIATION

We have recently reported a longitudinal study of healthy volunteers[86] which found that the longitudinal variation for fibrinogen within an individual (physiological + methodological variation) was 18%. Because the methodological variation was very small, its effect on this within-individual variation was negligible. Similar within-individual variations have been described in healthy volunteers by Thompson[87] and Marckmann[88]. Because the variations within individuals are comparable to the

variation between individuals, it will be necessary in order to classify groups with different thrombotic risks to study large groups or to calculate habitual levels from multiple samples.

Pathology

An overview of fibrinogen levels in different diseases and of acquired dysfibrinogen is given by Dang et al[55]. Hereditary dysfibrinogenemia may yield decreased levels with functional fibrinogen assays and normal or slightly decreased levels of fibrinogen with immunological assays. Most of these dysfibrinogens are either asymptomatic or cause a bleeding tendency. However, several families have now been identified in which the dysfibrinogen yields apparently low levels in functional assays in association with thrombophilia[overview: 89].

In epidemiological studies for haemostatic indicators of cardiovascular risk, the gravimetric assay[28,31,33,90], clotting assays[32,35,91,92] and immunologic assays[30,33,34] work equally well.

CONCLUSION

In this paper we have described the major categories of assays. The clotting rate assays give a functional fibrinogen level, are reproducible, fast, cheap and, in normal circumstances, not very easily affected by disturbing factors. The tests can be used in automatic coagulometers with turbidimetric end point assessment. The clottable protein assays also give fibrinogen levels based on the clotting capacity, but prolonged clotting is not detected by these tests. The advantage of the Ellis and Stransky derived methods is that they can easily be used in automatic coagulometers in combination with the prothrombin time.

A promising development in immunological methods is the introduction of the EIA that uses monoclonal antibodies specific for the HMW and LMW forms of fibrinogen. As these are the most clottable forms, this assay may eventually be valuable to assess thrombotic risk.

When using plasma fibrinogen levels to determine thrombotic risk, it is very important to have 1) a reliable method and 2) a good standard. When such a standard is used, the different methods give comparable results in healthy volunteers. Good comparability is also described for patients, but the evidence is less convincing. An international reference for fibrinogen is now available.

The value of the different assays to estimate thrombotic risk has not yet been described. Therefore, epidemiological studies are needed in which the value of fibrinogen as risk indicator is compared with different assays, to establish a standardized fibrinogen method.

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CHAPTER 2

THE SENSITIVITY AND SPECIFICITY OF SOME CLOTTING RATE AND IMMUNOLOGICAL FIBRINOGEN ASSAYS FOR HIGH, LOW AND LOW' MOLECULAR WEIGHT FORMS OF FIBRINOGEN

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ABSTRACT

Plasma fibrinogen levels are an important indicator for the risk of cardiac events. There is much diversity in the fibrinogen molecules that can be found *in vivo*. The methods that are available for the assessment of plasma fibrinogen levels have different specificities for these forms of fibrinogen, which suggests that they may not detect the cardiac risk equally well.

The heterogeneity of fibrinogen is partly explained by the degradation of the Aα chains, resulting different molecular weight (MW) forms, namely the high (HMW), the low (LMW) and the very low (LMW') forms that normally constitute 70%, 26% and 4%, respectively of the total fibrinogen in plasma. In this study we evaluated if and how these molecular weight forms influenced the results of fibrinogen assays, including clotting rate assays using either thrombin, peptidase or reptilase and an enzyme immuno assay (EIA) based on monoclonal antibodies an which detects fibrinogen that has both intact amino- and carboxyterminal ends of the Aα-chains.

The clotting rate assays showed complex clotting curves for the three forms at increasing concentrations. The sensitivity of the EIA for the HMW fibrinogen forms was found to be three times higher than for the LMW form, while as expected the LMW' form was hardly detected. It is therefore concluded that the fibrinogen assays need to be compared in prospective, epidemiological studies to select the assay(s) which give(s) optimal assessment of the risk of cardiac events.

INTRODUCTION

The plasma level of fibrinogen has been identified as a strong risk indicator for cardiovascular disease[1-8]. The mechanisms underlying this relation have not yet been identified. A possible mechanism might be that increased plasma fibrinogen levels will stimulate clot formation. Fibrinogen might also be directly associated with cardiac risk because of its important contribution to the plasma viscosity[9,10]; because fibrinogen stimulates the growth and migration of smooth muscle cells[11,12]; because it is involved in the aggregation of blood platelets[13] and because it is associated with plaque growth[14]. Another hypothesis is that atherosclerosis is due to a chronic inflammation of the vascular wall[15] and that this causes increased plasma levels of fibrinogen as a consequence of an acute phase response[16].

In the above mentioned epidemiological studies a variety of fibrinogen assays was applied, namely assays based on the clotting rate[5-8] or the amount of clottable protein[1,2,4] and immunological assays[3]. The association between the risk for cardiac events and the plasma fibrinogen level was observed with any of the methods. However, since each assay has its own characteristics and fibrinogen occurs in several different molecular weight forms[17] it is conceivable that the various assays will give different information and indicates that their value in risk assessment may vary. This suggests that it will be worthwhile to evaluate the fibrinogen assays for

optimal assessment of cardiac risk.

One of the reasons for molecular heterogeneity in the fibrinogen molecules is limited degradation of the COOH-terminal ends of the Aα chains, resulting in three molecular weight forms of fibrinogen in plasma[18-22]. Fibrinogen is assumed to be synthesized in the high molecular weight form (HMW), with both Aα-chains intact at the carboxyl-terminal ends. In plasma two degraded forms exist, one form in which one of the Aα-chains is degraded at the carboxyl end. This form is called the low molecular weight (LMW) form. In the other form both Aα-chains are degraded and this form is called the LMW' form. In a normal plasma 70% of fibrinogen is HMW, 26% is LMW and only 4% is LMW'[23]. The enzyme or enzymes, responsible for the degradation have not yet been identified, but analysis of the COOH-terminal end of the Aα-chain has excluded plasmin, gelatinase and trypsin[24,25].

The clotting behaviour of these three fibrinogen forms is different[18,20,22]. The thrombin clotting rate of the HMW forms is higher than that of the LMW forms. With the LMW' form the longest clotting times are observed. The difference in clotting times is more pronounced when reptilase is used[18]. It is expected that these different clotting characteristics may influence the functional fibrinogen assays that use thrombin[26] or reptilase when the ratios of HMW, LMW and LMW' differ from normal. The methods that measure the fibrinogen protein concentration, such as enzyme immuno assays with polyclonal antibodies will probably not be affected by a difference in clotting behaviour.

Moreover, these variations in characteristics of the three molecular weight forms suggest that their contribution to the cardiac risk might be different, and require a further definition of the specificity and sensitivity of assays for the three molecular weight forms of fibrinogen. The aim of this study was to define in more detail the sensitivity of various clotting (Clauss, peptidase and reptilase tests) and enzyme immuno methods (total fibrinogen, HMW+LMW fibrinogen) for measuring the different forms of fibrinogen and as a consequence for the measurement of total fibrinogen levels.

MATERIALS AND METHODS

Materials

Aprotinin (Trasylol®) was obtained from Bayer (Leverkusen, Germany), soybean trypsin inhibitor from Sigma (St Louis, MO, USA), diisoprophylfluorophosphate (DFP) and ß-alanine were obtained from Aldrich (Beerse, Belgium). Sepharose-lysine, Sepharose-heparin and Sepharose-6B-CL were obtained from Pharmacia (Woerden, the Netherlands). Acrylamide and bisacrylamide were obtained from BDH (Poole, England).

Buffers

0.15 M phosphate buffer contained 27 mmol/L ethylenedinitrilo tetraacetic acid

disodium salt (EDTA) and 20 KIU/L trasylol pH=7.45. Owren buffer contained of 0.029 mol/L sodium diethylbarbiturate, 0.126 mol/L NaCl, pH=7.35. Fibrinogen solvent consisted of 8 vol 0.3 mol/L NaCl, 1 vol Owren buffer and 1 vol 10 KIU/mL trasylol as described by Holm et al.[18].

Fibrinogen isolation

Nine volumes venous blood (250 mL) were collected of healthy volunteers in 1 volume sodium citrate (0.11 mol/L) with the protease inhibitors trasylol (400 KIU/mL), soybean trypsin inhibitor (1 mg/mL) and DFP (1 mmol/L) and immediately placed in melting ice. After centrifugation (30 min, 3000 g, 4°C) the plasma was collected and fibrinogen was isolated as previously described (27). Briefly, the plasma was diluted three times in phosphate buffer. The plasma was passed over a Sepharose-lysine column to remove the plasminogen. Then $(NH_4)_2SO_4$ was added to a final concentration of 25%. The precipitate was collected by centrifugation (20 min 3000 x g, 4°C), dissolved in the phosphate buffer and passed over a Sepharose-6B-CL column run in the same buffer. The fractions of the second eluded peak were pooled and precipitated with 50% $(NH_4)_2SO_4$. The precipitate was collected after centrifugation (20 min 3000 x g, 4°C), resuspended in fibrinogen solvent and subsequently dialysed against Owren buffer containing 0.011 M citrate. Finally, the fibronectin was removed by passing the fibrinogen solution over a Sepharose-gelatine column.

Alternatively, the fibrinogen was isolated using ß-alanine, essentially as described by Straughn[28]. Briefly, ß-alanine was added to a final concentration of 1 mol/L and incubated on ice for 30 min. The precipitate was removed by centrifugation for 30 min at 2000 x g, 4°C. To the supernatant ß-alanine was added to raise the final concentration to 3 mol/L, and incubated for 30 min on ice. The precipitated fibrinogen was collected by centrifugation for 20 min at 9000 x g, 4°C and dissolved in 154 mmol/L NaCl with 0.011 mol/L citrate. The fibrinogen was reprecipitated by adding ß-alanine to a final concentration of 3 mol/L and incubation for 30 min on ice. The fibrinogen was collected by centrifugation for 25 min at 2000 x g, 4°C and dissolved to a concentration of approximately 5 in Owren buffer with 0.011 mol/L citrate g/L, and then dialysed for 16 hour against the same buffer.

table 1. Precipitation fractions of fibrinogen.

fraction nr.	The expected main fibrinogen form in the fractions	Molecular weight
fraction 1	HMW-fibrinogen	340.000 Da
fraction 2	LMW-fibrinogen	305.000 Da
fraction 3	LMW'-fibrinogen	270.000 Da

Fractionation of molecular weight forms of fibrinogen by sequential $(NH_4)_2SO_4$ precipitation

The molecular weight forms of fibrinogen were separated as described previously[17]. Briefly, saturated $(NH_4)_2SO_4$-solution was added to a fibrinogen solution to a final concentration of 19% (v/v) and incubated for 10 minutes. The precipitate was collected by centrifugation (1200 x g, 10 min)(fraction 1) and resuspended 154 mmol/L NaCl in 1/3 of the original volume. The precipitate obtained between 22 and 24% $(NH_4)_2SO_4$ saturation was collected (1200 x g, 10 min.)(fraction 2) and resuspended in 154 mmol/L NaCl in 1/20 of the original volume; and finally the precipitate between 26 and 30% $(NH_4)_2SO_4$ saturation was collected by centrifugation (1200 x g, 10 min.)(fraction 3) and resuspended in 154 mmol/L NaCl in 1/40 of the original volume. The precipitates were resuspended in and dialysed against 154 mmol/L NaCl (table 1). The whole procedure was performed at room temperature.

Electrophoresis

SDS/polyacrylamide gel electrophoresis was performed according to the method of Laemmli, with resolving gels containing a gradient of 2.5 - 16 % (w/v) acrylamide and stacking gels of 2.5% acrylamide[29]. The individual chains of fibrinogen were examined on SDS/polyacrylamide gels under reducing circumstances, with resolving gels containing 10% (w/v) acrylamide and stacking gels of 5% acrylamide. After staining of the bands with Coomassie blue, the bands were scanned.

Fibrinogen assays

The fibrinogen concentrations in the purified fractions were estimated by the optical density at 280 nm, using the OD (1%, 280 nm) = 15.0, but we are aware that this factor might vary for the three forms of fibrinogen.

Total fibrinogen antigen was determined with an enzyme immuno assay (EIA) that uses a pool of rabbit anti human fibrinogen IgG's as catching antibodies[30] and peroxidase conjugated monoclonal antibodies against fragment DD (DD13)[31] as tagging antibodies.

(HMW+LMW)-fibrinogen levels were determined using an enzyme immuno assay (EIA)[32](Organon Teknika, Boxtel, the Netherlands). In this assay a monoclonal antibody against the intact carboxyl-terminal end of the fibrinogen Aα-chain is used as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain, including fibrinopeptide A (Y18) as the tagging antibody.

Clotting times of the three fibrinogen forms were determined using thrombin, according to Von Clauss[26], reptilase and peptidase according to the instructions of the manufacturer ((DIAMED, Morat-Murten, Switserland) in a dilution series of 0.1 - 0.5 mg/mL (concentrations determined by the total fibrinogen EIA). The clotting times were determined in the presence of 10% citrated plasma of a patient that is deficient in fibrinogen (functional and immunological levels <0.2 mg/mL)(kind gift

of Prof. Egbring, Marburg, Germany).

RESULTS

On SDS-PAAGE gels the bands of the fibrinogen forms were clearly different for HMW, LMW and LMW'- fibrinogen forms and showed the expected pattern (figure 1). Scanning of the density of the SDS-PAAGE gels, using the γ-band as an internal control (100%), gave a density of the Aα band in fraction 1 of 155% (SD 26%, n=4) while in fractions 2 the Aα band had decreased to 113% (SD 7%, n=4). In fraction 3 the Aα band had further decreased to 34% (SD 10%, n=4). Furthermore it is observed that several lower molecular weight bands appeared and that the peak of the Aα chain had broadened (table 2, figure 1) in the LMW' fraction. No difference was observed in the patterns between the fibrinogens that had been isolated using ß-alanine or using $(NH_4)_2SO_4$ precipitation followed by gel filtration.

Table 2. Mean (SD) of the density of the respective bands after scanning results of the SDS-PAAGE gel of fibrinogen forms under reducing conditions. The density of the Bß-chain has been used as internal standard.

	Aα-chain		Bß-chain	γ-chain	
fraction HMW	127%	(14%)	100%	101%	(27%)
fraction LMW	87%	(6%)	100%	84%	(9%)
fraction LMW'	41%	(9%)	100%	92%	(12%)

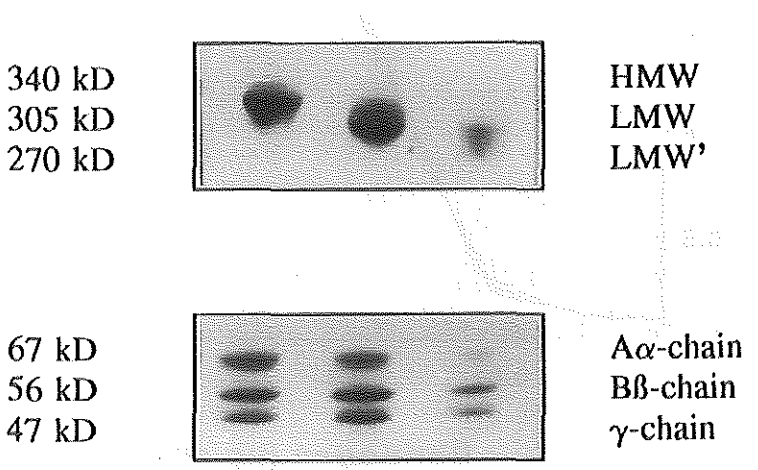

Figure 1. SDS/polyacrylamide gels of fibrinogen fractions 1 (mainly HMW), 2 (mainly LMW) and 3 (mainly LMW') under non-reducing and reducing conditions

Fibrinogen assays

The levels measured in the fractions with the EIA for total fibrinogen were virtually equal to the levels that were calculated from the optical density of the fibrinogen solution at 280 nm. Figure 2 shows that the dilution curves of the three fractions were parallel to each other and to pooled plasma. In the EIA for HMW+LMW fibrinogen the HMW fraction was 127% (SD 14%, n=3) of that of pooled plasma and LMW was detected approximately a factor three less sensitive with 41% (SD 22%) of pooled plasma (figure 3). Since the monoclonal antibodies in this assay were insensitive to LMW' fibrinogen, it was not expected that this fraction would be detected. In fraction 3 we measured 13 % (SD 7%) of pooled plasma which indicates contamination with fibrinogen forms that have intact Aα-chains (figure 3).

The results of the three clotting rate assays were very similar (figure 4). In the Clauss, the peptidase and the reptilase based assays the clotting rate of the HMW fraction was slightly higher than that of pooled plasma, the curve of the LMW fraction crossed that of the pooled plasma, while the LMW' fraction has longer clotting times than pooled plasma. It was remarkable that the dilution curves of the fibrinogen fractions were not parallel to each other or to pooled plasma (figure 4).

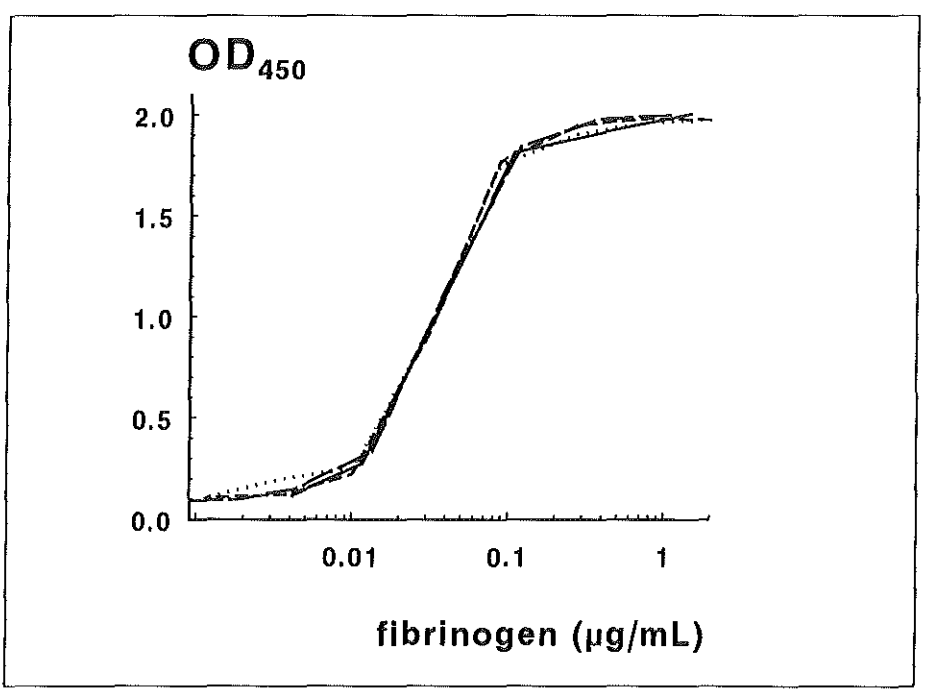

Figure 2. Typical example of dilution curves of pooled plasma (————) , HMW (– – – –), LMW (----), and LMW' (·····) fibrinogen in the enzyme immuno assay for total fibrinogen.

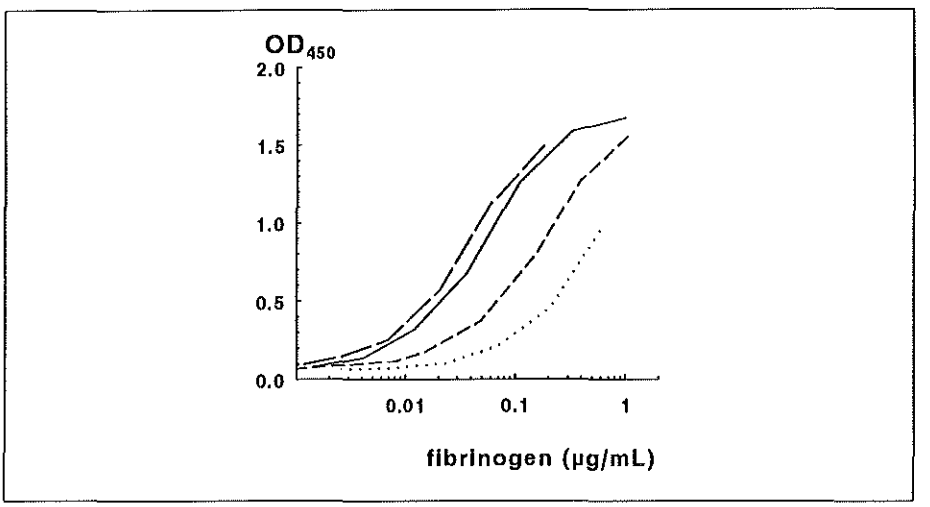

Figure 3. Typical example of dilution curves of pooled plasma (————), HMW (– – – –), LMW (----), and LMW' (·····) fibrinogen in the enzyme immuno assay for HMW+LMW fibrinogen.

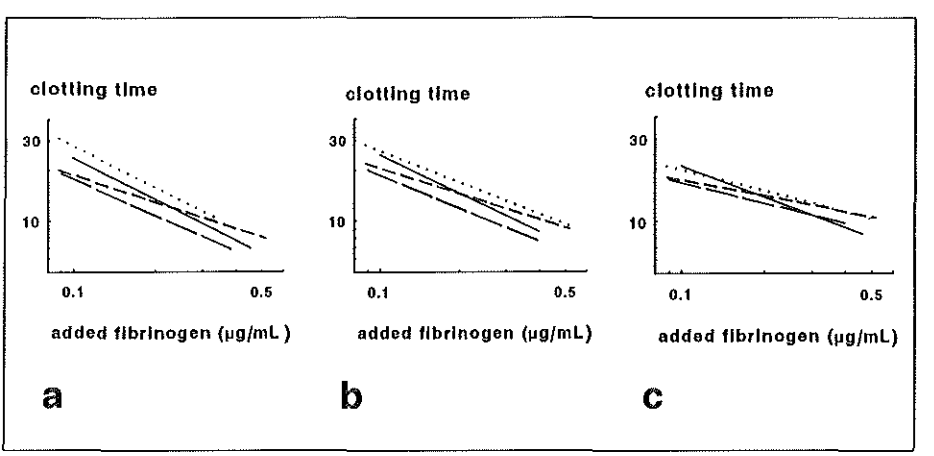

Figure 4. The clotting characteristics of pooled plasma (————), HMW (– – – –), LMW (----), and LMW' (·····) fibrinogen when thrombin (a), peptidase (b) and reptilase (c) are used as enzymes to start the clotting.

DISCUSSION

In the circulation fibrinogen can be found in many different forms. There are for example differences in the extent of degradation of the carboxyl-terminal ends of the Aα-chain, resulting in HMW, LMW and LMW' fibrinogen. This heterogeneity of fibrinogen makes it necessary to assess the specificity of different fibrinogen assays for the various forms.

The results of this study suggest only a slightly higher sensitivity for the HMW fibrinogen forms in clotting rate assays, independent of whether the clotting was initiated with thrombin, peptidase or reptilase. In the EIA for HMW+LMW fibrinogen the responses with the HMW fibrinogen forms are much higher than those for the other forms.

The HMW, LMW and LMW' fractions of fibrinogen isolated using ß-alanine precipitation had much longer clotting times with thrombin, peptidase or reptilase than the corresponding fractions prepared from fibrinogen isolated by the method described by Ruyven et al[27]. On the SDS-PAAGE gels the band pattern of the fractions obtained by the two isolation methods of fibrinogen were similar, and HMW, LMW and LMW' yielded the expected patterns. This suggests some denaturation of the fibrinogen using the ß-alanine precipitation method[28]. When we compared the clotting curves of the three fibrinogen forms that were derived from fibrinogen, isolated as described by Ruyven et al[27] we observed that the clotting time for LMW' was somewhat longer than that for HMW, which was somewhat longer than HMW. This might be ascribed to a decrease in the number of "b" binding site involved in the lateral polymerisation of fibrin[33].

To our surprise the clotting curves of the HMW, LMW and LMW' fractions were not parallel to each other or to those of pooled plasma in none of the three clotting assays. This indicates that the fibrinogen levels, measured with clotting rate assays rate assays, depends on a complex composite of relative contributions of the three fibrinogen forms, which will make the interpretation of these clotting rate assays complicated.

The fibrinogen levels, measured with the EIA for HMW+LMW fibrinogen, are mainly determined by the HMW level, since this form is detected three times more sensitive than the LMW form and since the LMW' form is hardly detected at all. Every LMW fibrinogen molecule has one epitope for the G8 monoclonal antibody and might therefore, theoretically, be detected as well as a molecule with two epitopes. Since there was a clear difference in sensitivity for the HMW and LMW forms, it might be suggested that the two intact carboxyl-terminal ends of the Aα-chain in the HMW form are cooperative. Because the LMW fraction is contaminated with HMW and the LMW' fraction contains some LMW fibrinogen, the actual differences in sensitivity of this HMW+LMW EIA for the three fibrinogen forms might be greater.

In normal plasma a distribution of 70% HMW, 26% LMW and 4% LMW' has

been reported. Using the relative sensitivities of the EIA towards the different chains (127%, 41% and 13% for HMW, LMW and LMW', respectively), the calculated fibrinogen concentration was 99.5%. This indicates that measurement in a mixture of the MW fibrinogen forms, i.e. plasma, gives a simple addition of the different forms in this assay.

If the clotting characteristics of fibrinogen determine the thrombotic risk, it will be expected that HMW fibrinogen form contributes most to the risk. A higher thrombotic risk by HMW fibrinogen has also been suggested by the study of Thorsen et al, who describe that the ADP-induced aggregation of blood platelets of the LMW fibrinogen was only 75% of the effect of the HMW form[34], while with a mixture of LMW and LMW' fibrinogen the aggregation was even further decreased to 50%. However, there are also indications that there is no increased tendency of HMW to thrombus formation because no difference was found in the amounts of ^{125}I labelled HMW and ^{131}I labelled LMW that had been incorporated in venous thrombi in patients that had undergone total hip arthroplasty[35].

It might also be possible that the LMW fraction is the most dangerous fibrinogen form, because clots made of LMW fibrin have thicker fibres[36-40] which makes the LMW clots more difficult to lyse than clots of HMW fibrin[41,42]. Increases in the LMW form would be best detected by the ratio of the fibrinogen levels measured with the HMW+LMW EIA over the total fibrinogen level: this ratio will be lower if there is relatively more LMW fibrinogen.

Our conclusion is that the clotting rate assays and the EIA for HMW+LMW fibrinogen are sensitive to the quality of fibrinogen. It would be interesting to study if the highest sensitivity for HMW fibrinogen forms makes the HMW+LMW EIA or a ratio of the EIA over the Clauss a better indicator of the thrombotic risk. The characteristics of method that identifies the most dangerous form of fibrinogen might also give the best indication of thrombotic risk.

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CHAPTER 3

MEASURING PLASMA FIBRINOGEN LEVELS IN PATIENTS WITH LIVER CIRRHOSIS. THE OCCURRENCE OF PROTEOLYTIC FIBRIN(OGEN) DEGRADATION PRODUCTS AND THEIR INFLUENCE ON SEVERAL FIBRINOGEN ASSAYS.

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ABSTRACT

In patients with liver cirrhosis the fibrinogen molecule is under constant attack of various proteolytic enzymes, which might affect results of the different assay systems for fibrinogen. We therefore studied the measurement of fibrinogen in the plasma of patients with mild, moderate and severe cirrhosis of the liver. Fibrinogen levels were measured with the Clauss method (functional fibrinogen); an enzyme immuno assay (EIA) for HMW+LMW fibrinogen; and an assay that measures the total clottable fibrinogen. With all three methods we found normal or slightly increased fibrinogen levels in patients with mild or moderate cirrhosis, whereas patients with severe cirrhosis had decreased levels. No evidence was found for increased partial fibrinogen proteolysis, resulting in increases of LMW'-fibrinogen in cirrhotic patients.

We also documented the fibrin(ogen) degradation, and we observed that *fibrinogen* degradation products levels increased slightly with the severity of the disease, but were still in the normal range in patients with severe cirrhosis. This indicates a very low level of primary fibrinolysis. Fibrin degradation products levels increased much stronger, which points to intravascular coagulation. The levels of the fibrin degradation products remained below the level where they are expected to influence the Clauss assay.

In patients with liver cirrhosis the measurement of plasma fibrinogen levels with the three studied methods give comparable results. However, the assay for total clottable protein can not accurately measure very low fibrinogen levels, which can be frequently observed in severe cirrhotics. We suggest to apply the Clauss assay in cirrhotic patients because of this and because it has a good reproducibility and because the test is cheap, quick and easy to perform.

INTRODUCTION

Fibrinogen is a symmetrical glycoprotein consisting of two Aα, two Bβ and two γ-chains. Under normal conditions fibrinogen occurs in three molecular forms[1-6]: 1) the high molecular weight form (HMW-fibrinogen) with both Aα-chains intact; 2) the low molecular weight form group (LMW-fibrinogen) with one Aα-chain intact and the other Aα-chain shortened in the carboxyl-terminal region to varying extent; and 3) the group of LMW'-forms of fibrinogen with both Aα-chains shortened to different degrees. The percentages of HMW-, LMW- and LMW'-fibrinogen in healthy individuals are about 70%, 26% and 4%, respectively. The LMW and LMW'-forms can be considered as partly proteolysed derivatives of the HMW-form of fibrinogen[1-4]. The enzyme or enzymes, responsible for this degradation, have not yet been identified[6]. Although fibrinogen degradation by plasmin *in vivo* can give degradation products of similar size, analysis of the amino acids of the C-terminal region of the Aα chains of LMW- and LMW'-fibrinogen has shown that these

fibrinogen forms do not result from plasmin degradation *in vitro*[6,7].

Fibrinogen is also subject to several other proteolytic processes. Firstly the conversion to fibrin by thrombin, the product of an activated coagulation system and secondly the degradation by plasmin, the product of an activated fibrinolytic system.

Several assays exist for the assessment of fibrinogen levels. Examples are the Clauss assay, based on the clotting rate[8]; an assay for clottable fibrinogen[9]; and a recently described EIA which quantitates the amount of the HMW+LMW forms of fibrinogen[10]. These assays are affected to different extents by the presence of fibrinogen derivatives and the amounts of the three molecular forms.

The *Clauss method* is influenced by the presence of anticoagulant *fibrinogen* degradation products (FgDP) and fibrin degradation products (FbDP)[11], which will lead to spuriously low fibrinogen levels. Soluble fibrin will lead to apparently increased fibrinogen levels. The amount of *clottable fibrinogen* will be increased by clottable degradation products. The *EIA for HMW+LMW fibrinogen* will not detect LMW' fibrinogen[10].

The aforementioned proteolytic processes may be strongly affected during cirrhosis of the liver[12-18], and the resulting fibrinogen derivatives may have effects on the recording of fibrinogen levels, depending on the method used. As a consequence conclusions as to the effects of the severity of the disease on fibrinogen levels may not be easy to draw.

We studied the measurement of fibrinogen levels with the functional Clauss method, the total clottable fibrinogen assay and the EIA for HMW+LMW fibrinogen in healthy individuals and in patients with mild, moderate and severe cirrhosis of the liver. We also documented the status of fibrin(ogen) degradation using assays for soluble fibrin[19], FgDP[20], FbDP[21] and TDP (total degradation products = FgDP+FbDP)[22] (table 1).

Our data show that in patients with liver cirrhosis there is an ongoing disseminated intravascular coagulation with concomitant reactive fibrinolysis which is more active than the primary fibrinolysis. The more severe the cirrhosis, the higher the FbDP and TDP levels in the patients. We evaluated to what extent these fibrinogen derivatives affected results in three different assays for fibrinogen and from this evaluation we suggest to apply the Clauss assay in patients with liver cirrhosis.

PATIENTS, MATERIALS AND METHODS

Patients

The study group comprised 44 patients with biopsy-proven cirrhosis of the liver. The patients were classified according to Pugh's modification of the Child classification[23] as mild (Child A, n = 20), moderate (Child B, n = 10) and severe (Child C, n = 14). Twelve apparently healthy volunteers were included as normal controls.

Informed consent was obtained from the patients and the volunteers. The study was carried out according to the principles of the Helsinki Declaration.

Blood samples
Nine volumes of venous blood were collected between 10.00 and 12.00 a.m. in plastic tubes containing one volume of cold trisodium citrate 0.11 mol/L, and placed immediately in melting ice. Plasma was prepared by centrifugation (2000 x g, 30 min, 4°C) and stored in small aliquots at -70°C.

Assays (an overview is given in table 1)
Total clottable fibrinogen (modification of Jacobsson[9]) was determined by allowing 1 mL plasma to clot with 25 IU of thrombin for two hours at room temperature. The clot was washed with 0.15 M NaCl, dried and subsequently suspended in 6.7 M alkaline urea. The fibrinogen concentration is calculated from the optical density at 280 nm (A(1cm,1%) = 15.75).

Functional fibrinogen levels were determined according to Clauss[8]. The standard curve was made using purified fibrinogen (Merz & Dade, Düdingen, Switzerland).

Table 1. Assays for fibrinogen and its degradation products

Method	What is measured
Clauss method	Functional fibrinogen assay
Jacobsson modification	Total clottable protein
EIA (HMW+LMW)-fibrinogen	HMW+LMW degradation products of fibrinogen, with two intact (HMW) or one intact and one degraded Aα-chain (LMW)
EIA FgDP	Fibrinogen degradation products
EIA FbDP	Fibrin degradation products
EIA TDP	Fibrin + fibrinogen degradation products (sum of FgDP + FbDP)
EIA soluble fibrin	Soluble fibrin

(HMW+LMW)-fibrinogen levels were determined using an enzyme immuno assay (EIA)[10]. In this assay a monoclonal antibody against the carboxyl terminal end of the fibrinogen Aα-chain is used as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain (Y18) as the tagging antibody. Thus the EIA will only measure HMW- and LMW-fibrinogen. LMW'-fibrinogen will not be detected, but can be calculated as the difference between the fibrinogen values determined with the assay for total clottable fibrinogen and fibrinogen as determined with this EIA. The standard curve was made from pooled

citrated plasma in which the fibrinogen concentration was determined by a gravimetric method[24].

Fibrinogen **degradation products** (FgDP) were measured using a test kit (Fibrinostika FgDP, Organon Teknika, Turnhout, Belgium)[20]. In this sandwich EIA, a monoclonal antibody (FDP-14), directed against an epitope on the stretch 52-114 of the fibrinogen Bß-chain[25], specific for FgDP and FbDP and not reactive with fibrinogen or fibrin is used as a capture antibody. A horse-radish peroxidase (HRP)-conjugated monoclonal antibody, specific for fibrinopeptide A (Y18/HRP) is used as a tagging antibody. As a consequence of the combined specificities of FDP-14 and Y18, the EIA is specific for FgDP and does not detect fibrinogen (HMW, LMW nor LMW'), fibrin of FbDP.

Fibrin degradation products (FbDP) were measured using a test kit (Fibrinostika FbDP, Organon Teknika, Turnhout, Belgium)[21]. In this sandwich EIA, FDP-14 (see above) is used as capture antibody. The tagging antibody (DD-13/HRP), elicited against D-dimer, makes this EIA specific for FbDP. Although the DD-13 antibody was directed against D-dimer, it does not discriminate between crosslinked and non-crosslinked forms of FbDP. It does not detect HMW-, LMW- or LMW'-fibrinogen, fibrin or FgDP.

Total degradation products (TDP) were measured using a test kit (Fibrinostika TDP, Organon Teknika, Turnhout, Belgium)[22]. In this sandwich EIA, FDP-14 (see above) is used as the capture antibody. The tagging step is done with a mixture of Y18/HRP and DD-13/HRP (see above). The assay assesses the total of FgDP + FbDP (= TDP) and does not detect HMW, LMW and LMW'-fibrinogen or fibrin.

Soluble fibrin was measured using a sandwich EIA (Fibrinostika Soluble Fibrin, Organon Teknika, Turnhout, Belgium)[19] in which monoclonal antibody anti-Fb-1/2 specific for soluble fibrin, is used as capture antibody. The tagging step is with G8 conjugated with HRP (see above). The assay assesses soluble fibrin and does not detect fibrinogen, FgDP or FbDP.

Immunoblotting: Aα-chain degradation was visualized by immunoblotting after polyacrylamide electrophoresis essentially as described by Grøn et al.[6], using Y18/HRP for immunostaining.

Statistical analysis was performed using non-parametric tests. Correlations were calculated with the Spearman rank correlation and the fibrinogen levels in the groups

were compared with the Kruskall Wallis test. Any probability less than 0.05 was considered to represent a significant difference. The assay variation was expressed by the standard deviation of the duplicate measurements and was calculated using the formula:

$$\sqrt{\frac{(differences\ of\ the\ duplicate\ measurement)^2}{2 * number\ of\ assays}}$$

RESULTS

Levels of fibrinogen and soluble fibrin

The median levels of functional fibrinogen (Clauss assay) were 1.8 g/L in the normal group (defined as 100%), 2.1 g/L (i.e. 117% of normal) in Child A, 2.4 g/L (137% of normal) in Child B and 1.3 g/L (74% of normal) in Child C (Fig. 1). The fibrinogen levels in patients with Child C cirrhosis were significantly lower than the levels in the other patients.

The median levels of total clottable fibrinogen were 2.1 g/L in the normal group (defined as 100%), 2.2 g/L (i.e. 103% of normal) in Child A, 2.2 g/L (103% of normal) in Child B and 0.86 g/L (41% of normal) in Child C. In this assay fibrinogen levels under 1 g/L gave too low fibrinogen levels, because there was incomplete recovery of the clotted fibrinogen.

With the EIA for (HMW+LMW)-fibrinogen we found 2.65 g/L in the normal group (defined as 100%), 2.65 g/L (i.e. 100% of normal) in Child A, 3.50 g/L (132% of normal) in Child B and 1.90 g/L (i.e. 72% of normal) in Child C.

The differences in absolute levels of fibrinogen are probably due to differences in calibration material (see Materials and Methods). There was a good correlation between the fibrinogen levels, measured with the three assays (between EIA and Clauss method $R = 0.88$, $p < 0.001$, between EIA and total clottable fibrinogen method $R = 0.89$, $p < 0.001$, between Clauss method and total clottable fibrinogen method $R = 0.88$, $p < 0.001$). There was no indication for systematic errors in these assays. The assay variation was 3% in the Clauss assay, 5% in the total clottable protein assay and 6% in the EIA.

The calculated LMW' levels (fibrinogen measured with the total clottable fibrinogen minus levels measured with the EIA) were comparable in the individuals in the four groups. This is supported by our finding that with immunoblotting no increases in the degraded Aα-chains could be demonstrated (results not shown). We conclude therefore that there are no increased LMW' fibrinogen levels in cirrhotic plasma.

Plasma levels of soluble fibrin in patients with a severe cirrhosis were comparable to levels in the normal group, i.e. below 10 μg/ml.

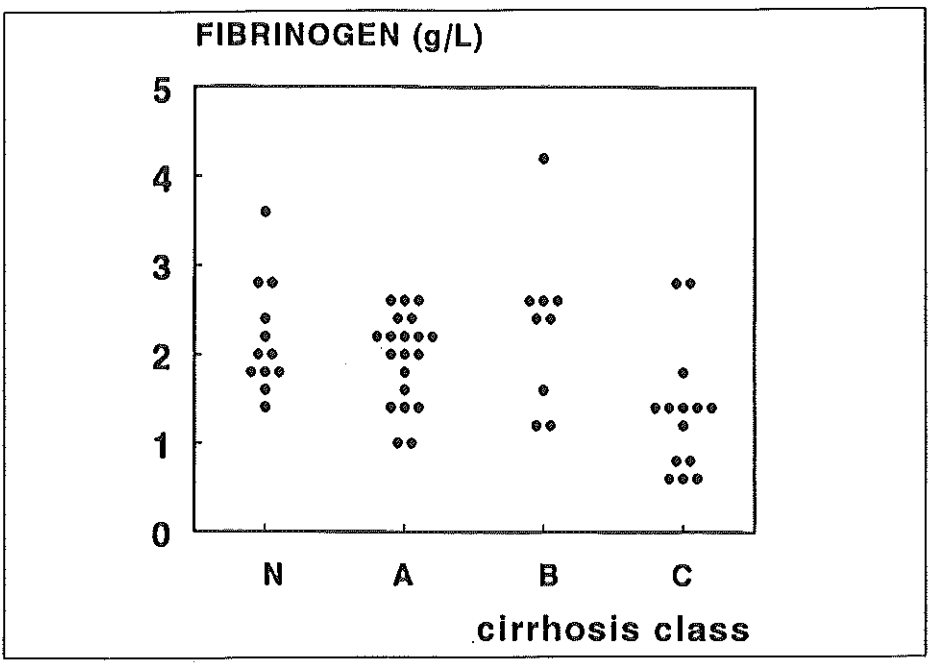

Figure 1. Plasma levels of functional fibrinogen (Clauss) in healthy volunteers (N) and in patients with mild (A), moderate (B) and severe (B) cirrhosis of the liver.

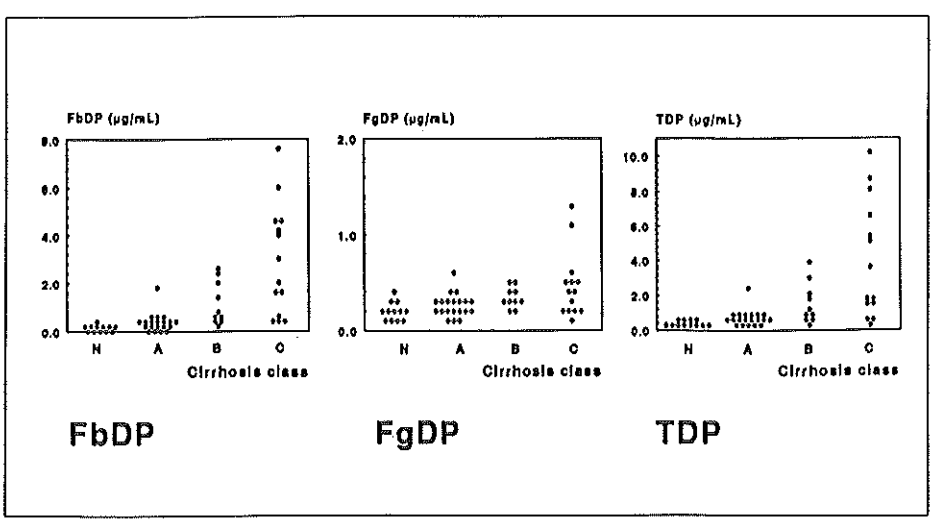

Figure 2. Plasma levels of fibrin (FbDP), fibrinogen (FgDP) and total (TDP) degradation levels in healthy volunteers (N) and in patients with mild (A), moderate (B) and severe (B) cirrhosis of the liver.

Plasma fibrin(ogen) degradation products

The levels of fibrin degradation products were different between groups of patients with different severity of the cirrhosis using the Kruskall Wallis test ($p<0.001$). The median in the normal group was 0.23 μg/ml, in Child A 0.33 μg/ml, in Child B 0.66 μg/ml and in Child C 2.45 μg/ml (Fig. 2).

The levels of *fibrinogen* degradation products (FgDP) also differed significantly ($P < 0.03$) in the four groups using the Kruskall Wallis test. In the normal group the median was 0.23 μg/ml, in Child A 0.26 μg/ml, in Child B 0.34 μg/ml and in Child C 0.40 μg/ml (Fig. 2).

As could be anticipated from the FgDP and FbDP levels, a similar difference between the patient groups could be found for the levels of the total degradation products (TDP)($P < 0.001$) using the Kruskall Wallis test. The median in the normal group was 0.41 μg/ml, in Child A 0.64 μg/ml, in Child B 1.19 μg/ml and in Child C 2.75 μg/ml (Fig. 2). The levels of the total degradation products corresponded well with the calculated sum of the separately determined levels of FbDP and FgDP ($R = 0.98$), with no indication for a systematic error.

DISCUSSION

In this study we assessed fibrinogen and some of its derivatives in plasma of patients with varying degrees of liver cirrhosis. We found normal or slightly increased levels of fibrinogen using the functional Clauss test, the EIA and with the assay for total clottable fibrinogen in patients with liver cirrhosis classified as Child A and B. With the three tests for fibrinogen we only found decreased levels of fibrinogen in Child C cirrhotics, which may be the result of an increased consumption or a decreased synthesis.

Partial fibrinogen proteolysis appears to be a normal physiological event, since slightly-proteolysed forms of fibrinogen occur under normal conditions. These slightly-damaged fibrinogen forms (LMW and LMW') are clottable and will contribute to the result of functional assays, such as the Clauss method. It was expected that in patients with liver cirrhosis there is a higher degree of proteolysis, because Lipinski[17] and Weinstein[18] described an increased proteolysis of fibrinogen in patients with liver cirrhosis. However, the results of our study indicate that the LMW' is not affected, because a good correlation was found between the fibrinogen levels measured with a recently-developed enzyme immuno assay (EIA) for (HMW+LMW)-fibrinogen and fibrinogen as assessed by the Clauss method or the total clottable fibrinogen method in plasma from patients with different stages of liver cirrhosis. The calculated LMW' levels did not change with increasing degree of cirrhosis. Also, immunoblotting experiments performed to visualize a possible increase of degraded Aα-chains showed no differences of degraded Aα-chains between the studied groups, which further supports the conclusion that there is no

significant increase in LMW' formation in liver cirrhosis.

The soluble fibrin levels found in severe cirrhotics were comparable with those found in the normal group (< 10 μg/ml), indicating that the haemostatic balance is not severely disturbed.

Activation of the coagulation system in patients with liver cirrhosis has been suggested by various authors[26-28]. The increased levels of FbDP found here indicates that some activation of the coagulation system takes place, but that the fibrinolytic system is able to cope with the generation of fibrin. We also found a correlation of the degree of cirrhosis with *fibrinogen* degradation products, which is expected when plasmin is formed without prior activation of the coagulation system. However, the levels of the *fibrinogen* degradation products show only a minor increase, which indicates that there is either a very low grade of primary fibrinogenolysis or that the small amount of FgDP is the result of spillover cleavage of fibrinogen by plasmin.

FbDP and TDP are known to have a significant influence in the Clauss test. However, in this group of patients the levels of FbDP and FgDP did not reach the levels that are known to affect the Clauss test indicating that even in severe cirrhotics, the Clauss method gives accurate results.

In conclusion: The level of activation of the coagulation system correlates with the level of liver cirrhosis in this group. The increases in *fibrinogen* degradation levels in the patients with more severe cirrhosis indicate a low level of ongoing primary fibrinolysis. We could not show an increased degradation of the Aα-chains of fibrinogen. In the Clauss, nor in the total clottable fibrinogen assay nor in the EIA could a disturbance be found of degradation products or fibrinogen or fibrin as they can be found in patients with mild, moderate or severe liver cirrhosis. Therefore, we believe that for measuring the fibrinogen levels, all three methods can be used. However, we suggest to use the Clauss assay, because it can accurately measure low fibrinogen levels, has a good reproducibility and because the test is cheap, quick and easy to perform.

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CHAPTER 4

REGULATION OF HISTIDINE-RICH GLYCOPROTEIN (HRG), FIBRINOGEN AND C-REACTIVE PROTEIN SYNTHESIS THROUGH CYTOKINES IN PRIMARY CULTURES OF HEPATOCYTES FROM CYNOMOLGUS MONKEY.

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ABSTRACT

Histidine-rich glycoprotein (HRG) is a human plasma protein that is synthesized by the liver. During the acute phase reaction the plasma levels of HRG are decreased. We could confirm this negative acute phase behaviour in cell culture experiments with cynomolgus monkey (Macaca fascicularis) hepatocytes that were cultured in the presence of conditioned medium from stimulated monocytes/macrophages. Both the protein and mRNA levels of HRG were decreased whereas the protein and mRNA levels of the positive acute phase proteins C-reactive protein (CRP) and fibrinogen were increased. To investigate the mechanism of the negative acute phase reaction of HRG in more detail, the effects of interleukin 6 (IL6), interleukin 1ß (IL1ß) and tumour necrosis factor-α (TNFα) were documented. Both the protein and the mRNA levels of HRG were markedly reduced by IL1ß and TNFα, whereas interleukin-6 (IL6) had no effect. Different effects were observed for the positive acute phase proteins fibrinogen and CRP, where secreted protein and production of mRNA were increased by IL6 and decreased by TNFα, and fibrinogen was additionally decreased by IL1ß.

Our observations indicate that hepatocytes from humans or cynomolgus monkeys can be considered a suitable model for studying the regulation of HRG. We showed that HRG is a negative acute phase protein and that the negative acute phase reaction in hepatocytes from the cynomolgus monkey is regulated by TNFα and IL1ß, and not by IL6.

INTRODUCTION

Histidine-rich glycoprotein (HRG) is a plasma glycoprotein that *in vitro* modulates both coagulation and fibrinolysis[1]. A range of possible functions arises from the capacity of HRG to interact with multiple ligands, including heparin[2], plasminogen[3], thrombospondin[4] and fibrinogen and fibrin[5]. An association between HRG levels and the risk of thrombosis is suggested by the high prevalence of elevated plasma HRG levels in patients with venous thrombosis[6]. In addition several thrombophilic families with familial elevation of HRG have been described[7-9]. Patients who develop deep venous thrombosis during the acute stage of a myocardial infarction have higher HRG levels than patients who do not develop this complication[10].

Only limited knowledge is available about the regulation of the plasma HRG levels. In several studies a significant decrease of HRG levels (17% to 36%) has been found upon administration of oral contraceptives[11,12]. A decrease is also reported in diseases like severe liver malfunction[13] and sepsis[14], or in response to immunosuppressive steroid therapy[15]. Furthermore HRG levels are negatively correlated with C-reactive protein (CRP) levels, indicating that HRG levels exhibit a negative response in the acute phase[16]. In addition, treatment of rabbits with turpentine results in a four to five fold decrease of the mRNA levels of HRG[17].

The acute phase synthesis of proteins in the liver ,is mainly regulated by the cytokines interleukin-6 (IL6), interleukin-1ß (IL1ß) and tumour necrosis factor-α (TNFα)[18-20]. These cytokines can be secreted by stimulated monocytes[21]. Since HRG is synthesized only in the liver[22] we decided to study the regulation of the HRG-synthesis by these three cytokines in cultured hepatocytes.

In the study of the regulation of HRG the cell culture model that most approaches the *in vivo* situation would be primary cultures of human hepatocytes. However, human hepatocytes are difficult to obtain and a large variation in the quality of the hepatocytes due to a high diversity in the donor lifestyle complicates this model. Several alternative cell culture systems can be suggested to study HRG synthesis. The first are the human hepatoma cell lines HepG2 and Hep3B, which are cell lines secreting many human liver proteins[23, overview: 24] and in which the regulation of the protein synthesis bears great resemblance to the regulation in primary hepatocytes. A disadvantage of the use of hepatoma cell lines is that the cells are dedifferentiated and thereby lost several functions. An alternative model may be primary monolayer cultures of hepatocytes from the cynomolgus monkey (Macaca fascicularis), a nonhuman primate. The HRG of old world monkeys has several antigenic domains which are similar to domains in human HRG[25,26]. Additionally, it has been shown that the cynomolgus monkey can be used as a model to study the development of atherosclerosis[27], which is often considered as a process of chronic inflammation[28], and to study the lipid and lipoprotein metabolism[29-32].

We studied the HRG secretion of the hepatoma cell lines HepG2 and Hep3B and of primary human and cynomolgus monkey hepatocytes. We then documented the acute phase regulation of HRG in primary hepatocyte cultures of Macaca fascicularis. We investigated the HRG secretion and mRNA levels in the presence of conditioned medium from monocytes/macrophages. The regulation mechanism was further studied in experiments where the hepatocytes were cultured in the presence of the cytokines IL6, IL1ß and TNFα. Key experiments with TNFα were performed with primary human hepatocyte cultures.

MATERIALS AND METHODS

Hepatoma cell lines. The established cell lines HepG2 and Hep3B, derived from human liver tumours, were obtained from Dr. B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA). The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) medium (Flow Laboratories, Irvine, Scotland, UK), supplemented with 10% heat-inactivated fetal bovine serum (30 min 56°C), 2 mmol/L L-glutamine, 100 μg/mL streptomycin and 100 IU/mL penicillin (Boehringer Mannheim, Mannheim, Germany) at 37°C in a 5% CO_2/95% air atmosphere. The medium was renewed twice a week. The experiments were started when the cells had grown to confluency.

Primary hepatocyte cultures. Isolation of parenchymal cells from human and

monkey liver was performed by a modification of the two step collagenase method[33] as described previously[34-36]. Human donor livers became available through the Rotterdam Auxiliary Liver Transplantation Program. Livers from Macaca fascicularis (1.5-3 years old) were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, the Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitic vaccine at this institute. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%) was 66-96%. The parenchymal cells were seeded on fibronectin coated culture dishes at a density of 2 x 10^5 viable cells per cm^2 and were maintained in Williams E (WE) medium, supplemented with 10% heat-inactivated fetal bovine serum (30 min 56°C), 2 mmol/L L-glutamine, 135 nmol/L insulin, 50 nmol/L dexamethason, 100 μg/mL kanamycin, 100 μg/mL streptomycin and 100 IU/mL penicillin at 37°C in a 5% CO_2/95% air atmosphere. After 14-16 hours the non-adherent cells were washed from the plates and the cells were maintained in the same culture medium.

Conditioned medium (CM) from monocytes/macrophages. Monocytes were isolated as described previously[37]. Briefly, mononuclear cells were isolated from the blood of healthy blood donors using density centrifugation over Ficoll (Pharmacia, Woerden, the Netherlands), resuspended in M199 medium and incubated in culture wells for 45 minutes. The monocytes, that had specifically attached to the plastic, were then cultured for 48 hours in M199 medium, supplemented with 10 μg/ml lipopolysaccharide from Escherichia coli (LPS)(serotype 0128:B12, Sigma), 10% fetal bovine serum (not heat-inactivated), 2 mmol/L L-glutamine, 100 μg/mL streptomycin and 100 IU/mL penicillin at 37°C in a 5% CO_2/95% air atmosphere as described previously[37]. Conditioned medium was collected and stored at -20°C until use. The concentrations of IL1ß in CM has recently been described to range from 2.5-500 U/mL and of IL6 from 500-10000 U/mL as determined by immunoassay[38].

Incubation of hepatocytes with cytokines. The effect of conditioned monocyte/macrophage medium was studied by culturing the cells during two consecutive 24 hours incubation periods in the presence of 10% conditioned monocyte/macrophage medium. The effects of the cytokines on protein secretion and mRNA levels were also studied by culturing the cells during two consecutive 24 hours periods in the presence of cytokines with final concentrations of 50 and 250 U/ml IL6; 50 and 500 U/ml IL1ß and 10,25,50,100 and 250 U/ml TNFα. The protein measurements and mRNA assays were performed after the second incubation period.

Assays

The antibodies that were used for the measurement of HRG, CRP and fibrinogen were raised against purified human proteins, but were also suitable for the measurement of HRG, CRP and fibrinogen in the media of hepatocytes from the

cynomolgus monkey. Human pooled plasma was used for the reference curve in the assays for HRG, fibrinogen and CRP, therefore the levels of these proteins are expressed as equivalents of the corresponding human proteins.

HRG secretion was measured in conditioned hepatocyte medium with a sandwich ELISA specific for HRG. Polyclonal rabbit anti-HRG antibodies raised against purified human HRG (Behringwerke, Marburg, Germany) were used for coating. Rabbit anti-HRG IgG was used as tagging antibody. The detection limit was 0.5 ng/ml.

Fibrinogen secretion was measured in culture medium with an enzyme immune assay[39], using monoclonal antibodies against the COOH terminus of the Aα chain as catching antibody and horseradish peroxidase labelled monoclonal antibodies against the NH_2 terminus of the Aα chain as tagging antibody.

C-reactive protein secretion was measured in conditioned medium with an enzyme immune assay, using horseradish peroxidase labelled rabbit polyclonal antibodies against human CRP (DAKO, Denmark) as catching and tagging antibodies.

Total cell protein. Cell protein was assessed with the Lowry method for total protein[40] using bovine serum albumin (BSA, Sigma) as reference.

mRNA analysis. RNA was isolated from the hepatocytes after the second 24 hours period using the method described by Chomzynski and Sacchi[41]. Equal amounts of RNA from different incubation experiments were separated on a denaturing 1.2% agarose gel containing 0.22 mol/L formaldehyde. The RNA was then transferred to a nylon membrane (Amersham, UK) by Northern blotting. The membranes were hybridized at 65°C in 7% sodium dodecyl sulphate with 0.5 mol/L $NaHPO_4$ and 10 mmol/L EDTA, pH=7.2 using ^{32}P labelled probes which were labelled with a random primer method (Amersham, Bucks, UK). The complete 2.1 kb cDNA fragment of human HRG[42], cDNA for the Bß chain of fibrinogen (gift of Dr. S. Lord, Chapel Hill, USA), cDNA of CRP[43], and a 1.2 kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Kindly provided by Dr. R. Offringa)[44] were used as probes. The blots were routinely washed at 65°C two times with 2 x SSC (1 x SSC = 0.15 mol/L NaCl/0.015 mol/L sodium citrate, pH=7.0), 1% SDS and two times with 0.3 x SSC, 1%SDS. Autoradiograms were prepared using Kodak XAR-5 film and intensifying screens at -70°C. The mRNA bands were scanned using a Hewlet Packard ScanjetPlus, after which quantitation of the relative amounts of mRNA was conducted using the density of the 28S RNA band, stained by ethidium bromide, as an internal standard.

Statistical analysis. The effects of conditioned monocyte/macrophage medium and of the individual cytokines on the protein synthesis of HRG, CRP and fibrinogen were studied with the Student's t-test. The statistical package SOLO for personal computers was used to do the calculations. P levels under 0.05 were considered significant.

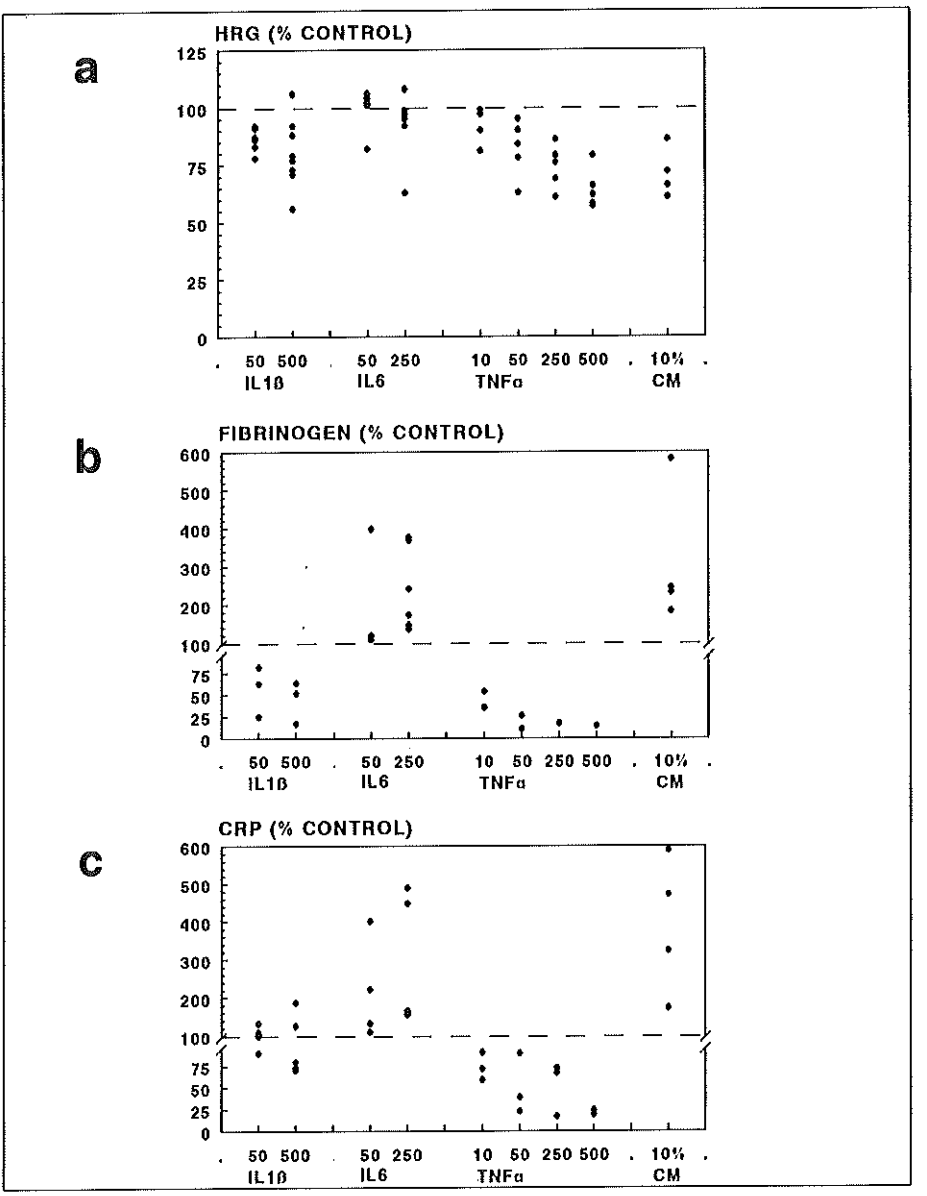

figure 1. Effect of 2 times 24 hours treatment with interleukin-6 (IL6), interleukin-1ß (IL1ß), tumour necrosis factor α (TNFα) or conditioned medium from LPS-stimulated monocytes/macrophages on the secretion of histidine-rich glycoprotein (HRG)(A), C-reactive protein (CRP)(B) and fibrinogen (C) by cynomolgus monkey hepatocytes. An average of 4 experiments was performed for each concentration of a cytokine. The levels are expressed as a percentage of the control. The mean absolute synthesis of HRG under basal culture conditions was 100 ng/mg cell protein/24 hours (SD 98, range 17-359, n=12), of CRP this was 63 ng/mg cell protein/24 hours (SD 57, range 8-194, n=13) and of fibrinogen 2.24 μg/mg cell protein/24 hours (SD 0.93, range 0.86-4.06, n=12).

RESULTS

HRG production by hepatoma cell lines and primary hepatocytes

HRG was analyzed in the media of HepG2 and Hep3B cells cultured either with serum free medium or with medium supplemented with 10% FCS or 1% (w/v) HSA. However, no HRG could be detected in the conditioned media after 24 and 48 hours with our assay that has a detection limit of 0.5 ng/ml HRG. Also, no HRG mRNA could be demonstrated in the hepatoma cells.

In primary cultures of simian hepatocytes a mean basal HRG secretion of 100 (SD 98, range 17-359, n=12) ng/mg cell protein/24 hours was found, whereas in human hepatocytes a basal level of HRG secretion of 173 (SD 188, range 12-430, n=4) ng/mg cell protein/24 hours was observed. The mean basal CRP synthesis in the simian hepatocytes was 63 ng/mg cell protein/24 hours (SD 57, range 8-194, n=13) and for fibrinogen it was 2.24 μg/mg cell protein/24 hours (SD 0.93, range 0.86-4.06, n=12). In both simian and human hepatocytes substantial amounts of mRNA could be detected with the human cDNA probe for HRG. The length of the human and simian mRNA was 2.4 and 2.9 kb, respectively. The human HRG mRNA length was in agreement with published data[42].

Effects of conditioned monocyte/macrophage medium

After culturing simian hepatocytes for two consecutive 24 hours periods in the presence of 10% conditioned medium from lipopolysaccharide (LPS) stimulated monocytes/macrophages the HRG secretion had decreased to 71% of the control (SD 11, range 61-86%, p=0.01, n=4). A marked increase of the CRP secretion to 390% (SD 156%, range 173-589%, p=0.07, n=4) and of the fibrinogen secretion to 310% (SD 156%, range 183-581%, p=0.05, n=4) of the control levels were observed (figure 1). The density of the HRG mRNA band was compared with the density of the 28S RNA band, which was therefore used as a measure of the amount of RNA that was analyzed. With the conditioned medium a decrease of the HRG mRNA level to 15% of the control was observed while the mRNA levels of CRP and fibrinogen had increased (figure 2).

Effect of individual cytokines on secreted protein and mRNA levels

When the hepatocytes had been cultured for two times 24 hours in the presence of IL1ß or TNFα, the HRG secretion had decreased in a dose dependent way, showing a decrease to 79% of the control at 500 U/ml IL1ß (SD 14%, p=0.004, n=9) and a decrease to 66% of the control at 500 U/ml TNFα (SD 9%, p<0.001, n=6) (figure 1). The HRG secretion was not significantly influenced by IL6 at concentrations up to 250 U/ml. In 3 key experiments with human hepatocytes the HRG secretion was decreased 30% by 500 IU/mL TNFα, comparable to the effects in simian hepatocytes.

The secretion of the acute phase protein CRP was dose dependently elevated by IL6 (380% of control levels with 250 U/mL IL6), but not influenced by 500 IU/mL IL1ß. A dose-dependent decrease was observed with TNFα (22% of the control

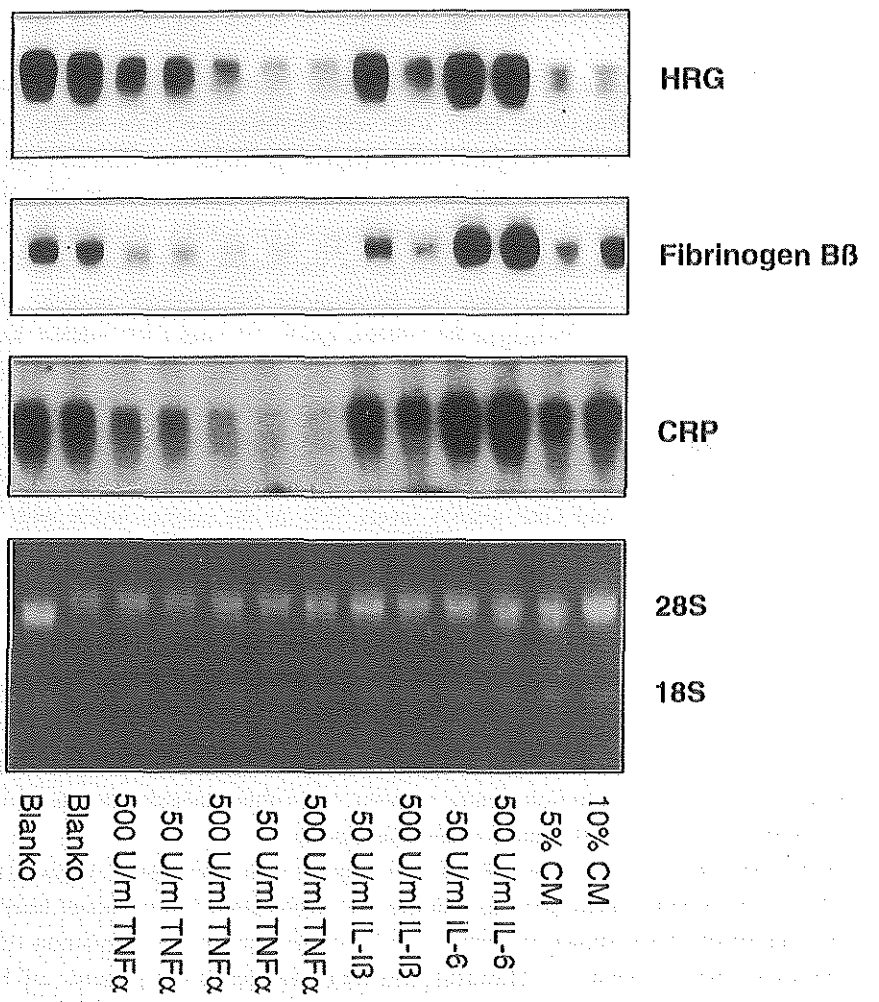

figure 2. Effect of 2 times 24 hours treatment with interleukin-6 (IL6), interleukin-1ß (IL1ß), tumour necrosis factor α (TNFα) or conditioned medium from LPS-stimulated monocytes/macrophages on the mRNA levels of histidine-rich glycoprotein (HRG), fibrinogen and C-reactive protein (CRP) in cynomolgus monkey hepatocytes (n=1). The intensity of the 28S RNA bands was used as an internal standard to correct for differences in the amount of total RNA applied to the gel.

levels with 500 U/mL TNFα)(figure 1).
The synthesis of fibrinogen was dose-dependently decreased by IL1ß (50% of control levels with 500 U/mL) and TNFα (14% of control levels with 500 U/mL TNFα). Culturing of the simian hepatocytes in the presence of IL6 showed a dose-dependent increase of the fibrinogen secretion (225% of the control levels with 250 U/mL IL6)(figure 1).

To investigate the regulatory mechanism of HRG, CRP and fibrinogen secretion, the effects of the cytokines on the HRG, CRP and Bß-fibrinogen chain mRNA levels were assessed by Northern blot hybridization (figure 2). Since the GAPDH mRNA levels were increased by TNFα (results not shown), the density of the 28S RNA band was used as an internal control. The decrease of the mRNA levels of the three proteins after culturing the simian hepatocytes in the presence of TNFα was dose-dependently and much stronger than the decrease of the secreted protein. HRG mRNA levels were 10%, fibrinogen Bß mRNA levels 2% and CRP mRNA levels 33% of the control levels with 500 U/mL TNFα. Culturing the hepatocytes with IL1ß also showed a dose-dependent decrease of the mRNA of the three proteins that was much stronger than the effect of the secreted proteins, but the effect is less pronounced for HRG and Bß fibrinogen. HRG mRNA levels were 49%, fibrinogen Bß mRNA levels 38% and CRP mRNA levels 75% of the control levels with 500 U/mL IL1ß. No effect of IL6 on the HRG mRNA level was observed.

DISCUSSION

Most acute phase proteins are synthesized in the liver. Therefore, cultured hepatocytes might be used as a model system for studying the acute phase behaviour of several proteins such as HRG. Primary cultures of human hepatocytes can be considered as the most physiologically relevant cell culture system, but since their availability is limited, we evaluated alternative models.

Firstly, the hepatoma cell lines HepG2 and Hep3B were considered, because they are known to produce many proteins that are also synthesized in the human liver[23,24]. It has repeatedly been shown that there is also consistency in the regulation of the synthesis of these proteins[45,46]. However, we could not detect the presence of HRG in conditioned medium of these cell lines, making HepG2 and Hep3B cells unsuitable for studying the regulation of HRG. The absence of HRG in conditioned media of HepG2 cells has previously been described by Fair et al[47]. The acute phase protein CRP is also not produced by HepG2 cells[24,48]. However, hepatoma cell lines have been described as being useful for the study of a number of other acute phase proteins, like fibrinogen, haptoglobin and albumin[23,24].

Secondly, primary monolayer cultures of hepatocytes from the cynomolgus monkey were evaluated. In these cells mRNA encoding for HRG was detected and they secreted HRG in the culture medium in comparable quantities to primary human

hepatocyte cultures. Therefore, primary cultures of simian hepatocytes were used to study the acute phase regulation of HRG. We started by culturing simian hepatocytes in the presence of conditioned medium (CM) from LPS stimulated monocytes/macrophages which contains high levels of the cytokines IL6, IL1ß and TNFα. CM induced a marked decrease of the HRG synthesis and exerted even larger effects on the level of HRG mRNA. This confirms that HRG is a negative acute phase reactant, as has previously been suggested by studies in patients with inflammatory diseases[16] and shown in studies performed in cultured rabbit hepatocytes[17]. CM significantly increased the secretion of the positive acute phase proteins fibrinogen and CRP, that were used as controls for the stimulation of the acute phase of the cultured hepatocytes.

The decreasing effects of CM, TNFα and IL1ß on the mRNA levels of HRG, Bß fibrinogen and CRP was much more pronounced than their effects on the protein secretion. The secretion of the proteins is the accumulated secretion of a 24 hour period, while the mRNA levels represent the situation in the hepatocytes at the end of this 24 hour period. This might be the explanation for the differences in the magnitude of the effects that we observed. Another explanation might be that the cytokines affect RNA transcription or other steps in the protein synthesis.

To characterize the regulation of the HRG synthesis by the acute phase the effects of individual cytokines on the HRG secretion were studied. Our results with the primary cultured simian hepatocytes identified TNFα and IL1ß as the cytokines that regulate the HRG synthesis. The cytokine that is the strongest determinant of the synthesis of most positive acute phase proteins, IL6, produced only a small, but not significant decrease of the HRG secretion. IL1ß and TNFα have also been described to decrease the synthesis in hepatoma and primary human hepatocytes of albumin and transferrin, two other negative acute phase proteins[18,45,49,50]. However, although IL6 has been described as decreasing the secretion of these two proteins in hepatoma and primary human hepatocytes, it did not affect the HRG secretion in simian hepatocytes in our study. This suggests that there are either species differences in the regulation of acute phase proteins or that the regulations of the negative acute phase proteins do not always parallel each other. Differences in the regulation of acute phase proteins are further indicated by our observations on the secretion of fibrinogen which was increased by IL6 and decreased by IL1ß and TNFα, while CRP secretion was increased by IL6, decreased by TNFα and not affected by IL1ß.

The models to study the regulation of protein synthesis in hepatocytes have their own advantages and limitations. For example, the effects of cytokines on the synthesis of acute phase proteins in human hepatoma cell lines are not consistent in the different cell lines. Also, the effects are not always identical to the effects in primary human hepatocyte cultures because hepatoma cells may have dedifferentiated[49], resulting either in different regulation of some proteins or in a

loss of the capacity to synthesize specific proteins like HRG and CRP. On the other hand, the advantage of human hepatoma cell lines is that they yield a reproducible model. The preferred primary hepatocyte model would be human hepatocytes. Since their availability is limited, hepatocytes from other species are regularly used. The disadvantage here is the possibility of species differences. Especially for acute phase proteins, these differences have been described. For example, CRP is an acute phase protein in human, but not in goat and cow that have a constitutional high level of CRP which do not rise following injury[51,52] indicates that the acute phase reaction needs to be studied in a species that is as closely related as possible. Since the cynomolgus monkey is an old world monkey, it is relatively close to human.

In our study we only had the opportunity to compare the effect of TNFα in human and simian hepatocytes. In both models, the HRG secretion was decreased by TNFα. Furthermore, the positive acute phase effects of cytokines on CRP and fibrinogen in hepatocytes from the cynomolgus monkey were comparable to the effects that have been described for these cytokines in human hepatocytes[45,53]. The similarity in the observations in human and simian hepatocytes suggests that primary cultures of hepatocytes from cynomolgus monkeys may be considered a suitable model system for studying acute phase proteins. However, care must be taken in extrapolating the results of simian hepatocytes to the human situation, because differences between the two systems are also observed, for example, in human hepatocytes where both IL1ß and IL6 stimulate the CRP synthesis[51], while we observed no effect of IL1ß on CRP secretion in simian hepatocytes. This requires further comparative studies in human and simian hepatocytes.

The results of this study showed that TNFα and IL1ß downregulated the HRG secretion and mRNA levels in primary cultures of simian hepatocytes. IL6 does not appear to be an important cytokine for the regulation of the HRG synthesis, as opposed to its strong effect on the synthesis of most positive acute phase proteins, such as fibrinogen and CRP.

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CHAPTER 5

CORONARY ARTERY DISEASE AND INFLAMMATION: ASSOCIATION OF CORONARY ARTERY DISEASE WITH FIBRINOGEN, C-REACTIVE PROTEIN, CYTOKINES AND SMOKING

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ABSTRACT

The plasma levels of fibrinogen and C-reactive protein (CRP) are associated with the risk for ischaemic heart disease (IHD). Both these proteins are significantly increased by the acute phase reaction. The cytokines that have a major role in the regulation of synthesis of acute phase proteins by the liver are interleukin 6 (IL6), interleukin 1ß (IL1ß), and tumour necrosis factor α (TNFα). The production of these cytokines will be increased by an inflamed vascular wall. It has therefore been suggested that plasma fibrinogen and CRP levels reflect the inflammatory condition of the vascular wall as well.

Smoking is another well-known risk factor for cardiovascular disease. Both fibrinogen and CRP have been reported to correlate with smoking habits. If the inflammatory state of the vascular wall is increased by smoking, it will result in increased plasma cytokine levels and consequently in increased levels of acute phase proteins.

In this study we evaluated the inflammatory state of 34 patients with severe coronary artery disease (CAD)(with complaints of angina and scheduled for PTCA) and 30 healthy controls comparable for age and smoking habits. We measured fibrinogen, CRP, IL1ß, IL6 and TNFα-levels and also combined the individual proteins in an index which we denominate as the inflammatory index.

Our data indicate an elevated inflammatory status in the patient group compared to the healthy controls. When we discriminate between smokers and non-smokers, we find a significant higher inflammatory status: in non-smoking patients than in non-smoking controls; in smoking patients than in smoking controls; in smoking than in non-smoking patients; and in smoking than in non-smoking controls.

Our results indicate an increased inflammatory condition of the vascular wall in patients with severe CAD compared to healthy controls. Furthermore, we demonstrate that in patients with CAD and in healthy controls, smoking increases the inflammatory state as reflected by an index consisting of fibrinogen, CRP and IL6. Remarkably, the increase of fibrinogen in CAD patients could not be fully explained by increased inflammation.

INTRODUCTION

Elevated plasma fibrinogen levels have been identified as an independent risk indicator for coronary artery diseases in healthy populations[1-6]. Recently, in the ECAT-Angina Pectoris study on patients with angina pectoris both plasma fibrinogen and C-reactive protein (CRP) levels were higher in the event group[7]. In this study both the fibrinogen and the CRP level were well within the clinical normal range in both the high and the low risk groups. Other inflammatory markers, such as leucocyte count[6,8,9], monocyte count[10], cytokines in the atheroma[11], expression of granulocyte and monocyte receptors[12], and inflammatory infiltrates in cardiac

arteries[13] have also shown a correlation with the pathogenesis of coronary artery disease.

In general, during inflammation the levels of the acute phase proteins fibrinogen and CRP are increased. A contribution of inflammation to atherosclerosis has been suggested[14-18]. Ross et al.[19] postulates that advanced lesions of atherosclerosis result from excessive inflammatory - fibroproliferative response to numerous different forms of insults. The resulting low grade inflammation of the vascular wall is even suggested to be the main "mechanism" of atherosclerosis[19]. The plasma fibrinogen and CRP levels might then very well represent the inflammatory condition of the vascular wall, because an association has been found between the number of diseased vessels and the plasma fibrinogen levels[20-22].

Smoking increases the risk for cardiovascular disease[23-28]. In general, the idea is that smoking enhances the platelet aggregation[29] or reduces the oxygenation status of the blood, by an increase in the tonus of the coronary arteries and by the binding of carbomonoxide to haemoglobin[30-33]. However, both fibrinogen and CRP show a correlation with smoking habits as well[7,26,27,34-38] which may suggest that smoking induces an inflammatory reaction of the coronary arteries, finally leading to aggravation of the coronary artery disease. The correlation with smoking might also be mediated through the acute phase reaction. Therefore, we established in this study the relation between smoking habits of patients with severe coronary artery disease (CAD) and healthy controls on the one hand and the inflammatory status, as reflected by the levels of fibrinogen, CRP, interleukin-1ß (IL1ß), interleukin-6 (IL6) and tumour necrosis factor-α (TNFα), on the other.

PATIENTS AND METHODS

Patients and controls

Thirty-four patients (26 male, 8 female) with complaints of angina and scheduled for percutaneous transluminal coronary angioplasty (PTCA) were enroled in this study. On the diagnostic coronary angiogram only one target vessel was considered responsible for the complaints. The mean (1 SD) age was 54.2 (8.3) years. Fifteen patients had a history of myocardial infarction, 6 patients had undergone PTCA earlier and in 2 patients a coronary artery bypass graft (CABG) operation had been performed before their participation in the study. The severity of anginal complaints was scored according to the New York Heart Association classification: eight patients were in Class II, 17 patients were in Class III and 9 patients were in Class IV. The patients did not take any medication known to influence fibrinogen levels.

On coronary angiography one vessel disease (VD) was documented in 29 patients, two VD in 4 patients and three VD in 1 patient. The target vessel was the left descending artery (LAD) in 17 patients, the left circumflex artery in 7 patients and

the right coronary artery in 10 patients. Thirty healthy male blood donors, comparable for age (54.0 (6.4) years)[39,40] and smoking habits, formed the control group.

The study was conducted in accordance with the Declaration of Helsinki: approval was given by the Medical Ethical Committee of the Erasmus University, Rotterdam and written consent was obtained from the patients.

Smoking

The participants were interviewed for their smoking status. If smoking was stopped within one month before blood sampling the participant was considered a smoker. The ex-smokers who had stopped smoking at least 6 months before blood sampling were considered non-smokers. No patients stopped smoking between 1 and 6 months before the start of the study. Sixteen patients and 15 controls were smokers.

Blood Sampling

Blood was collected and anticoagulated with 5.4 mM EDTA in melting ice. After centrifugation at 2000 x g for 15 min the plasma was collected and stored at -80°C until use. The buffy-coat fraction was used for monocyte isolation.

Cytokine secretion of monocytes

Monocytes were isolated from the buffy-coat fraction which was reconstituted with phosphate buffered saline (PBS) to 20 ml, layered on top of a Lymphoprep (Nycomed, Haarlem, The Netherlands) gradient, and centrifuged at 800 x g for 10 min at room temperature. The fraction containing the mononuclear leucocytes was washed with PBS. Next, the erythrocytes were lysed in 155 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 0.1 mmol/L EDTA. The remaining mononuclear leucocytes were washed once with ammonium chloride solution, once with PBS and were then resuspended in RPMI-medium (Flow Laboratories, Irvine, UK) with 2% fetal calf serum. The percentage of monocytes in this fraction was determined from cytospin preparations and in general amounted to 25%. 6.3 x 10^5 cells were transferred into 96-wells culture plates (Costar, Badhoevedorp, The Netherlands) and incubated at 37°C in a humidified environment of 5% CO_2/95% air in the presence or absence of 5 ng/ml lipopolysaccharides[41]. After 24 h supernatants were collected and stored at -70°C.

Fibrinogen

Fibrinogen is measured with an enzyme immuno assay that uses the monoclonal antibody G8 (directed against the COOH-terminal part of the fibrinogen α-chain) as a catching antibody and peroxidase conjugated - Y18 (directed against the NH_2-terminal part of the fibrinogen α-chain) as tagging antibody[42].

C-reactive protein

C-reactive protein is measured with a sensitive enzyme immuno assay that uses rabbit anti-human C-reactive protein (Dako) as a catching and a tagging antibody.

Cytokines

IL6, IL1ß and TNFα were measured with enzyme immuno assays according to the instructions of the manufacturer (Medgenix, Amersfoort, the Netherlands).

Inflammatory index

Because the study groups are small and with a large variation, we combined the inflammatory markers in an inflammatory index to study the total acute phase condition of the patients. The inflammatory index was composed by replacing the data for each variable with their ranks and adding those ranks. Eventually, the inflammatory index consisted of fibrinogen, CRP and IL6. We did not include IL1ß and TNFα because they did not contribute.

Statistical analysis

The distribution of the variables was positively skewed. Logarithmic transformation gave a normal distribution for fibrinogen, CRP and TNFα, but not for IL-1ß and IL6 and therefore we used non-parametric tests (Mann-Whitney, median and range) to study the single variables. The inflammatory index was studied using analysis of covariance with adjustment for age and gender as described by Conover[43], who shows that parametric tests can be assessed for analyzing rank transformed data.
Because the fibrinogen levels were normally distributed after a logarithmic transformation, we studied the effect of smoking and CAD on fibrinogen in healthy controls and patients after adjusting the fibrinogen levels for CRP and IL6 in a multiple regression analysis.

RESULTS

When we compared the plasma levels of acute phase proteins in the patient group with those in the control group we found that the fibrinogen levels in the patients were significantly higher than those in the healthy controls (table 1). We found no difference for CRP. When comparing the plasma levels of the individual cytokines, we observed only a difference in IL6 and not for IL1ß and TNFα. We constructed a parameter that represents the inflammatory markers by replacing the data for fibrinogen, CRP and IL6 with their ranks and adding these ranks of the grouped variables, IL1ß and TNFα were not included in this index because they did not contribute. We then noticed that this inflammatory index is significantly higher in patients (mean 114) than in controls (mean 79)($p=0.0002$) using the Mann-Whitney test (table 1).

Table 1. Median and (range) of acute phase markers in healthy controls and patients.

	Controls	Patients	p
Fibrinogen (g/L)	2.09 (1.34-3.05)	2.87 (1.7-4.7)	0.0002
C-reactive protein (mg/L)	1.99 (0.27-20)	1.3 (0.1-20)	n.s.
Interleukin-1ß (pg/ml)	0 (0-46.0)	0.22 (0-50.3)	n.s.
Interleukin-6 (pg/ml)	0 (0-40.8)	1.81 (0-38.9)	0.0001
Tumour necrosis factor-α (pg/ml)	8.8 (3.1-82.8)	8.7 (0.7-43.2)	n.s.
Combined marker index	79 (26-135)	114 (45-183)	0.0002

p gives the significance between the control and patient group as found with the Mann-Whitney test.

When the patient and control groups were subdivided into smokers and non-smokers the acute phase protein fibrinogen levels were higher in smoking than in non-smoking controls (2.3 g/L and 1.9 g/L, respectively), but similar in non-smoking and smoking patients (both 2.9 g/L)(table 2). The plasma fibrinogen levels were higher in non-smoking patients than in non-smoking controls, and also higher in smoking patients than in smoking controls. We further observed that smoking gives a comparable increase of the CRP levels in CAD patients and healthy controls (0.9 vs 3.0 g/L and 1.0 vs 4.0 g/L, respectively). The levels of the IL6 and IL1ß were

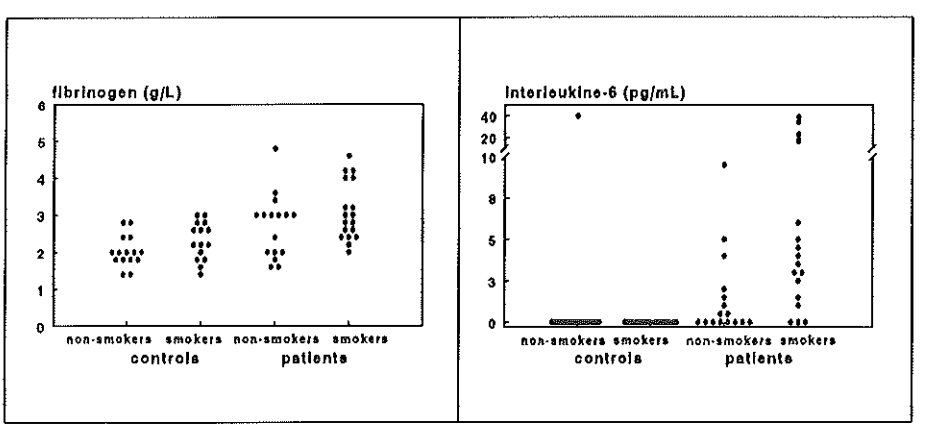

Figure 1. Fibrinogen levels for non-smoking and smoking controls; and non-smoking and smoking patients.

Figure 2. Interleukin-6 levels for non-smoking and smoking controls; and non-smoking and smoking patients.

Table 2. Acute phase markers (median and range) in healthy controls and patients, considering their smoking status.

	controls		patients					
	non-smokers	smokers	non-smokers	smokers	A	B	C	D
Fibrinogen (g/L)	1.93 (1.34-2.74)	2.29 (1.35-3.05)	2.87 (1.65-4.73)	2.86 (2.00-4.55)	0.07	n.s.	0.02	0.008
C-reactive protein (mg/L)	1.00 (0.27-20)	3.97 (0.27-20)	0.85 (0.11-5.68)	3.01 (0.31-20)	n.s.	0.02	n.s.	n.s.
Interleukin-1ß (pg/ml)	0 (0-46.0)	0 (0-46.0)	0 (0-7.3)	0.87 (0-50.3)	n.s.	0.08	n.s.	n.s.
Interleukin-6 (pg/ml)	0 (0-40.8)	0 (0-0)	0.1 (0-9.5)	3.8 (0-38.9)	n.d.	0.02	0.06	<0.001
Tissue necrosis factor-α (pg/ml)	6.3 (3.1-82.8)	10.3 (3.1-24.5)	8.5 (0.7-42.5)	9.3 (2.4-43.2)	0.10	n.s.	n.s.	n.s.
Combined marker index	69 (27-135)	89 (26-130)	93 (45-152)	132 (66-183)	0.03	0.002	0.04	0.007

Significance levels in columns

A: between non-smoking and smoking controls
B: between non-smoking and smoking patients
C: between non-smoking controls and non-smoking patients
D: between smoking controls and smoking patients

n.s. Significance level >0.1 in the Mann Whitney test
n.d. not determined (too many very low levels)

very often below the detection limit, which hampered the study of the control group. The levels of these cytokines were highest in the smoking patient group. No significant effects on the TNFα levels were found.

Combining fibrinogen, CRP and IL6 in an inflammatory index resulted in highly significant differences between non-smokers and smokers in both the patients and the controls. The inflammatory index was also significantly elevated in smoking patients in relation to smoking controls and in non-smoking patients in relation to non-smoking controls (table 2).

When we adjusted the fibrinogen levels for CRP and IL6 we still observed significantly higher values in non-smoking patients than in non-smoking controls ($p<0.001$) and in smoking patients when compared to smoking controls ($p=0.03$). The effect of smoking on the adjusted fibrinogen levels in patients and in healthy controls was not significant (data not shown).

We also studied the secretion of cytokines by normal and LPS-stimulated monocytes of non-smoking and smoking patients. The results showed that smoking did not affect the basal cytokine secretion or the LPS-induced cytokine production (table 3). There was no relation between the cytokine secretion of normal and LPS-stimulated monocytes and the cytokine levels we measured in plasma.

Table 3. Median and range of cytokines secreted by lipopolysaccharide (LPS) stimulated and unstimulated monocytes of patients.

		non-smokers	smokers	p
unstimulated monocytes	Il1ß	31 (0-272)	63 (0-210)	n.s.
	Il6	340 (1-1381)	794 (0-2230)	n.s.
	TNFα	47 (2-218)	61 (0-293)	n.s.
LPS-stimulated monocytes	Il1ß	113 (19-461)	125 (0-565)	n.s.
	IL6	891 (19-1952)	1106 (3-2284)	n.s.
	TNFα	84 (24-266)	64 (0-457)	n.s.

p gives the significance between the control and patient group as found with the Mann-Whitney test.

DISCUSSION

We studied the inflammatory markers fibrinogen, CRP and the cytokines IL6, IL1ß and TNFα in patients with severe PAD and in healthy volunteers. The patients had significantly higher levels of fibrinogen and IL6 than controls. As anticipated, the

inflammatory index was higher in patients than in controls. Even though the majority of the levels would clinically not be considered increased, our results indicate that patients with CAD have an increased habitual inflammatory status.

When we then studied the effect of smoking on the plasma fibrinogen levels in the two groups, we found an effect of smoking in healthy controls, but not in patients. Previously, it has already been reported in individuals without cardiovascular disease that smoking increased fibrinogen levels in a dose dependent way[26,37]. The absence of an increase of fibrinogen levels in patients suggests that the fibrinogen-increasing effect of smoking is eliminated in patients where the fibrinogen is already increased by CAD. The PLAT study on patients with vascular disease also described the absence of an effect of smoking on the plasma fibrinogen levels[21].

The CRP levels were equally elevated by smoking in the controls and the patients. The effects of smoking on CRP have repeatedly been reported in healthy controls[34,38] but the effect of smoking on CRP in CAD patients has not been studied before.

The association between smoking and the acute phase proteins fibrinogen and CRP suggests that smoking induces a low-grade, continuous inflammatory status. This does not necessarily mean that the vessel wall is more inflamed, but the identification of smoking as a risk factor for ischaemic heart disease strongly suggests that smoking has an effect on the condition of the vascular wall.

The regulation of the fibrinogen and CRP synthesis in the liver is mainly by IL6, IL1ß and TNFα[44-46]. In this study the levels of IL6 and IL1ß are higher in smoking than in non-smoking patients, but no conclusions can be drawn for the controls because in too many individuals the levels are below the detection limit. TNFα is not influenced by smoking.

Because of the modest sample size of this study, we combined fibrinogen, CRP and IL6 in an inflammatory index. IL1ß and TNFα were not included in this index, because they did not contribute. We found a comparable increasing effect of smoking on the inflammatory index in controls and atherosclerotic patients. This further supports the hypothesis of an increased inflammatory status in smokers[7,26,27,34-38].

In this study we only had one blood sample from each patient. It is known that acute phase proteins, especially CRP, react very strongly to inflammatory reactions. Even a light common flu gives a marked elevation. This process should be separated from the levels that were increased as a result of the inflammatory status of the blood vessels[47]. Therefore it is advisable to use multiple samples in order to determine a basal, constant level for acute phase proteins and exclude the outliers. In this study, a few CRP levels were above the clinical normal value of 10 mg/L and this increase might be caused by some other disease. Because we used nonparametric statistics or parametric statistics on rank transformed data our results are not biased by these patients, but more precise results will be obtained if habitual levels can be determined.

Monocytes are a major source of various cytokines. Because smoking patients had a higher inflammatory index than non-smokers we studied whether the basal or the LPS-stimulated production of cytokines in monocytes of smokers was increased. This was not the case. This might indicate that smoking does not bring circulating monocytes in an activated condition. Although there is also no association between plasma levels of cytokines and the basal or LPS-induced cytokine production of monocytes it is still possible that smoking has an effect on local cytokine release by monocytes or has an effect on other cytokine-releasing cells, like the endothelial cells that line the (coronary) blood vessels.

To evaluate whether the acute phase reaction was the only regulatory mechanism that contributed to the observed increase in the levels of fibrinogen, we also studied the fibrinogen levels when we adjusted those for CRP and IL6. The effect of smoking in controls was reduced, indicating that smoking indeed induces an inflammatory response. However, we still found ,both smokers and non-smokers, a significant contribution of atherosclerosis to the fibrinogen levels. This implies that another mechanism may also be involved here, which may be at least partly genetic, as it has been reported that up to 51% of the fibrinogen level is genetically determined[48,49].

In summary, we have shown that 1) patients with CAD have a higher inflammatory status than healthy controls; 2) that the inflammatory status is increased by smoking both in CAD patients and in healthy controls; and 3) that the increase of the plasma fibrinogen levels in patients with coronary artery disease cannot be fully explained by an increased inflammatory status.

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CHAPTER 6

INTER- AND INTRAINDIVIDUAL VARIABILITY IN PLASMA FIBRINOGEN, PLASMINOGEN ACTIVATOR INHIBITOR (PAI) ACTIVITY, C-REACTIVE PROTEIN AND HISTIDINE-RICH GLYCOPROTEIN IN YOUNG HEALTHY VOLUNTEERS

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ABSTRACT

We investigated the intraindividual variability in plasma levels of fibrinogen, plasminogen activator inhibitor (PAI) activity, C-reactive protein (CRP) and histidine-rich glycoprotein (HRG) in 20 healthy, young individuals and compared this with the interindividual variability in a comparable group. For each of these parameters the intraindividual variation (13% for fibrinogen, 4% for log(PAI-activity), 14% for log(CRP), 1% for HRG of the total variance) is smaller than the interindividual variation (87%, 96%, 86%, 99% of the total variance, respectively).

The results in this group indicate that for assessment of an individual level multiple sampling will not be needed for HRG and PAI-activity. For fibrinogen duplicate sampling will be recommended and for CRP triplicate sampling is recommended because that will make the identification of incidental peak values possible. In an epidemiological study the sample sizes, based on the variance of this transversally studied group of healthy young individuals, needed to detect a 15% difference between two groups will be 142 for fibrinogen, 162 for PAI-activity, 412 for CRP and 86 for HRG.

Additionally, we studied the contribution of genetic polymorphisms of the Bß-fibrinogen (*Bcl*I and G/A^{-455}) and PAI-activity (*Hind*III and CA-repeat) genes to the intra- and the interindividual variation. The fibrinogen genotypes were associated with plasma fibrinogen levels. However, no effects of fibrinogen or PAI polymorphisms on intraindividual variation were observed in this group of young, healthy individuals. One has to be conscious that different variations might be possible in groups of older or diseased individuals.

INTRODUCTION

Fibrinogen[1-7] as well as plasminogen activity inhibitor-1 (PAI-1)[8] and C-reactive protein (CRP)[5] are variables that have been identified as risk indicators for cardiovascular disease in various epidemiological studies. Elevated plasma levels of histidine-rich glycoprotein (HRG) are associated with venous thrombosis[9] and patients who develop venous thrombosis during the acute phase of a myocardial infarction have higher HRG levels[10]. Therefore interest is developing in the measurement procedures of these factors, because accurate and specific knowledge of plasma fibrinogen, PAI-activity, CRP and HRG levels will increase their value as risk indicators.

The plasma levels of fibrinogen, PAI-activity, CRP and HRG are influenced by various life style aspects. Fibrinogen is raised by smoking, obesity, increasing age and by oral contraceptive use[11,12]. A negative association has been found between mild alcohol intake and the plasma fibrinogen concentration[13]. PAI-activity is raised by smoking[14], increasing age[15,16], pregnancy[17], regular alcohol intake[18,19] and fish oil consumption[20]. Training[21] and use of oral contraceptives or anabolic steroids[22] can

reduce plasma levels. Furthermore, PAI-activity levels in plasma are subject to diurnal variation[23]. CRP levels are increased in smokers[5,24,25], with physiological stress[26] and are associated with obesity[5]. HRG is decreased by estrogens[27,28] and anabolic steroids[29].

The acute phase reaction can increase the plasma fibrinogen levels two to fourfold and CRP levels can increase even a hundredfold. PAI-1 and HRG are also an acute phase proteins, but the effect on their plasma levels is much smaller. PAI-activity will also increase in acute phase situations, but HRG is a negative acute phase protein[30-32]. When using these factors as risk indicators we are interested in habitual levels and not in short-time fluctuation by transient acute phase reactions. In most epidemiological studies one blood sample is taken, which is then accepted as representing the habitual level.

Several investigators have reported genetic polymorphisms of the fibrinogen and PAI-1 genes. The genotypes were associated with plasma fibrinogen and PAI-levels[33-36]. Humphries et al[34] also reported that individuals carrying the rare allele of the B*cl*I polymorphism of the Bß-fibrinogen gene have a larger longitudinal variation of their fibrinogen levels.

In this study we have investigated the value of single and multiple sampling in assessing the habitual levels of fibrinogen, PAI-activity, CRP and HRG in young, healthy individuals. We also documented the contribution of genetic polymorphisms of fibrinogen and PAI to their habitual plasma levels and to their longitudinal variation in plasma levels of these factors.

VOLUNTEERS, MATERIALS AND METHODS

Healthy individuals

In the longitudinal study 20 apparently healthy volunteers were included with a median age of 31 years range (24-58). Of the 10 males and 10 females, two were smokers. The median body mass index (BMI) was 22.3 kg/m^2 range (16.9-28.7). Blood was collected every three weeks during 6 months (from december 1990 to june 1991). At each visit the volunteers completed a questionnaire on factors influencing fibrinogen, PAI-activity, CRP and HRG levels (smoking, diet, alcohol use, medication, disease).

For the transversal study the first blood sampling from the volunteers of the longitudinal study was used, extended with blood samples from 39 healthy male volunteers from whom only one blood sample was drawn. In the transversal group (n=59) the median age was 38 years (range 24-58), the median BMI 25.1 kg/m^2 range (16.9-31.5) and 22 were smokers.

Blood collection

Venous blood was collected into CTAD (Becton Dickinson, Meylan Cedex, France)

or sodium citrate (final concentration 0.011 mol/L) under strictly standardized conditions[37]. Plasmas were stored at -70°C.

Assays

Fibrinogen levels were determined according to Clauss[38] and expressed as g/L using as a standard pooled citrated plasma in which the fibrinogen level was determined with a gravimetric method[39]. PAI-activity levels were obtained from the CTAD plasma using the Verheijen method[40] and expressed as IU/ml, using pooled plasma (7.6 IU/mL) as a standard. CRP levels were measured with an enzyme immuno assay that used rabbit antibodies to CRP (DAKO, Denmark) as both catching and tagging antibody and expressed as mg/L. CRP standard serum (Behringwerke, Marburg, Germany) was used for calibration. HRG levels were determined by single radial immuno diffusion[41] with rabbit antibodies raised against purified HRG (Behringwerke, Marburg, Germany) and the levels are expressed as percentage of a plasma pool of 20 healthy volunteers (%PP).

The within-day and between day coefficients of variation were 1.7% and 6.3% for the fibrinogen assay, 6% and 12% for the PAI-activity assay, 2.9% and 7.2% for the CRP-EIA and 9% and 11% for the HRG-assay, respectively.

Detection of polymorphisms

The *Bcl*I restriction fragment. length polymorphism (RFLP) of the Bβ-fibrinogen gene was assessed by Southern blot analysis of *Bcl*I digested genomic DNA using a β-fibrinogen cDNA probe (courtesy Dr. S. Lord) as previously described[34].

The G/A^{-455} RFLP of the Bß-fibrinogen gene was determined by amplification of the polymorphic region by polymerase chain reaction (PCR), followed by digestion with the restriction enzyme *Hae*3 as described by Thomas et al[33].

The *Hind*III RFLP of the PAI-1 gene[42] was assessed by Southern blot analysis of *Hind*III digested DNA using a PAI-1 cDNA probe (courtesy of Dr. P. Bosma).

Determination of the genotype of the polymorphic (CA)n region in the PAI-1 gene was performed as described before[35]. The most frequent allele was designated "z" and the other alleles were designated by their base pair differences from z. Thus, the following allele types z, z+2, z+4, z+8 and z+10 can be identified.

Statistical evaluation

The (geometrical) mean level of the nine samplings was used as the habitual level. Due to skewed distribution of PAI-activity and CRP levels, we used logarithmical transformed data. The distribution of fibrinogen and HRG levels was not significantly deviant from normal and therefore not transformed. The arithmetical mean is given for fibrinogen and HRG, where the geometrical mean is given for PAI-activity and CRP.

Table 1. Estimates of components of variation in plasma fibrinogen, PAI-activity, CRP and HRG levels of 20 healthy volunteers followed over 6 months.

	n_{ind}	n_{obs}	mean		total	interindividual	intraindividual
fibrinogen (g/L)	20	164	2.64	variance	0.807	0.706	0.101
				% of total variance		87%	13%
log (PAI-activity) (IU/L)	20	164	0.805	variance	5.11	4.91	0.20
				% of total variance		96%	4%
log (CRP) (mg/L)	20	164	-0.125	variance	4.04	3.46	0.58
				% of total variance		86%	14%
HRG (%PP)	20	164	113	variance	7173	7074	99
				% of total variance		99%	1%

n_{ind} number of volunteers

n_{obs} number of observations up to 9 per volunteer)

In the longitudinal study one-way analysis of variance (ANOVA) was performed to separate the sources of variation. We applied an additive variance component model in which: Y_{ij} (the value of person i at time j) = $\mu + \alpha_i + \varepsilon_{ij}$
In this model μ is the true mean of the population, a_i, is the deviation from the true mean of the *i*th person ($i = 1....20$). The residual, ε_{ij}, thus comprises the intra-personal variation and intra-serial analytical variation. The random terms a_i and ε_{ij} were assumed to be independent and normally distributed with zero expectations. The measurement number was not added as a factor, because the deviations from the true mean are assumed to be independent from the sample time.

The within subject component can be reduced by using the average of *m* repeated measurements, using the formula: $\sigma^2_{tot} = (\sigma^2_{\alpha} + \sigma^2_{\varepsilon}/m)$.

In the transversal study analysis of covariance (ANCOVA) using multiple linear regression analysis was performed to evaluate the effect of genetic Bß-fibrinogen and PAI genotypes on plasma levels of fibrinogen and PAI-activity, respectively. Age, body mass index (BMI) and gender were added as covariables and mean (standard error of the mean) are given. From those individuals that also participated in the longitudinal study, results from the first sampling were used in the transversal analysis.

The statistical package SOLO was used for the analysis and p-values below 0.05 were considered significant.

RESULTS

Figures 1 to 4 show the data of plasma fibrinogen, PAI-activity, CRP and HRG in 20 individuals (sampled repeatedly up to 9 times) over a period of 6 months. Both intra- and interindividual variation is obvious for all variables. The estimated contributions of the intra- and interindividual variation to the total variance is given in table 1. The contributions of the intraindividual variation of a parameter to its total variation were 1% for HRG, 4% for log(PAI-activity), 13% for fibrinogen and 14% for log(CRP), which was much smaller for each parameter than the interindividual variation, which ranged between 86% and 99%. Information from the questionnaire that the participants of the longitudinal study filled in at every visit could explain only a limited number of the peak values.

The descriptive statistics of the transversal analysis are presented in table 2. The plasma levels observed in this enlarged group (n=59) are comparable to the levels in the longitudinal group (n=20).

In table 3 the influence of the *Bcl*1 and G/A^{-455} polymorphisms on the plasma levels of fibrinogen are shown. Individuals with the rare allele have higher fibrinogen levels. The *Bcl*1 and G/A^{-455} fibrinogen polymorphism were closely linked in this group. Taking the habitual levels (mean of all longitudinal samplings) from

Table 2. Mean range (central 95%) of plasma fibrinogen, PAI-activity, CRP and HRG levels in the transversal study with 59 healthy volunteers. The arithmetical mean is given for fibrinogen and HRG, the geometrical mean for PAI-activity and CRP.

	n_{obs}	mean	(range of central 95%)
fibrinogen (g/L)	59	2.64	(1.85-3.43)
PAI-activity (IU/ml)	59	8.81	(2.57-30.21)
C-reactive protein (mg/L)	59	0.83	(0.12-6.00)
HRG (%PP)	59	99.2	(49.4-149)

Table 3. Influence in the transversal study and on the habitual level of the longitudinal study of genetic polymorphisms on plasma fibrinogen levels, adjusted for age, gender, and BMI.

		fibrinogen (g/L)			habitual fibrinogen (g/L)		
RFLP		n_{obs}	mean	SEM	n_{obs}	mean	SEM
BclI	B1B1	42	2.58	0.06	15	2.54	0.05
	B1B2/B2B2	14	2.91*	0.08	5	2.98***	0.09
G/A^{-455}	G/G^{-455}	36	2.57	0.06	15	2.54	0.05
	$G/A^{-455}/A/A^{-455}$	18	2.81**	0.10	5	2.98***	0.09

* significantly different from individuals with genotype B1B1 ($p=0.006$)
** significantly different from individuals with genotype G/G^{-455} ($p=0.03$)
*** significantly different from individuals with genotype B2B2 or genotype G/G^{-455} ($p=0.002$). Since the genotypes were in complete linkage disequilibrium, the data are the same for both RFLP.
n_{obs} number of individuals
SEM standard error of the mean

the individuals in the longitudinal study a slightly larger effect of the polymorphism on the plasma fibrinogen levels was observed. In the longitudinal study we found no association between the intraindividual variation and the fibrinogen genotypes using ANOVA analysis.

The *Hind*III PAI polymorphism was not associated with the levels or the intraindividual variation of PAI-activity. The CA repeat polymorphism of the PAI gene showed many different genotypes, as expected for a multiple allele polymorphism. No effects of any of the alleles were detectable for either the (habitual) level or the intraindividual variation of PAI-activity.

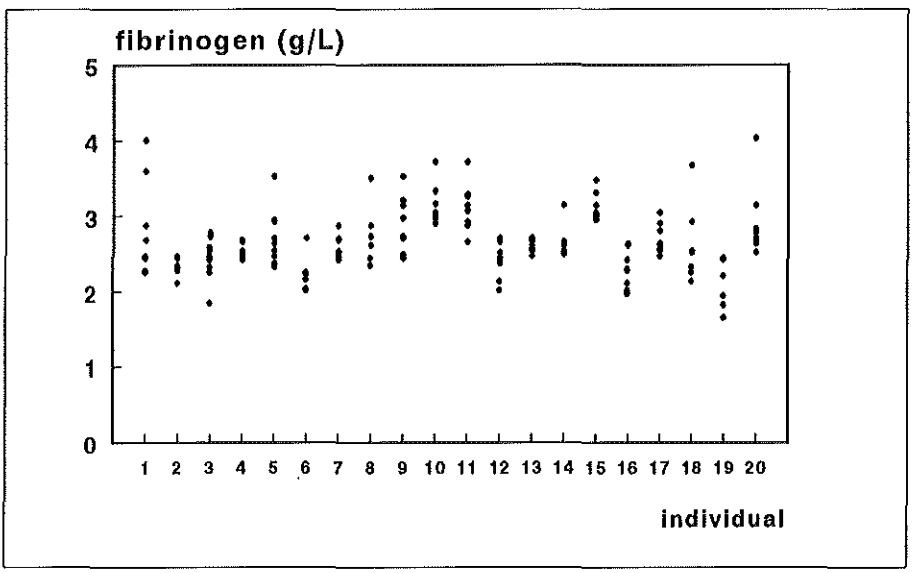

Figure 1. Plasma fibrinogen levels in 20 individuals on up to 9 samplings over a 6 months period. Each dot represent the level of one sampling.

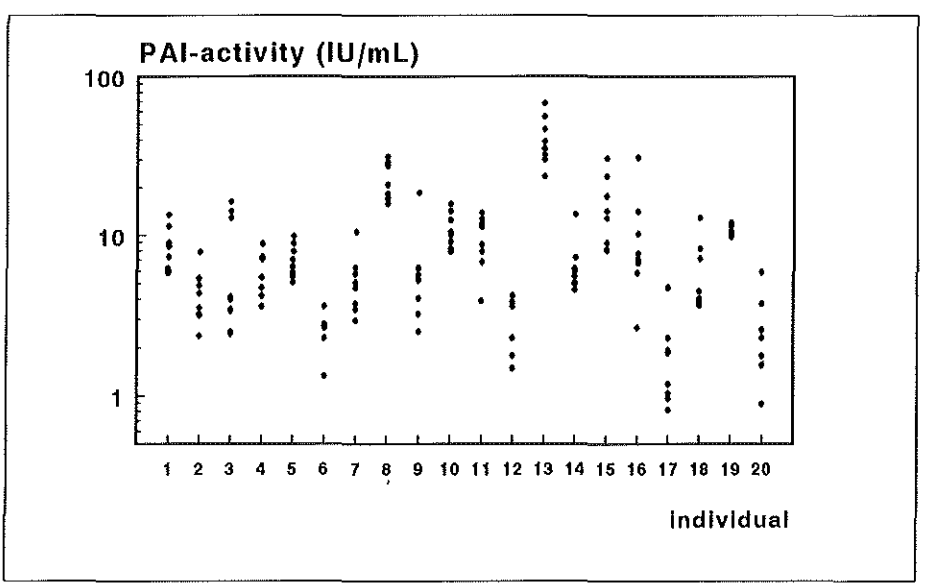

Figure 2. Plasma plasminogen activator inhibitor (PAI)-activity levels in 20 individuals on up to 9 samplings over a 6 months period. Each dot represent the level of one sampling.

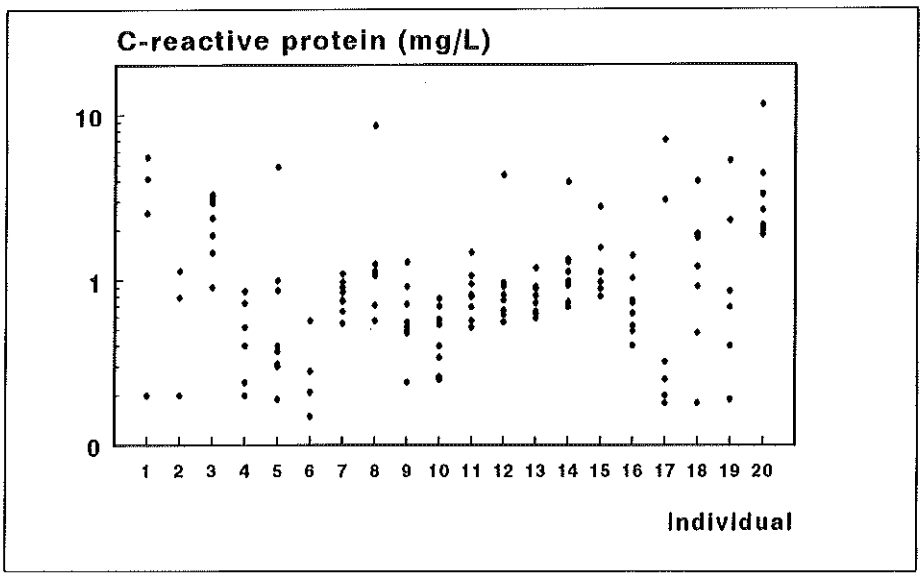

Figure 3. Plasma C-reactive protein levels in 20 individuals on up to 9 samplings over a 6 months period. Each dot represent the level of one sampling.

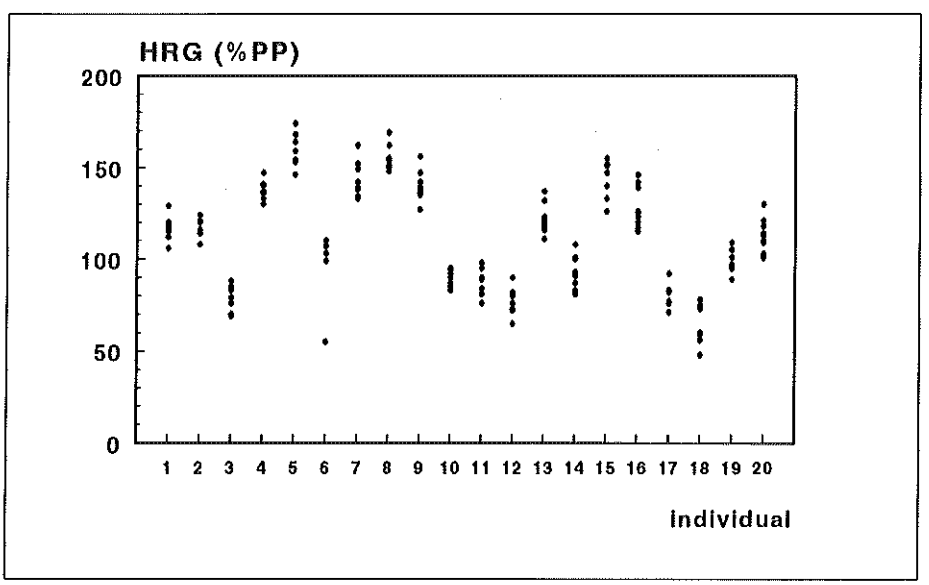

Figure 4. Plasma histidine-rich glycoprotein (HRG) levels in 20 individuals on up to 9 samplings over a 6 months period. Each dot represent the level of one sampling.

DISCUSSION

Because elevated fibrinogen, PAI-activity and CRP levels have gained interest as risk indicators for cardiovascular disease[1-8] and elevated HRG is associated with thrombosis[9,10] it has become important to accurately know the habitual levels of these parameters. Until now, single fibrinogen, PAI-activity, CRP and HRG measurements are accepted to represent habitual levels. This might be inaccurate as the plasma levels of these proteins are for instance influenced by a transient acute phase reaction, which may lead to temporary variations in the plasma levels. In the present study we documented the inaccuracy of estimating habitual levels when only 1 plasma sample is studied and how many samples should be taken to obtain estimates of habitual plasma levels of fibrinogen, PAI-activity, CRP and HRG.

In a longitudinal study of 9 blood samplings in 20 individuals we showed that for plasma fibrinogen levels the intraindividual variation has an contribution of 13% to the total variance. It is required to perform multiple sampling in order to decrease the contribution of this intraindividual variation and to obtain an estimate of the habitual level that is accurate when compared to the interindividual variation. With 2 random samplings of plasma fibrinogen levels the contribution of the individual variation will be reduced to less than 10%, indicating a correlation between the interindividual variance and the total variance above 0.90, which we used as an arbitrary unit. In this study the period between the samplings was 3 weeks. Peak values were not consistent in consecutive samplings, indicating that a period between samplings of 3 weeks is sufficient for disposing of peak effects.

To compare the interindividual variation in our group with the variation reported in other studies, we calculated the coefficient of variation (CV) in a transversal part of the study on 59 healthy individuals (single sampling). We found a CV of 0.15, which is in good agreement with the results of Marckmann et al[43] and Thompson et al[44]. It was calculated that a sample size of 142 (2 groups of 71) individuals will be needed to differentiate two groups with a difference of 1 standard deviation of the mean. We choose this example of 1 standard deviation because in the Northwick Park Heart Study[1] it was shown that this difference will give a 84% increase for the risk of cardiac events in the next five years.

When we studied the variation in the logarithmically transformed PAI-activity data, we observe that almost all variance can be ascribed to the interindividual variation. The intraindividual variation is well below 10% and therefore single sampling is sufficient. The sample size required to measure a difference of 15% of the log(PAI-activity) between two groups is 162 individuals. For PAI-activity, CRP and HRG the association between increase and cardiac risk is not so well established as for fibrinogen. Since 1 standard deviation of fibrinogen is 15% of the mean level, we calculated the sample size for 15% differences between the groups.

For the logarithmically transformed CRP levels we find a 14% contribution of the intraindividual variation to the total variance. The mean of two samplings will give a

good estimation of the habitual level. However, if we study figure 3, we frequently observe outliers. Therefore, in assessing the habitual level, we advise to perform three of more measurements to be able to identify and exclude these outliers. For the same reasons as for fibrinogen, a 3 week period between sampling can be used. The sample size required to measure a difference of 15% of the log(CRP) between two groups is 412 individuals. In this study the contribution of the analytical variance was not analyzed separately, but it is included in the intraindividual variation. For fibrinogen measurements with the Clauss method it has been described[44] that the analytical variance is only a small part of the intraindividual variation. For the other methods the variation is already below 10% so that reduction of the analytical variation will not be required.

The intraindividual variation of plasma HRG level is very small (only 1% of the total variance) and can therefore be neglected. Multiple sampling is thus not warranted to determine the habitual HRG level. The sample size required to measure a difference of 15% of the HRG level between two groups is 86 individuals.

If duplicate sampling will be performed, the required sample size for fibrinogen will go from 142 to 133, while for CRP it will go from 412 to 381. This implies that when the intraindividual variation is relatively low, multiple sampling will hardly influence the number of individuals that need to be studied in an epidemiological study. However, for the reliable determination (10% criterium) of the habitual level of an individual, multiple sampling will be necessary.

The genetic polymorphisms of the Bß-fibrinogen gene showed an association with the plasma fibrinogen levels obtained by simple sampling in the transversal group. These findings are in agreement with other studies in healthy volunteers[33,34]. The association between the habitual levels (mean of up to nine samplings) of the individuals of the longitudinal study and genotypes of the Bß-RFLP were more significant and the difference between the levels was somewhat larger as expected. Since the association was already significant with single sampling, multiple sampling will not give an essential improvement.

No association was found between the genotypes and the intraindividual variation in the longitudinal group. The genotypes of *Hind*III and CA-repeat polymorphisms of the PAI gene did not associate with either habitual plasma levels or intraindividual variation. The association was also not significant when the habitual log(PAI-activity) levels of individuals in the longitudinal study were evaluated. It might be interesting to study the contribution of genetic elements to the variation of fibrinogen and PAI-activity in a larger study, because there are reports about different reaction to acute phase stimulation (e.g. smoking) in individuals with different G/A^{-455} genotypes of the Bß-fibrinogen RFLP.

In conclusion, we observed that the intraindividual contribution of the total variance in a longitudinal study of fibrinogen, PAI-activity, CRP and HRG is lower than the interindividual variation. However, multiple sampling is advised for

fibrinogen to decrease the contribution of the intraindividual variation under 10% and for CRP to exclude the outliers. A contribution of genetic polymorphisms of the Bß-fibrinogen gene to the interindividual, but not the intraindividual, variation was observed.

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CHAPTER 7

EFFECTS OF FISH OIL AND VITAMIN E ON THE CARDIOVASCULAR RISK INDICATORS FIBRINOGEN, C-REACTIVE PROTEIN AND PAI ACTIVITY IN HEALTHY YOUNG VOLUNTEERS.

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ABSTRACT

High plasma levels of fibrinogen, C-reactive protein (CRP) and plasminogen activator inhibitor (PAI) activity are associated with an increased risk of ischaemic heart disease and stroke. There are indications that N-3 fatty acids are involved in the production of these proteins, but only in the presence of an adequate amount of the antioxidant vitamin E.

In this study we have evaluated the effect on plasma levels of fibrinogen and CRP of fish oil containing N-3 fatty acids and the natural antioxidant vitamin E. 11 healthy young volunteers with fibrinogen and CRP levels in the normal range were supplemented for one week with 30 g/day fish oil, containing 60 IU vitamin E. No effects on fibrinogen and on CRP were found, indicating that fish oil has no effect on basal levels of fibrinogen and CRP. The effect of vitamin E on fibrinogen, CRP and PAI activity levels was evaluated in 20 healthy volunteers (age 21-31) who received vitamin E (dose doubling every 2 weeks from 25 to 800 IU/day) for 12 weeks. No significant changes in fibrinogen, CRP and PAI activity levels were found.

From these studies we conclude that it is unlikely that short term supplementation with fish oil or vitamin E influences the levels of the acute phase reactants fibrinogen, CRP or PAI activity in healthy young volunteers with normal levels of these risk indicators.

INTRODUCTION

High plasma levels of fibrinogen[1,2], C-reactive protein (CRP)[1] and plasminogen activator inhibitor (PAI)[3] are associated with an increased risk of myocardial infarction and stroke. Plasma levels of the three proteins are increased during the acute phase reaction. It has been suggested that the actual factor which may increase the risk of heart disease and stroke is a chronic local inflammatory state and that the plasma levels of fibrinogen, CRP and PAI are indicators thereof and as such positively associated with risk[1,4].

The major initiators of increased synthesis of acute phase proteins in the liver are the cytokines interleukin-6 (IL6), interleukin-1ß (IL1ß) and tissue necrosis factor-α (TNFα)[5]. Activated monocytes and other activated cells of the reticulo-endothelial system are major sources of these cytokines. N-3 fatty acids reduce the response of monocytes to stimuli which may result in a reduced production of cytokines[6]. On the other hand, polyunsaturated fatty acids in LDL, if not well protected by antioxidants, are more susceptible to oxidation. There is accumulating evidence that oxidation of LDL will lead to activation of cells in the vascular wall to produce cytokines, resulting in a local inflammatory reaction[4]. Vitamin E protects LDL against oxidative modification[7] which may explain the observation in epidemiological studies that there is an inverse relation between intake and plasma levels of dietary

antioxidants and the risk of myocardial infarctions[8,9].

Recently, Haglund et al[10] reported effects of fish oil supplemented with vitamin E on fibrinogen levels also in healthy volunteers rendering it possible that basal synthesis of fibrinogen is influenced. The aim of this study was to evaluate in healthy volunteers the effect of fish oil and the antioxidant vitamin E on fibrinogen and in addition PAI activity and to document the acute phase condition by measuring CRP.

VOLUNTEERS AND METHODS

Volunteers

To evaluate the effect of fish oil containing a moderate dose of vitamin E, 11 healthy volunteers (age 18-22) were supplemented with fish oil (Maxepa: 30 gram daily containing 5.4 g EPA, 3.6 g DHA, 60 mg Vitamin E) for one week.

To evaluate the effect of vitamin E, 20 healthy volunteers (age 21-31) were given vitamin E (consecutively 25-50-100-200-400-800 mg/day, each for 2 weeks) for 12 weeks.

Methods

(HMW+LMW) Fibrinogen levels were determined in EDTA plasma using an enzyme immuno assay (EIA)[11]. In this assay a monoclonal antibody against the carboxyl terminal end of the fibrinogen Aα-chain is used as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain (Y18) as the tagging antibody. Thus the EIA will only measure HMW and LMW fibrinogen.

C-reactive protein was measured with an EIA based on rabbit polyclonal antibodies against human CRP. One volunteer with CRP levels > 10 mg/l was excluded, because this high level is an indication of substantial inflammation.

PAI activity was measured according to Verheijen[12].

Statistical methods

Because CRP and PAI activity were positively skewed, the data were transformed logarithmically before analysis. Mean (for CRP and PAI the geometrical mean) and its 95% confidence interval are presented. The effects of fish oil and vitamin E treatment were studied with the paired Student t-test. Correlations were calculated with the Pearson correlation test. All statistical methods were performed with the SOLO computer program.

RESULTS

In the eleven young volunteers the one week treatment with 30 g fish oil plus 60 mg vitamin E per day had marked effects on plasma fatty acids (mean decrease of 39%)

and on triglyceride levels (mean decrease 36%), as reported previously[13]. In contrast, neither fibrinogen nor CRP levels did show a significant change (table 1). In accordance with the differences in behaviour of fibrinogen and CRP versus triglyceride and fatty acids, no relations were observed between these two categories of variables.

In the twenty healthy young volunteers receiving up to 800 mg/day vitamin E, plasma vitamin E levels showed an increase from 23.6 ± 3.6 μM to 61.2 ± 11.8 μM. Triglyceride and total cholesterol levels did not change (data not shown). No significant changes in fibrinogen or CRP activity were found (table 2); in accordance, using the Pearson correlation test, significant correlations of 0.74 and 0.78 between the levels before and after treatment were found for fibrinogen and CRP, respectively. For PAI activity we also found no significant changes, but a correlation between pre- and post-values was absent.

Table 1. Mean (95% confidence interval) of plasma fibrinogen and CRP levels before and after 1 week fish oil administration in 11 healthy young volunteers.

	before fish oil	after fish oil
Fibrinogen (g/L)	1.9 (1.7 -2.1)	1.7 (1.6-1.9)
CRP (mg/L)	0.83 (0.47-1.5)	0.91 (0.54-1.5)

Table 2. Mean (95% confidence interval) of plasma fibrinogen, CRP and PAI activity levels before and after 12 weeks vitamin E supplementation in 20 healthy young volunteers.

	before vitamin E	after vitamin E
Fibrinogen (g/L)	1.8 (1.6 -2.0)	1.8 (1.5-2.1)
CRP (mg/L)	0.37 (0.19-2.0)	0.29 (0.16-0.56)
PAI (IU/mL	5.0 (4.6-6.1)	6.2 (5.3-7)

DISCUSSION

One requirement for reducing plasma fibrinogen levels by fish oil treatment on fibrinogen levels might be an adequate amount of vitamin E. Haglund et al[10] showed in 12 healthy volunteers that fibrinogen levels decreased after supplementation with 30 ml fish oil containing 45 mg vitamin E per day, whereas no effects were found when the volunteers were given 30 ml fish oil with 9 mg vitamin E per day for a three week period.

We found no effects on fibrinogen levels after administration of fish oil with relatively high vitamin E levels or after administration of moderate to high doses (up to 800 mg/day) of vitamin E for 12 weeks. Although our studies had a different

design, our results can not confirm the results of Haglund.

A possible explanation for the absence of fibrinogen decrease in our fish oil study might be the duration of our fish oil administration, which was only one week in our study, while Haglund gave his volunteers fish oil for three weeks. The treatment period with vitamin E, however, should have been sufficient (12 weeks).

An analysis of the literature reveals that the studies showing a decrease of fibrinogen levels concerned patients with increased fibrinogen levels. A prolonged low grade acute phase reaction is the main mechanism known to increase habitual fibrinogen levels. Therefore we documented in our volunteers the level of the acute phase reactant CRP. Our volunteers had no increased fibrinogen or CRP levels at the start of the study. Our findings of absence of a change in both fibrinogen and CRP would therefore support the above analysis that fish oil or vitamin E might be mainly active in case of elevated levels of these components.

We conclude that in young, healthy volunteers without signs of a stimulated acute phase reaction, supplementation with vitamin E has no effect on the mentioned variables. It remains to be tested whether effects can be found in individuals with elevated levels of fibrinogen and/or CRP, e.g. smokers.

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CHAPTER 8

THE EFFECT OF TICLOPIDINE UPON PLASMA FIBRINOGEN LEVELS IN PATIENTS UNDERGOING SUPRAPUBIC PROSTATECTOMY

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ABSTRACT

The mechanisms of the antithrombotic effect of the platelet aggregation inhibiting agent Ticlopidine might include a decrease of the plasma fibrinogen level. The effect of ticlopidine on increased fibrinogen synthesis following trauma, such as surgery, is however not known. 46 patients who underwent suprapubic prostatectomy were randomized to receive either (group A) Ticlopidine (2 x 250 mg daily) from the second preoperative day until the seventh postoperative day or (group B) placebo up till the day of surgery and further acenocoumarol against post-operative thrombosis.

We measured the plasma fibrinogen levels pre- and post-operatively and observed that the level and in particular the rise of the plasma fibrinogen concentration was not different in the two groups. It is concluded that compared with the standard treatment in group B ticlopidine does not influence trauma-induced fibrinogen increase.

INTRODUCTION

Ticlopidine has been reported to reduce the number of thromboembolic events and of restenosis in patients undergoing haemodialysis[1] and cardiac surgery[2,3]. It also improves the walking ability in patients with intermittent claudication[4,5].

The main effect of Ticlopidine appears to be on the first and second phase of the blood platelet aggregation induced by ADP, collagen and platelet aggregation factor[6,7]. Varying effects are reported concerning the effect on the aggregation through the arachidonic pathway and in contrast to aspirin, ticlopidine does not inhibit cyclo-oxygenase[8]. The activity is suggested to be mediated through blocking platelet fibrinogen receptors[9] or altering platelet ADP-binding[10,11]. However, no exact biochemical mechanism of ticlopidine has been established, and in view of discrepancies between in vitro and in vivo effects, the formation of at least one active transient metabolite is surmised[12].

Recently, an additional effect of Ticlopidine in patients with peripheral atherosclerotic disease or ischaemic cerebrovascular disease has been reported; namely a decrease in the plasma fibrinogen level[13-17]. This finding is particularly interesting because an elevated plasma fibrinogen level is a risk factor for arteriosclerosis, stroke and myocardial infarction[18]. The fibrinogen decreasing effect has sofar only been observed in patients, with the exception of diabetic subjects[19] and no information of effects in healthy volunteers is available as yet.

It remains possible therefore that the effect of ticlopidine is confined to situations with induced increases in fibrinogen. In this context we studied the effects of ticlopidine in a group of patients who underwent an urologic operation, viz suprapubic prostatectomy[20]. Post-operatively there always is an increase in the plasma fibrinogen levels, induced by surgical trauma.

PATIENTS AND METHODS

Patients

Fifty consecutive patients admitted to the urological ward for open prostatectomy were - with informed consent - randomly allocated to one of two groups (A or B). Group A received Ticlopidine, 250 mg twice daily from the second pre-operative until the seventh post-operative day; group B received a placebo from the second pre-operative day until the day of surgery. Post-operatively, patients in group B were treated with acenocoumarol like the other patients on the ward not participating in the study. The patients and technicians performing the leg scanning were not informed about the medication.

Excluded from the study were patients with serious liver or renal disease, haemorrhagic diathesis, or patients who received drugs that could interfere with platelet function. Post-operatively, all patients were treated with Baralgin^R to prevent painful bladder spasms. Patients taking part in this trial did not receive ϵ-aminocaproic acid. Of the fifty patients who entered the study, four could not be evaluated. Two patients appeared on return from the operation room to have undergone a trans-urethral prostatectomy, one patient turned out to suffer from a bladder carcinoma. One patient was excluded from the trial because of technical errors in the leg scanning (belonged to group B).

Eventually group A consisted of 26 patients, group B of 20. There were no differences between the two groups regarding risk factors for heart and vessel disease. More information on this patient group, platelet aggregation inhibition, bleeding complications, and isotopic DVT can be found in ref. 21.

Methods

Plasma fibrinogen levels were measured on day -3 (start of the study), day 0 (day of the operation) and day +3 by the Clauss method[22] in platelet-poor plasma (PPP).

Patient compliance was assessed by platelet ADP-aggregation. Aggregation was measured in a Peyton aggregometer: 9 ml of blood was collected in 1 ml 0.109 M Na citrate, centrifuged for 15 min at 100 x g for the preparation of platelet-rich plasma (PRP). Two ml of this PRP was centrifuged for 10 min at 1500 x g in order to obtain PPP. The aggregation tests were performed between 1 and 2 hours after collection of the blood. To induce aggregation, ADP (Sigma Chemie) was added to PRP with a final concentration of 0.5 μg/ml. Aggregation was expressed as percent of maximal aggregation.

Thrombosis was detected by ^{125}I-fibrinogen uptake test[23].

Data analysis was performed by non-parametric tests for both independent and paired samples. Any probability equal or less than 0.05 is considered to represent a significant difference between the data studied.

RESULTS

The biological effect of Ticlopidine was monitored by following the ADP-induced aggregation of the patients' blood platelets. Ticlopidine inhibited the ADP-induced aggregation as shown in Fig. 1. In the placebo group, some impairment of aggregation was also seen, but on day 0, the ADP-induced aggregation was already significantly more reduced in the Ticlopidine group compared with the control group.

Both the Ticlopidine group and acenocoumarol group showed significantly increased fibrinogen levels on day 3 compared with preoperative levels ($P < 0.001$) (Fig. 2). The levels were not significantly different in the two groups ($P > 0.20$). The individual rise in postoperative fibrinogen levels was also not different in the two groups.

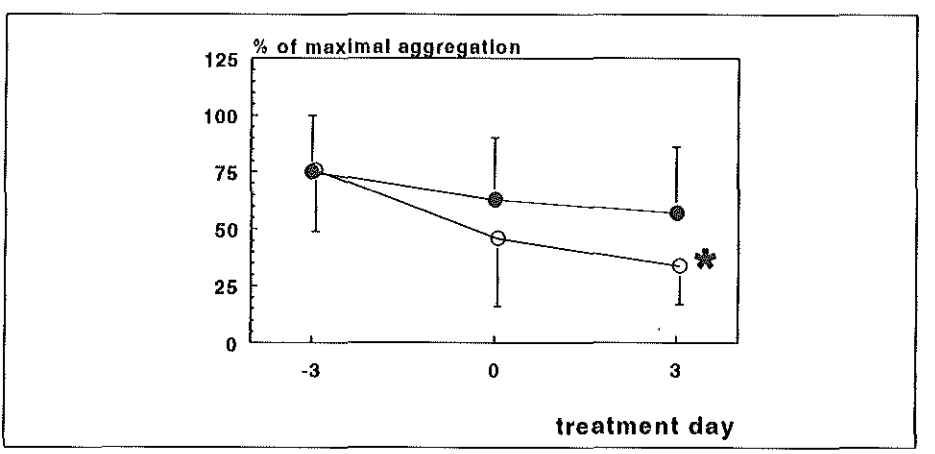

Figure 1. ADP induced aggregation in group A (●) and group B (○). (*$p<0.01$)

During the whole study only one case of clinically detectable thrombosis was encountered; this was a patient in the placebo group who showed transient signs of deep vein thrombosis (DVT) in his left calf. By means of the ^{125}I-fibrinogen uptake test DVT was detected in four patients of group A (12.7%) and in seven patients in group B (28.5%), including the patient with clinical thrombosis, all within the first three postoperative days. This difference is not significant ($p = 0.23$). In group A only one case of bilateral thrombosis was found, in group B four cases[21].

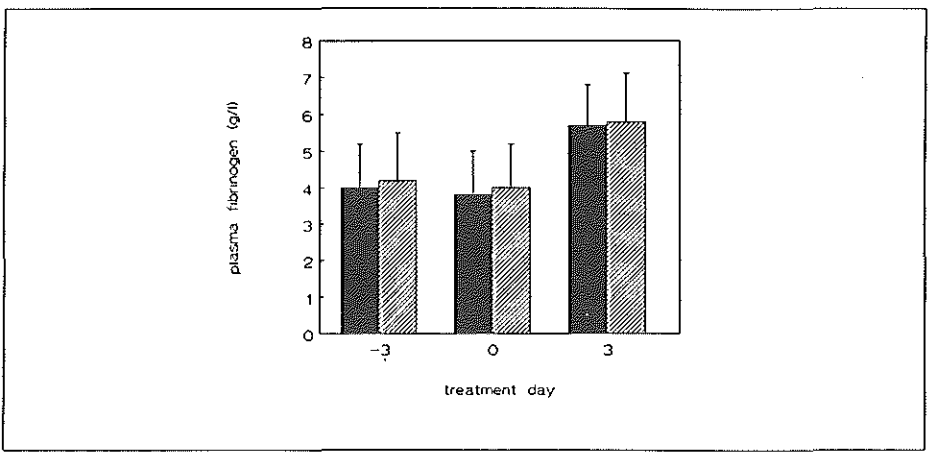

Figure 2. Mean plasma fibrinogen levels on days -3, 0, and +3 in group A (●) and group B (○).

DISCUSSION

A significant reduction in the ADP-induced platelet aggregation from the day of surgery onwards witnessed the biological effect of ticlopidine in the treatment group, where ticlopidine was given from day -3 onwards. As it is unethical to withhold anticoagulants after surgery the control group was postoperatively treated with acenocoumarol.

The fibrinogen levels in the ticlopidine group and the placebo-acenocoumarol group were similar at the start of the study (day -3) and no effect on these basal levels was apparent at day 0 pre-operatively. It indicates that ticlopidine had no effect on the constitutive synthesis of fibrinogen in this group of men. In men of this age group an enlarged prostate is considered normal, therefore they can be considered healthy. Until now, all reports of ticlopidine effects on plasma fibrinogen levels dealt with patient groups[13-17]. It is therefore possible that the observed fibrinogen lowering only occurs in disease and is, in fact, a normalization of the elevated fibrinogen levels. On the other hand, these studies describe fibrinogen decrease after a treatment period of several months[13-17]. It is possible that our treatment period was too short to show an effect on the base-line levels as the biological half life of fibrinogen is 2½ days.

The post-operative rise in fibrinogen concentration is a measure for the acute phase stimulated production. This rise was not different in the two groups, indicating that ticlopidine is not capable of preventing the post-operative rise of the fibrinogen concentration. In other studies[14,24] it has been suggested that the action of ticlopidine on the fibrinogen level is an anti-inflammatory effect. Randi[24] hypothesized that ticlopidine acts by inhibiting the exposure of fibrinogen receptors on platelets which results in a decrease in the fibrinogen levels. After major surgery there is important

platelet stimulation, but we could not observe a reduction of the fibrinogen rise. Therefore, it is unlikely that this mechanism plays a major role in the acute increase of fibrinogen levels after surgery.

Palereti et al[13] report a longitudinal study where the fibrinogen levels increased gradually in the placebo group. The end of the 21 month follow-up study coincided with the winter months. The elevated incidence of illness in the winter may contribute to this increase. In the ticlopidine group, this relation was not seen. Therefore it is possible that a reduction of disease induced fibrinogen elevation may be the underlying mechanism.

Further studies that measure other acute phase proteins (e.g. C-reactive protein) and cytokines (e.g. interleukin-6) may elucidate the effect of ticlopidine on the acute phase induced increase of fibrinogen levels.

We can only conclude that, after operations with a high post-thrombotic risk, the fibrinogen increase is not significantly altered by ticlopidine in its magnitude, in comparison with the acenocoumarol treatment. That both treatments have reduced the fibrinogen increase to a similar extent is not probable, since no effect of acenocoumarol on fibrinogen levels has been described in the literature.

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CHAPTER 9

MODULATION OF PLASMA FIBRINOGEN LEVELS BY TICLOPIDINE IN HEALTHY VOLUNTEERS AND PATIENTS WITH STABLE ANGINA PECTORIS.

Contributions of the acute phase reaction, fibrin(ogen) degradation and genetic polymorphisms of fibrinogen.

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ABSTRACT

Elevated plasma fibrinogen levels represent an increased risk for cardiac events. Ticlopidine is a drug that inhibits the ADP-induced aggregation of blood platelets and it also has been described that ticlopidine can decrease the plasma fibrinogen level in patients with vascular diseases. The mechanism of this decrease has not yet been elucidated and therefore mechanisms that are known to affect fibrinogen levels were studied, viz. the acute phase reaction, total fibrin and fibrinogen degradation (TDP) levels and the fibrinogen G/A^{-455} Bß-chain polymorphism.

The fibrinogen lowering effect of ticlopidine was studied in 26 healthy volunteers and in 26 patients with stable angina pectoris in a double blind, placebo controlled, randomized cross-over study. Plasma fibrinogen levels were measured with the Clauss assay and enzyme immuno assay for high plus low molecular weight (HMW+LMW) forms of fibrinogen. C-reactive protein and TDP levels were measured with an EIA.

In the healthy volunteers the functional fibrinogen levels had decreased 0.20 g/L (9%, $p<0.05$) after 4 weeks of ticlopidine administration, whereas the HMW+LMW fibrinogen levels, the CRP and the TDP levels were not significantly decreased. In the stable angina pectoris patients the baseline fibrinogen, CRP and TDP levels were significantly higher than in the volunteer group. After four weeks ticlopidine administration the functional fibrinogen levels had decreased with 0.39 g/L (11%, $p<0.005$), whereas the HMW+LMW fibrinogen, the CRP and the TDP levels were not significantly changed. Neither in the volunteers or the patients was the effect of ticlopidine on the fibrinogen levels associated with the fibrinogen G/A^{-455} genotype.

Therefore, the fibrinogen lowering effect of ticlopidine is unlikely to be regulated by the acute phase reaction, TDP-levels or the fibrinogen G/A^{-455} Bß-chain polymorphism.

INTRODUCTION

It has been shown repeatedly that elevated plasma levels of fibrinogen indicate a risk for the development of cardiovascular diseases[1-7], peripheral arterial disease[8] and stroke[9]. After adjusting for other risk factors such as cholesterol levels and smoking the fibrinogen levels remained significantly associated with risk of atherothrombotic events. Additionally, the plasma levels of fibrinogen are associated with the severity of atherosclerosis[10-12]. Since it has been suggested that atherosclerosis is an inflammatory process of the vascular wall[13], the increased plasma levels of the acute phase protein fibrinogen that are observed in patients with cardiovascular disease[12-16] might reflect an elevated inflammatory status of the vascular wall of these patients.

Although a causal relation between elevated plasma fibrinogen levels and increased risk of atherothrombotic events has not yet been definitively established, much

interest has developed for agents that can lower the fibrinogen levels. An agent that has shown to have a decreasing effect on fibrinogen levels in patients with coronary diseases is ticlopidine[17-25]. The main effect of ticlopidine is the inhibition of platelet aggregation and for that reason it is consequently used in the reduction of atherothrombotic events[26-28]. Ticlopidine is not functional in itself, but is transformed in an active, not yet identified, metabolite[26].

Although the additional fibrinogen lowering effect of ticlopidine has repeatedly been observed, the underlying mechanism still has to be elucidated. Studying patients that already have elevated plasma fibrinogen levels, makes it impossible to differentiate between normalization and systemic decrease of the basal fibrinogen levels. Such a separation of mechanisms can only be made when both patients and healthy volunteers are studied, but these studies have not yet been performed.

The most important mechanism that regulates the plasma fibrinogen levels is the acute phase reaction, which can lead to a two to fourfold increase. In patients with vascular disease a modest increase of the inflammatory state has been observed, demonstrated by increased plasma levels of several markers of inflammation, such as fibrinogen, interleukin-6, leucocyte receptors on vascular endothelium, complement[16,30-32]. If ticlopidine influences the acute phase status, it may involve the cytokine secretion of activated cells, resulting in a normalization of the plasma fibrinogen levels. Additionally, the plasma levels of other acute phase markers, like the strong acute phase reactant C-reactive protein, are then expected to decrease.

Upregulation of fibrinogen synthesis in response to increased fibrin(ogen) degradation was indicated by a correlation between plasma fibrinogen levels and fibrin(ogen) degradation products (FDP)[33]. Ticlopidine is known to inhibit the aggregation of blood platelets by preventing the exposure of the glycoprotein IIb/IIIa complex, which is the receptor of fibrinogen on platelets[34]. This decrease of the fibrinogen binding to platelets might be a mechanism eventually leading to lowering of the production of FDP and consequently to lowering of the plasma fibrinogen levels, since it has been reported that FDP stimulate the fibrinogen synthesis of cultured human hepatocytes[35-37]. This possible mechanism would imply that ticlopidine administration lowers fibrinogen through lowering of the FDP levels.

The studies that have been performed until now have all measured the functional fibrinogen levels. A lowering of the fibrinogen levels that are measured with functional tests (viz. the Clauss test[38]) might also be explained by a decrease of the clotting characteristics of the fibrinogen molecules. Methods that measure the fibrinogen antigen levels, like enzyme immuno assays (EIA) will not be altered if it is the functionality of the fibrinogen that is affected by ticlopidine.

Another contribution to the fibrinogen regulation might be genetic. Different genotypes of DNA polymorphisms of the fibrinogen Bß-chain are known to be associated with plasma fibrinogen levels[39-42]. Besides higher fibrinogen levels, it has been described that individuals with the rare allele of these fibrinogen

polymorphisms also have a larger longitudinal variation of their plasma fibrinogen concentration[39]. In individuals with a low grade inflammatory state, e.g. smokers, the effect of the polymorphism is more pronounced[43]. This suggests that the rare allele might be more sensitive to environmental influences which increase fibrinogen synthesis. Consequently, the effect of ticlopidine administration of plasma fibrinogen levels might also depend on the genotypes.

In this study we analyzed the effect of ticlopidine on fibrinogen activity and HMW+LMW antigen levels in a group of healthy volunteers and in a group of patients with stable angina pectoris. We tested the hypothesis that the decrease of plasma fibrinogen levels by ticlopidine a) results from an effect on the acute phase reaction by analyzing also C-reactive protein, b) is an effect on the fibrin(ogen) degradation by studying the correlation between the effect of ticlopidine on fibrinogen and the effect on levels of circulating fibrin(ogen) degradation products and c) whether genetic polymorphisms of the fibrinogen genes contribute to the effect of ticlopidine on plasma fibrinogen levels.

PATIENTS AND METHODS

Subjects

One hundred male Caucasian blood donors of the Rotterdam Red Cross Blood Bank were asked to participate in this study. The first thirteen with the B1B1 genotype and the first thirteen consecutive donors with the B1B2 genotype formed the healthy volunteer group. The men were between 30 and 50 years of age and had no history of vascular disease. Volunteers were not included if they suffered from any disease and did not use any non-study medication. Twenty-six consecutive patients that visited the cardiology outpatient departments of the Medical Centre Alkmaar (n=18) or the University Hospital Rotterdam (n=8), having angina pectoris (NYHA 2-3/4) with evidence of coronary artery disease by either 1) coronary angiography, 2) documented myocardial infarction or 3) a positive exercise test formed the patient group. Patients were not included if they had other diseases than coronary artery disease. The patients only used medication for their cardiac complaints. The stable angina pectoris patients and the healthy volunteers were excluded from the study if they ever had had illnesses with bleeding risk, recent surgery or previous allergic reactions to medication.

Three individuals in the healthy volunteer group and one in the patient group had to leave the study because they developed pruritus, these individuals were replaced. No individual left the study for another reason. The demographic characteristics of the two groups are described in table 1. The study was approved by the Medical Ethical Committees of the Medical Centre Alkmaar and the University Hospital Rotterdam and written consent was obtained from the volunteers and patients in accordance with the Declaration of Helsinki.

Table 1. Baseline data of the healthy volunteers and the stable angina pectoris group. Mean (SD) are given for age and BMI.

	healthy volunteers	patients
age	41.6 (5.2)	65.3 (9.2)
gender	26 male	24 male/ 2 female
BMI	25.3 (4.4)	25.8 (0.6)

Design of the study

This study was a randomized double-blind AB/BA cross-over study. First all groups received placebo for two weeks to let the participants get used to taking the medication. Then the volunteers and the patients were ramdomized and either ticlopidine (250 mg 2 dd) or placebo were administered for four weeks, followed by a wash-out period of two weeks and the second test period in a cross-over design. A wash-out period of two weeks was used, since the platelet aggregation had then returned to normal[26] and because the half life of fibrinogen is 100 hours[44]. Blood was sampled at the start (visit 1), after the two-week adaptation period (visit 2), after 2 (visit 3) and 4 (visit 4) weeks in the first treatment period, after the two-week wash-out period (visit 5) and after 2 (visit 6) and 4 (visit 7) weeks in the second treatment period (see figure 1).

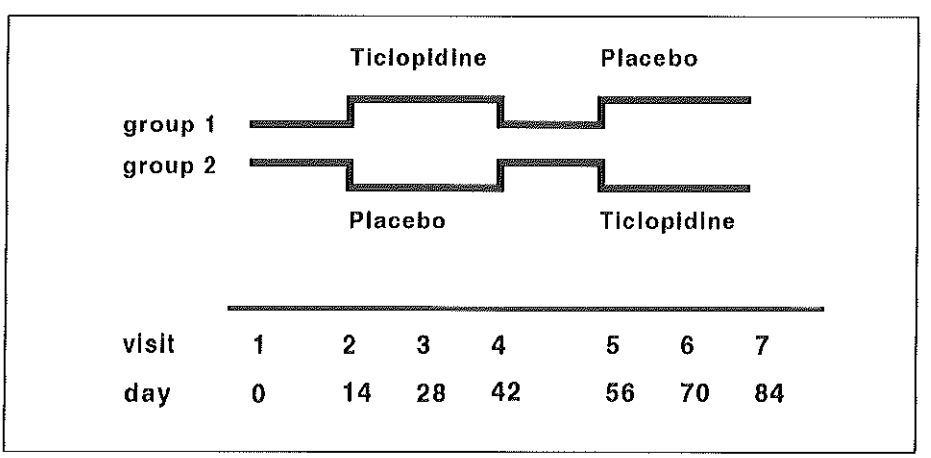

Figure 1. Treatment schedule for the healthy volunteers and the stable angina pectoris patients.

Assays

Platelet count and total white blood cell count were performed at each visit as safety measurements, platelet count had to remain > 150,000/mm^3 and total white blood cell count > 1,800/mm^3. No volunteer or patient ever had levels that were below these limits. The maximal platelet aggregation in platelet rich plasma after induction by ADP 5 μM was determined using a Peyton aggregometer (Salm en Kipp, Breukelen, the Netherlands) at visits 4 and 7 to check the compliance.

Plasma fibrinogen levels were functionally determined by the Clauss method[38].

High plus low molecular weight (HMW+LMW) fibrinogen levels were determined with an enzyme immuno assay (Fibrinostika intact fibrinogen, Organon Teknika, Boxtel, the Netherlands) according to the instructions of the manufacturer. In this assay a monoclonal antibody against the carboxyl-terminal end of the fibrinogen A-chain is used as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain as the tagging antibody (Y18). Thus the EIA will only measure HMW plus LMW fibrinogen, and not LMW' [45].

Total fibrin(ogen) degradation products (TDP) were determined with an enzyme immuno assay (Fibrinostika TDP, Organon Teknika, Boxtel, the Netherlands) according to the instructions of the manufacturer. In this EIA, a monoclonal antibody specific for the *degradation products of fibrinogen and fibrin*, and not reactive with fibrinogen or fibrin, is used as the capture antibody (FDP-14) and the tagging step is done with monoclonal antibodies against fibrinopeptide A (Y18) and D-Dimer (FDP-13), whereby it assesses the total of degradation products of fibrinogen and fibrin[46].

C-reactive protein was determined with an EIA that used rabbit antibodies against human CRP (DAKO, Denmark) as catching and tagging antibody.

DNA polymorphisms (*BclI*, *TaqI*, G/A^{-455}) were determined as described before[42,47]. Briefly, genomic DNA was amplified with the appropriate primers, after which the reaction product was incubated with the respective restriction enzyme. After separation on 2% agarose gels with ethidium bromide the fragments of different length were visualized under ultraviolet light.

Statistical analysis

Because CRP and TDP were positively skewed, the data were logarithmically transformed before analysis. The distribution of the resulting variables, called logCRP and logTDP, was not different from normal. The first analysis addresses the question whether any systematic differences in the composition of the treatment and status groups occur. This was done by (ANOVA) of the baseline levels for both visits 1 and 2 of functional and HMW+LMW antigen fibrinogen, logCRP and logTDP. Adequacy of the length of the wash-out period was determined by ANOVA applied to the data of visit 2 (baseline period 1) and visit 5 (baseline period 2). The fixed factors were visit, group and visit*group, the random factor was patient nested

by group. See Senn page 63[48] for more details. If the visit*group interaction was not significant, the wash-out period was regarded as adequate. The analyses were performed separately for patients and volunteers.

The effect of ticlopidine on the plasma fibrinogen levels in patients and volunteers separately was estimated using a paired student's t-test. The effect of genetic polymorphisms on the plasma fibrinogen levels was assessed using ANOVA with polymorphism group and status as fixed factors.

All computations were done using the statistical package SAS.

RESULTS

Healthy volunteers

The mean (SD) baseline level of functional plasma fibrinogen was 2.35 g/L (SD 0.35) and for LMW+HMW fibrinogen this was 1.97 g/L (SD 0.53)(table 2). The geometrical mean (central 95% range) of CRP was 0.21 mg/L (0.02-2.36) and for TDP this was 0.18 μg/mL (0.03-1.00). The baseline functional and HMW+LMW antigen levels of fibrinogen were not significantly correlated with logCRP or logTDP levels using Pearson's correlation analysis.

The design of this study was AB/BA cross-over and a 14 day wash-out period was applied. Using a general linear model procedure it was observed that the levels after the wash-out period were comparable to the baseline data.

After a 2 week administration of ticlopidine these levels had decreased to 2.18 g/L and after 4 weeks the mean functional fibrinogen levels was 2.16 g/L (SD 0.57) with an average decrease of 0.20 g/L (SD 0.33) (=mean increase of 9%) while the HMW+LMW levels were unchanged. There were no significant changes in the CRP or TDP levels.

The ratio of the functional fibrinogen over the HMW+LMW fibrinogen at baseline and after 4 weeks ticlopidine administration were comparable (1.23 and 1.16, respectively) suggesting that the function of the fibrinogen molecules has not been changed by ticlopidine in the volunteers.

No relation between the *Bcl*1, G/A^{-455} or *Taq*1 polymorphisms and the plasma fibrinogen level was observed. Also no difference in the effect of ticlopidine on fibrinogen levels was observed in individuals with the different genotypes of the *Bcl*1, G/A^{-455} and *Taq*1 polymorphisms.

Patients with stable angina pectoris

The mean (SD) baseline levels in the patient group of functional plasma fibrinogen were 3.44 g/L (SD 0.61) and of LMW+HMW they were 2.70 g/L (SD 0.60)(table 3), both significantly higher than in the healthy volunteers ($p<0.001$). The geometrical mean (central 5-95% range) of CRP was 1.45 mg/L (0.15-14.44) and for TDP this was 0.28 μg/mL (0.05-1.62), also significantly higher than in the

Table 2. (geometric) mean (central 95% range) of functional fibrinogen and HMW+LMW fibrinogen antigen, C-reactive protein (CRP) and total fibrin plus fibrinogen degradation products (TDP) in the healthy volunteers at baseline and after two and four weeks of ticlopidine administration.

		volunteers		
		baseline	2 weeks	4 weeks
functional fibrinogen (g/L)	treatment	2.35 (1.55-2.95)	2.18* (1.09-3.33)	2.16** (1.02-3.30)
	placebo	2.25 (1.25-3.25)	2.17 (1.05-3.29)	2.19 (1.27-3.11)
HMW+LMW fibrinogen (g/L)	treatment	1.97 (0.91-3.03)	2.07 (0.41-3.73)	1.94 (0.68-3.20)
	placebo	2.17 (0.77-3.57)	2.08 (0.78-3.38)	2.26 (0.78-3.74)
CRP (mg/L)	treatment	0.21 (0.02-2.36)	1.19* (0.12-11.59)	0.83 (0.12-5.87)
	placebo	0.69 (0.13-3.78)	0.81 (0.06-11.82)	0.74 (0.08-6.82)
TDP (μg/mL)	treatment	0.18 (0.03-1.00)	0.15 (0.05-0.50)	0.17 (0.03-1.00)
	placebo	0.16 (0.03-0.87)	0.15 (0.03-0.64)	0.14 (0.05-0.40)

* significantly different from the level at baseline ($p<0.05$) using a paired Student's t-test
** significantly different from the level at baseline ($p<0.005$) using a paired Student's t-test

healthy volunteers ($p=0.003$ and $p=0.05$, respectively). The baseline functional fibrinogen levels were strongly correlated with the logCRP levels ($R=0.73$, $p<0.0001$) but not with the logTDP levels. The baseline HMW+LMW fibrinogen levels were not correlated with the logCRP and the logTDP levels.

Analogous to the healthy individuals, it was observed that in the patients the 14 day wash-out period was also sufficient.

After a 2 week administration of ticlopidine the fibrinogen levels were not changed but after 4 weeks the functional fibrinogen levels had decreased to 3.18 g/L (SD 0.62)($p=0.01$) with an average decrease of 0.39 g/L (SD 0.60) (= mean decrease of 11%) while the HMW+LMW levels were unchanged.

The ratio of the functional fibrinogen over the HMW+LMW fibrinogen at baseline was 1.23 and after 4 weeks ticlopidine administration it had slightly increased to 1.28, which was not significant, suggesting that the function of the fibrinogen molecules has not changed by ticlopidine in the patient group.

No relation between the *Bcl*1, G/A^{-455} or *Taq*1 polymorphisms and the plasma fibrinogen level was observed. Also no difference in the effect of ticlopidine on fibrinogen levels was observed in individuals with the different genotypes of the *Bcl*1, G/A^{-455} and *Taq*1 polymorphisms.

Table 3. (geometric) mean (central 95% range) of functional fibrinogen and HMW+LMW fibrinogen antigen, C-reactive protein (CRP) and total fibrin plus fibrinogen degradation products (TDP) in the angina pectoris patients at baseline and after two and four weeks of ticlopidine administration.

		patients		
		baseline	2 weeks	4 weeks
functional fibrinogen (g/L)	treatment	3.56 (2.22-4.66)	3.39 (2.15-4.63)	3.18** (1.94-4.42)
	placebo	3.41 (1.97-4.85)	3.33 (1.85-4.81)	3.42 (2.08-4.76)
HMW+LMW fibrinogen (g/L)	treatment	2.70 (1.50-3.90)	2.68 (0.94-4.42)	2.76 (0.68-4.84)
	placebo	2.75 (0.69-4.81)	2.73 (1.23-4.23)	2.77 (0.81-4.73)
CRP (mg/L)	treatment	1.45 (0.15-14.44)	3.18 (1.94-4.42)	1.86 (0.24-14.30)
	placebo	1.57 (0.13-19.49)	1.40 (0.15-12.94)	1.13 (0.16-8.00)
TDP (μg/mL)	treatment	0.28 (0.05-1.62)	2.36 (0.24-23.57)	0.29 (0.05-1.57)
	placebo	0.29 (0.04-0.61)	0.28 (0.05-1.60)	0.28 (0.06-1.36)

* significantly different from the level at baseline ($p < 0.05$) using a paired Student's t-test
** significantly different from the level at baseline ($p < 0.005$) using a paired Student's t-test

DISCUSSION

In this study possible mechanisms for the fibrinogen decrease by ticlopidine were analyzed in a group of healthy volunteers and in a group of patients with stable angina pectoris. In the healthy volunteers the functional fibrinogen levels decreased 0.20 g/L (9%) after 4 weeks ticlopidine administration and in the patients with stable angina pectoris the decrease was 0.39 g/L (11%).

In the group of healthy volunteers there was no association at baseline between the functional fibrinogen levels and the levels of CRP and TDP. This might suggest that the fibrinogen levels in the healthy volunteers are "true" baseline levels, without an elevation due to stimulation of the fibrinogen synthesis by an acute phase reaction or increased TDPs. A basal state is further suggested by the low levels of CRP and TDP. After 4 weeks of ticlopidine administration a reduction of the functional fibrinogen levels with 0.20 g/L (9%) was observed. In the Northwick Park Heart Study[1] an increase of the plasma fibrinogen level with 1 standard deviation relative to the mean gave a 82% increase for the risk of a myocardial infarction within 5 years. This 1 standard deviation was ± 15% of the mean value, which suggests that the 9% decrease that we observed as a result of ticlopidine administration might have therapeutic significance.

In the stable angina pectoris patients the fibrinogen levels were higher than in the healthy volunteers. This increase is much larger than the 5% increase per 10 years that might be explained by the age difference between the groups. In the patient group the absolute reduction of the functional fibrinogen was much larger than in the volunteers, but the percentage reduction was comparable (11%).

Only the functional fibrinogen levels, and not the CRP and TDP levels were influenced by ticlopidine. This is in accordance with the results of Drouet et al, who found no alterations of the CRP levels or leucocyte counts in patients with peripheral arterial disease who were treated with ticlopidine for three months[25].

Ticlopidine was more effective than aspirin in the prevention of stroke in patients who had experienced transient ischemic attacks were compared in the Canadian-American Ticlopidine Study (CATS)[49]. Fibrinogen lowering could not be observed with the platelet aggregation inhibitor aspirin in healthy controls[50] or patients with coronary artery disease[51,52]. The difference in the effect of ticlopidine and aspirin on the plasma fibrinogen levels might contribute to this difference in efficacy.

The fibrinogen lowering effect of ticlopidine is only observed with the functional Clauss test and not with the HMW+LMW EIA. However, the ratio of these two assays is not changed by the ticlopidine administration in the volunteers or the patients. This suggests that the functionality of the fibrinogen molecule is not affected by ticlopidine. The variability of the EIA is larger (between day CV = 12%) than that of the Clauss assay (between day CV 4.2%), which might explain why no decrease of HMW+LMW fibrinogen is found while functional fibrinogen levels decreased and the ratio of functional over HMW+LMW fibrinogen was not changed.

Both in the healthy volunteers and the stable angina pectoris patients no correlation was observed between the genotypes of the Bß-chain polymorphism and the fibrinogen levels. This is in contrast to several other observations[39,42,53], but some other studies also found no effects[41,54]. This might be explained by the low number of smokers in this study, since only 3 volunteers and 1 patient smoked. It has been reported that the relation between the G/A^{-455} fibrinogen polymorphism and fibrinogen levels in much stronger in smokers[16,43,54]. We further did not observe a relation between the decrease of the fibrinogen levels and this polymorphism.

Since the percentage reduction of functional fibrinogen levels in the volunteers and in the patients was comparable after 4 weeks ticlopidine administration, and since an effect of ticlopidine on the acute phase reaction, the TDP levels or the ratio functional/HMW+LMW fibrinogen were not changed by ticlopidine, it is suggested that the functional metabolite of ticlopidine might directly affect the fibrinogen synthesis by human hepatocytes. A change of quantity rather than quality is also indicated by Drouet et al[25], who found 10% reduction of the fibrinogen levels with several methods (immunoreactive, clottable protein, Clauss, kinetic methods) in patients with peripheral arterial disease that had been treated with ticlopidine.

We conclude that since ticlopidine decreases the fibrinogen levels both in healthy volunteers and in patients with stable angina pectoris, and a contribution of the acute phase reaction and fibrin(ogen) degradation products is not suggested. No effect of DNA-polymorphisms of the fibrinogen Bß-chain was observed. However, further and particularly longer studies will be necessary to further elucidate the mechanism of the fibrinogen decrease by ticlopidine.

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CHAPTER 10

MODULATION OF PLASMA FIBRINOGEN LEVELS BY CIPROFIBRATE AND GEMFIBROZIL IN PRIMARY HYPERLIPIDAEMIA.

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ABSTRACT

Elevated plasma fibrinogen levels are getting increasingly accepted as independent risk indicators of cardiovascular disease. This has enhanced the interest in identifying agents that can normalize elevated plasma fibrinogen levels. One group of agents with this capacity consists of the lipid lowering fibric acid derivatives, e.g. ciprofibrate and gemfibrozil.

We studied the fibrinogen lowering effect of 12-week treatment with ciprofibrate (n=48) and gemfibrozil (n=51) in hypercholesterolemic patients. The correlation of the decrease in fibrinogen with the lipid lowering effects and the contribution of the acute phase and genetic polymorphisms to this decrease were evaluated.

After 12 weeks treatment the fibrinogen levels were significantly decreased ($p < 0.0005$) with both drugs, although the decrease in the ciprofibrate group (3.4 pre-treatment to 2.4 g/L after 12 weeks) was larger than in the gemfibrozil group (3.4 to 3.0 g/L). The lipid lowering effect of the two drugs was comparable and not parallel to the fibrinogen lowering effect. A decrease in the major regulating mechanism of plasma fibrinogen levels, the acute phase reaction, was invoked as underlying mechanism because the increased pre-treatment fibrinogen levels were normalized after treatment. However, pre-treatment C-reactive protein levels were not increased and did not change after treatment. No effects of the polymorphisms of the Bß-fibrinogen chain on the decrease of the plasma fibrinogen levels were observed. This suggests that a new, yet unknown, mechanism is involved in fibrinogen lowering by fibrates.

INTRODUCTION

An elevated plasma fibrinogen level is an important independent risk indicator for the development of cardiovascular disease[1-7]. Prospective studies like the Northwick Park Heart Study[1] and the Framingham Study[2] have shown that the predictive value of elevated plasma fibrinogen levels for developing cardiac events is of the same magnitude as that of elevated cholesterol levels. In the PROCAM study[3] it was shown that the highest cardiac risk was present in individuals with both high fibrinogen and high cholesterol levels.

In this respect it is of great interest to study ways to reduce fibrinogen in individuals with elevated levels. No agents have yet been identified that can selectively affect fibrinogen, but some drugs have been shown, as an additional effect, to decrease plasma fibrinogen levels. For example there are the fibric acid derivatives whose main effect is a reduction of triglyceride and cholesterol levels[8]. Increased plasma fibrinogen levels[9,10] can be decreased by about 20% after treatment with bezafibrate and clofibrate[11-15]. One study with ciprofibrate showed that this drug also decreased plasma fibrinogen by 21% in dyslipidemic patients[16]. Gemfibrozil also belongs to the fibric acid derivatives, but reports on its effect on fibrinogen are

conflicting[13,17-20].

The mechanism of the fibrinogen lowering effect of fibrates is still unknown. The most important mechanism that regulates the plasma fibrinogen levels is the acute phase reaction[21,22]. A role for the acute phase in fibrinogen lowering by fibrates is suggested by the results of Pickart et al who describe that clofibrate suppresses the acute phase stimulated synthesis of fibrinogen in the rat[23]. If the fibrinogen lowering effect of fibrates is a result of a diminished acute phase reaction it is expected that the plasma levels of other acute phase proteins, like C-reactive protein (CRP), will also change.

Regulation of the fibrinogen level can partly be accounted for by genetic factors. Two DNA-polymorphisms (G/A^{-455} and *Bcl*I) of the Bß-chain of fibrinogen have been reported[24-26], and healthy individuals with the rare A^{-455} allele have the highest plasma fibrinogen levels[24,27-29] and the greatest longitudinal variability of these fibrinogen levels[24]. If we use the longitudinal variation as a measure of the sensitivity to factors that regulate the fibrinogen levels, it might be suggested that the fibrinogen lowering effect of lipid lowering drugs (e.g. ciprofibrate) in individuals with the rare allele will be the largest.

In this study we documented the relation between plasma fibrinogen levels and factors that are known to influence these levels in hypercholesterolemic patients and evaluated the fibrinogen lowering effect of ciprofibrate and gemfibrozil and the contributions of the acute phase (reflected by CRP-levels) and of genetic factors.

PATIENTS AND METHODS

Patients

Ninety-nine patients with primary hyperlipidaemia (type IIa and b according to the Fredrickson classification) with a total cholesterol level equal to or higher than 6.5 mmol/L were randomly allocated to treatment with 100 mg/day ciprofibrate (n=48) or 2 times 600 mg/day gemfibrozil (n=51).

The study consisted of a dietary washout period and a placebo period of four weeks each, followed by an active twelve week treatment period. Five visits took place, the first visit was prior to the four week dietary period, the second visit took place prior to the placebo period. The other three visits were planned for, after 6 weeks and after 12 weeks of active treatment.

Age, body mass index (BMI) (weight divided by height^2), gender and smoking habits of the patients were carefully documented. Smokers were defined as individuals who had never smoked or who had stopped smoking more than 10 years ago. Information on smoking habits, however, could not be obtained from all patients; table 1 provides the available data. Basic characteristics are given, for each treatment group, in table 1.

Thirty healthy male blood donors, comparable for age (54.0 (SD 6.4) years) were

used as a reference group for the pre-treatment fibrinogen and CRP data.

At visits two, three, four and five blood samples were collected from fasting patients in trisodium citrate (final concentration 11 mmol/L). The blood samples were immediately put into melting ice. After centrifugation (30 min. 3000 g, 4°C) plasma was collected and stored at -80°C. DNA was isolated from the white blood cells and stored at 4°C.

The study was approved by the Medical Ethical Committee of the Slotervaart Hospital, Amsterdam and written consent was obtained from the patients in accordance with the Declaration of Helsinki.

Table 1. Baseline characteristics of the individuals in the ciprofibrate and the gemfibrozil group. Given are mean (1 SD).

	Gemfibrozil	Ciprofibrate	p
men/women	27/21	32/19	n.s.
age (years)	52.4 (12.5)	51.4 (11.4)	n.s.
smokers/non smokers	17/25	24/16	p=0.07
BMI	25.6 (3.4)	27.1 (4.4)	n.s.
cholesterol (mmol/L)	8.9 (1.8)	8.9 (2.0)	n.s.
Familial combined hypercholesterolemia	8	6	n.s.
Familial hypercholesterolemia	25	33 .	n.s.
polygenetic hypercholesterolemia	15	12	n.s.

BMI is body mass index (weight in kg divided by (length in m)^2)
n.s not significant with $p>0.10$ in the χ^2-test
p gives the difference between the ciprofibrate and the gemfibrozil group

Assays

Functional plasma fibrinogen levels were determined according to Clauss[30].

C-reactive protein levels were determined with an enzyme immune assay, using rabbit anti-human C-reactive protein (Dako, Copenhagen, Denmark) as the catching and the tagging antibody.

The **G/A^{-455} polymorphism** of the ß-fibrinogen gene is assessed as described by Thomas et al.[25]. Briefly, genomic DNA was amplified by polymerase chain reaction (PCR) and digested with the *Hae*III restriction enzyme (Boehringer Mannheim GmbH, Mannheim, Germany). The DNA fragments were then visualized under UV light after separation on 2% agarose gels containing 0.5 μg/mL ethidium bromide. Due to the small number of individuals in this study that were homozygous for the rare A^{-455} allele we combined the individuals with G/A^{-455} and A/A^{-455} in one group.

Statistical evaluation

The distribution of the CRP levels was skewed and therefore we used logarithmical transformation. The fibrinogen levels were normally distributed. The pre-treatment fibrinogen and CRP levels were the average of the levels at the first and the second visit. The pre-treatment characteristics of the two treatment groups were compared with the Chi^2-test. The pre-treatment associations between fibrinogen and CRP levels with age, BMI, smoking, gender and G/A^{-455}-RFLP were evaluated using multiple regression analysis. The effects of treatment with ciprofibrate and gemfibrozil on the changes of plasma fibrinogen and CRP levels was tested using the paired Student t-test. The effects were tested separately in individuals with or without the rare A^{-455}-allele and in men and women.

The results are given as mean (central 90% range) or mean (1 standard deviation). The statistical package SOLO was used and p-values less than 0.05 were considered significant.

RESULTS

Pre-treatment data

In the total patient group the mean (central 90% range) plasma pretreatment fibrinogen levels (average of the first two visits) was 3.4 g/L (2.7-4.2), whereas in the healthy reference group plasma levels of 2.1 g/L (1.3-3.1) were found. For CRP, levels were 0.98 mg/L (0.26-4.2) in the patient group and 2.0 mg/l (0.3-11) in the healthy volunteers. The pre-treatment levels of fibrinogen and C-reactive protein (CRP) were comparable in the ciprofibrate and gemfibrozil treated groups, with a Spearman correlation of 0.38 ($p<0.01$).

The pre-treatment plasma fibrinogen levels also revealed an increase of 0.15 g/L per 10 years of age ($R=0.30$, $p=0.002$). However, no significant effects of gender, BMI or smoking were observed. When we studied the relation between CRP and these variables, however, we found significant correlations with BMI ($R=0.39$,

Table 2. Mean (1 SD) levels of pre-treatment plasma fibrinogen levels for the genotypes of the G/A^{-455} polymorphism in smokers and non-smokers

	n	G/G^{-455}	n	G/A^{-455} and A/A^{-455}
non-smokers	20	3.33 (0.53)	20	3.47 (0.62)
smokers	30	3.23 (0.47)	11	3.88 (0.44) *

n gives the number of individuals

* significant higher ($p<0.05$) fibrinogen level than in the G/G^{-455} smokers group

p=0.0005) and smoking (0.3 mg/L higher in smokers with R=0.23 and p=0.03), but not with age and gender.

The pre-treatment fibrinogen levels in the patients with the rare allele of the G/A^{-455} RFLP were not significantly different when we studied the total patient group. However, when we studied smokers and non-smokers separately, higher fibrinogen levels were observed in smokers who possessed the G/A^{-455} allele whereas in the non-smokers this difference was not found (table 2). The effect of the G/A^{-455} RFLP on the fibrinogen levels was similar in men and women.

Effects of ciprofibrate and gemfibrozil treatment

After 6 weeks treatment with ciprofibrate the fibrinogen levels were already significantly reduced, whereas fibrinogen levels in the gemfibrozil group had not changed significantly (table 3). After 12 weeks of ciprofibrate treatment the fibrinogen levels had further decreased (mean total decrease 0.91 g/L, $p<0.0001$), and now the fibrinogen levels in the patients treated with gemfibrozil were also decreased. Although the reduction was smaller (0.41 g/L), it was significant ($p<0.0001$). In the last six weeks of treatment, both treatment groups showed a reduction comparable to the first six weeks (0.21 and 0.47 g/L, respectively).

The plasma levels of CRP were reduced after six weeks treatment with ciprofibrate (-0.11 mg/L, n.s.) and gemfibrozil (-0.29 g/L, p=0.002) but the levels had returned to pre-treatment levels after 12 weeks of treatment in both groups (table 3). After adjustment for CRP the decrease of the fibrinogen levels after 12 weeks treatment with ciprofibrate were 1.21 g/L ($p<0.0001$) and with gemfibrozil 0.81 g/L ($p<0.0001$).

No associations could be found between the changes in fibrinogen or CRP and the changes in total, LDL-, HDL-cholesterol or triglycerides (levels of lipid variables previously published by Knipscheer et al[31]). After 12 weeks treatment with ciprofibrate the plasma fibrinogen levels in the smokers were 0.30 g/L higher than in the non-smokers (R=0.35, p=0.03). In the patients treated with gemfibrozil for 12 weeks, this association was not found.

A somewhat larger decreasing effect after 12 weeks ciprofibrate treatment was observed in individuals who possessed the rare allele of the G/A^{-455} ß-fibrinogen polymorphism (table 4), though this was not significant. When non-smokers and smokers were analyzed separately, here too no effect of the RFLP was found, nor was any effect of the RFLP found when men and women were analyzed separately.

Table 3. Mean (central 90% range) of fibrinogen and CRP levels in the ciprofibrate and the gemfibrozil group at the different visits.

	Fibrinogen (g/L)				C-reactive protein (mg/L)			
	gemfibrozil		ciprofibrate		gemfibrozil		ciprofibrate	
	mean	central 90%	mean	central 90%	geometrical mean	central 90%	geometrical mean	central 90%
pre-treatment	3.44	2.27-4.61	3.38	2.19-4.57	0.93	0.13-6.7	1.28	0.15-10.6
6 weeks	3.24	2.08-4.44	2.89*	1.01-4.77	0.51*	0.02-13.8	0.94	0.04-19.7
12 weeks	3.01*	1.75-4.27	2.42*	1.61-3.24	1.25	0.11-14.4	1.32	0.20-8.7

* $p < 0.0005$ from the pre-treatment level using the Student-t test

Table 4. Mean (1 SD) levels of fibrinogen for the genotypes of the G/A^{-455} polymorphism of the Bß-chain of fibrinogen at the pre-treatment visit, after 6 and after 12 weeks treatment with ciprofibrate or gemfibrozil.

	gemfibrozil		ciprofibrate	
	G/G^{-455}	G/A^{-455} and A/A^{-455}	G/G^{-455}	G/A^{-455} and A/A^{-455}
pre-treatment	3.42 (0.41)	3.55 (0.71)	3.27 (0.57)	3.62 (0.53)
6 weeks	3.14 (0.49)	3.33 (0.69)	2.76 (0.56)	3.21 (1.55)
12 weeks	2.96 (0.72)	3.07 (0.55)	2.39 (0.41)	2.50 (0.42)

DISCUSSION

In hyperlipidemic patients we found significantly higher plasma fibrinogen levels than in the healthy volunteers. As the acute phase is a main regulatory mechanism of the fibrinogen synthesis, increased levels might be a reflection of a low grade inflammatory status in these patients. Therefore a relation between the plasma levels of fibrinogen and those of CRP was expected. We did observe a significant Spearman correlation of 0.38 ($p<0.01$). However, we also observed that the pre-treatment CRP levels in patients with hyperlipidaemia and in healthy controls were comparable. This discrepancy between fibrinogen and CRP levels indicates that the regulation of the synthesis of fibrinogen is partly, but not entirely the effect of a low grade acute phase reaction.

We documented factors that are known to be associated in healthy volunteers with the plasma fibrinogen and CRP levels, e.g. age, gender, BMI and smoking[1,32]. In our study fibrinogen was only significantly related to age, whereas CRP levels were associated with BMI and smoking. This further suggests that plasma fibrinogen and CRP-levels in these patients are regulated differently.

To our surprise, no relation existed between smoking and the plasma fibrinogen levels. In this study we included persons who had stopped smoking less than 10 years ago in the smoking group, but also when we compared current smokers with persons who had never smoked or had stopped smoking more than 10 years ago, we found no correlation between smoking and the plasma fibrinogen levels. We previously made a similar observation in patients with severe coronary artery disease[33], where we found no association between smoking and plasma fibrinogen levels in the patient group, while we observed an association in the healthy control group. Also, in the PLAT study, performed on patients with vascular disease, an effect of smoking on the plasma fibrinogen levels was absent[34]. This suggests that smoking will not give an additional elevation in patients with an increased plasma level of fibrinogen, both in patients with vascular disease and with type II hyperlipidaemia.

The relation between the G/A^{-455} polymorphism of the Bß-chain of fibrinogen and

the pre-treatment plasma fibrinogen levels was not significant in the total patient group. This confirms the reports on other patient groups. No associations could be found between the polymorphisms in Bß-fibrinogen chain and plasma fibrinogen levels in peripheral artery disease patients[35,36], cardiovascular disease patients[37] or thrombosis patients[38]. Because an association between this RFLP and fibrinogen levels is frequently reported in healthy individuals[24,27,29], it might be suggested that this association between ß-fibrinogen polymorphisms and plasma fibrinogen levels is confined to healthy individuals. However, in the smoking patients we did find a clear relation between the G/A^{-455} polymorphism and plasma fibrinogen levels. In the non-smoking patients this relation was absent. This confirms the results of Green et al[28], who described that in young survivors of myocardial infarction the relation between this RFLP in the gene of the fibrinogen Bß-chain and plasma fibrinogen levels can only be found in smokers. Also in the ECTIM study[37], there was an association between the G/A^{-455} RFLP and plasma fibrinogen levels but only in smokers.

In this study, we observed that the effect of fibrates on fibrinogen levels is quite slow: already after 6 weeks treatment with ciprofibrate, patients had already a significant decrease in plasma fibrinogen, but this effect was more pronounced after 12 weeks. With gemfibrozil no significant decrease was found after six weeks, but after twelve weeks the decrease was significant, although much smaller than after ciprofibrate treatment. This delayed effect suggests that there is no direct effect of the fibrates on the fibrinogen synthesis. Since the fibrinogen levels after 12 weeks treatment are lower than those after 6 weeks, a greater fibrinogen decrease might be achieved after prolonged treatment. A fibrinogen decrease was also found in the study of Avellone et al in patients with primary hypertriglyceridemia[19]. Unfortunately, this study was not placebo-controlled, which makes it impossible to differentiate drug effects from disease-associated changes in fibrinogen levels. Other studies with gemfibrozil, however, reported an increase of the fibrinogen levels in hypercholesterolemic patients type IIa and IIb[13] or found no significant effect in coronary artery disease patients[17,18,20].

No associations between the changes in plasma fibrinogen levels and those in total, LDL- and HDL-cholesterol were found in either the ciprofibrate or the gemfibrozil treated patients. This was anticipated, as the effect of the two drugs on fibrinogen was different, while the effect on the lipid parameters was comparable. This indicates different mechanisms for the fibrinogen decrease and the lipid changes of ciprofibrate and gemfibrozil.

We studied whether the genetic polymorphism of the Bß-fibrinogen gene might be associated with the fibrinogen decrease. Recently, it has been suggested that the Bß-fibrinogen polymorphisms are mainly important in the acute phase regulation of the fibrinogen synthesis, because the correlation between fibrinogen levels and genotype was only found in smoking survivors of myocardial infarction[28]. In this study we

could not confirm that the Bß-polymorphisms are associated with acute phase induced fibrinogen synthesis. At baseline, the individuals with the rare allele have slightly higher fibrinogen levels, though not significantly so. Although there is a trend, the decrease of fibrinogen is not significantly larger in individuals with the rare allele, but the significance might have been missed because the group that was homozygous for the rare allele was too small to be studied separately.

In summary, we have shown in this study that ciprofibrate decreases fibrinogen levels significantly, and that the levels that are found after 12 weeks of treatment are comparable with those in healthy individuals. Gemfibrozil gave a smaller fibrinogen decrease, which was only significant after 12 weeks. In attempts to elucidate the mechanism by which ciprofibrate lowers fibrinogen we found that the low grade acute phase stimulation of the fibrinogen synthesis contributes only minimally and that there must be another mechanism. The Bß-fibrinogen G/A^{-455} polymorphism was not associated with the reduction of the fibrinogen level by fibrates.

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CHAPTER 11

GENDER RELATED ASSOCIATION BETWEEN ß-FIBRINOGEN GENOTYPE AND PLASMA FIBRINOGEN LEVELS, AND α- AND ß-FIBRINOGEN GENOTYPE LINKAGE IN GREENLAND INUIT

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ABSTRACT

Elevated plasma fibrinogen levels represent an increased risk for cardiovascular disease, but the mechanism explaining this association is still not clear. Genetic differences of the fibrinogen genes might play a role, since it has been shown that individuals who carry the rare alleles of polymorphisms in the gene for the Bß-chain (*Bcl*1 and G/A-455) and the Aα-chain (*Taq*1) of fibrinogen have higher plasma fibrinogen levels, and patients with peripheral arterial disease have a higher frequency of the rare allele of the *Bcl*1 polymorphism than healthy controls. We studied in Greenland Inuit, a population with a low incidence of ischaemic heart disease, the polymorphisms of the fibrinogen gene and their association with the plasma fibrinogen level. The group studied has a small age range (30-34), 97% were smokers, 62 were men and 71 women.

We observed differences in the fibrinogen polymorphisms in the Inuit when compared to Caucasian populations: firstly, in the Inuit the frequencies of the rare alleles of the Bß-chain and the common alleles of the Aα-chain polymorphisms were lower than that in other published populations (all Caucasian). Accordingly, these distribution patterns give a higher frequency in the Inuit of alleles that are associated with lower plasma fibrinogen levels. Secondly, we observed linkage disequilibrium between Bß and Aα polymorphisms, which has not been observed in other healthy populations. In the Inuit men the rare allele of the *Bcl*1 and G/A^{-455} fibrinogen polymorphisms was associated with the plasma fibrinogen levels, comparable to the association described in Caucasian populations. In women, however, we did not find a significant association, supporting the desirability of separate analysis in men and women of the influence of genetic factors on atherosclerotic disease.

In conclusion: in the Inuit the association of fibrinogen polymorphisms with fibrinogen levels is comparable to that in Caucasians, but the genes that are associated with lower fibrinogen levels are more frequent in the Inuit than in Caucasians.

INTRODUCTION

In several epidemiological studies it has been shown that an increased plasma fibrinogen level is an independent risk indicator for cardiovascular disease[1-6]. The Northwick Park Heart Study showed that an increase of the fibrinogen level at one standard deviation predicted an increase in the risk for cardiovascular events within the next 5 years of 84%[1].

The mechanism of the association of fibrinogen with risk has not yet been elucidated. It might be that an increased amount of fibrinogen in the circulation gives an increased propensity for thrombosis[7] or directly contributes to the development of the atherosclerotic lesion[8]. There are also indications that increased plasma fibrinogen levels reflect the inflammatory condition of the vascular wall.

This theory is supported by the results of the ECAT-angina pectoris study[9], where increased fibrinogen levels and increased C-reactive protein (CRP) levels are both risk indicators for cardiac events in patients with angina pectoris. The PROCAM study has recently reported comparable results for a healthy population[5]. Another cardiovascular risk factor that is closely linked to inflammation is smoking, which increases the levels of fibrinogen and other acute phase reactants[10,11]. It is conceivable that smoking contributes to risk also because of its acute phase inducing properties.

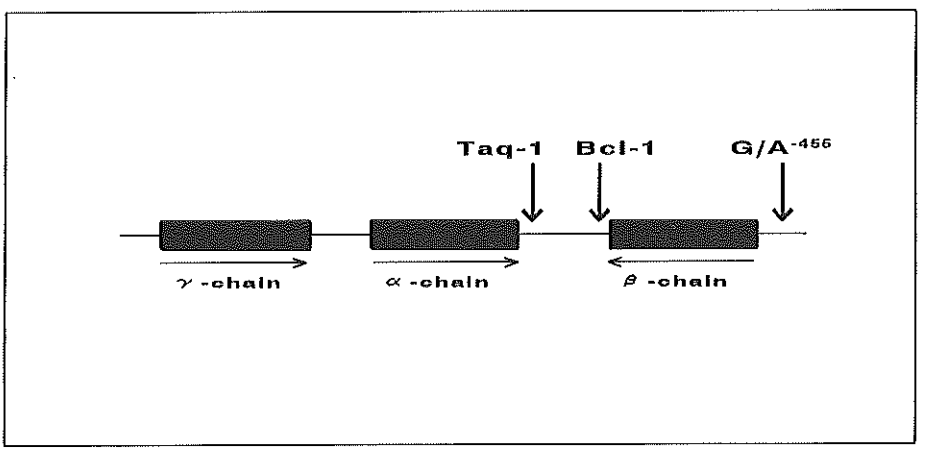

Figure 1. The fibrinogen genes of the α, ß and γ chain of fibrinogen with the location of the genetic polymorphisms that were applied in this study. The direction of transcription of the genes is represented by the arrows under the boxes.

Genetic variation may also play a role in determining plasma fibrinogen levels. An association between polymorphisms in the genes for the Aα and Bß chain and plasma fibrinogen levels (figure 1) has been described[12,13]. Recently, Green et al[14] showed that the association between G/A^{-455} genotypes and fibrinogen levels was observed only in smokers, suggesting that the increase of fibrinogen as the result of a low grade acute phase reaction, e.g. by smoking, might depend on polymorphisms of the fibrinogen Bß chain. It has also been reported that the binding of nuclear proteins to DNA fragments was influenced by the genotype at the G/A^{-455} and C/T^{-148} polymorphic sites of the Bß-fibrinogen gene[15,16], the last of which is located close to the interleukin-6 responsive element of the promoter. If these findings are combined, they imply an involvement of the ß-fibrinogen polymorphisms in the cytokine stimulated regulation of fibrinogen synthesis.

The association between fibrinogen genotypes and plasma levels has not been

confirmed in all studies. There is, however, much diversity in the composition of the population samples and in the fibrinogen assays used. One of the variant factors is the number of smokers. Since both the ECTIM study[17] and Green et al[14] reported that the association between genotypes and fibrinogen levels is stronger in smokers, part of the reported difference in the relation between fibrinogen polymorphisms and fibrinogen levels might be ascribed to this variation.

A fibrinogen restriction fragment length polymorphism (RFLP) at the 3' end of the α-fibrinogen chain has been described (*Taq*I)[12]. No significant correlation between these polymorphisms and plasma fibrinogen level has been found, but when the average excess of G/A^{-455} and *Taq*I combined genotype is estimated[18], the presence of functionally distinct genotype combinations is suggested[19].

We performed a study of fibrinogen polymorphisms in Greenland Inuit, a population with a low incidence of ischaemic heart disease[20]. We determined the allele frequencies of the G/A^{-455}, *Bcl*I and *Taq*I polymorphisms and calculated the associations between the different polymorphisms. In addition, we estimated the association between genotype and plasma fibrinogen levels (functional and immunological methods) in men and women.

METHODS

Population

One hundred and ninety two Inuit, aged 30-34 years, living in Nanortalik, in the southwest of Greenland, were invited to participate in this study. This group was studied because in individuals aged 30-34 no selection by coronary heart disease will be observed. A complete set of data, consisting of blood specimens, a filled-in questionnaire and anthropometric measurements was obtained from 133 individuals (62 men and 71 women). Upon medical investigation these 133 Inuit appeared to be healthy and did not show signs or symptoms indicating the presence of severe atherosclerosis. Characteristics of the population have been described elsewhere in detail[21]. We considered individuals who never smoked (n=4) and those who stopped smoking more than 10 years ago (n=2) as non-smokers, and current smokers and those who stopped less than 10 years ago as smokers (n=6, stopped between 3 months and 3 years before sampling). Of the smokers, 13% smoked less than 5 cigarettes/day, 60% smoked 5-14 cigarettes/day and 27% smoked more than 15 cigarettes/day. The Inuit had smoked for 17.5 (SD 3.6) years. There was no difference in smoking habits between men and women.

C-reactive protein and fibrinogen levels were available from all 133 subjects, polymorphisms analysis could not be performed in all samples, due to a poor quality of some DNA samples (see table 1 for number of analyzed samples). Body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

Fifty two Danes, comparable for age (30-34 years) and gender (28 men and 24

women), were the control group for plasma fibrinogen and CRP-levels. Danes were chosen as a reference group, because 1) it was not possible to compose a large enough group of Greenland inhabitants without Inuit among their ancestors, and 2) the way of living, plasma lipid and (apo)lipoprotein levels and apparently lack of ischaemic heart disease, and therefore the Danish control population is comparable with the Inuit[21].

Blood sampling

Blood was collected in sodium citrate (final concentration 14 mmol/L) and immediately placed in melting ice. After centrifugation (30 min, 2000 g, 4°C) the plasma was collected and frozen at -70°C. The blood cells were stored at -20°C.

Polymorphism analysis

Each 50 μl polymerase chain reaction (PCR) reaction contained 100-400 ng genomic DNA, 100 ng of each appropriate primer, 10 mmol/L Tris/HCl pH=9.0, 1,5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.01 (w/v) gelatin, 0.1% Triton X-100, 0.02 mmol/L dNTP, 0.1 U Taq polymerase (HT Biotechnology LTD, Cambridge, England). The reaction components were incubated at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min in a DNA thermal cycler (Perkin Elmer Cetus).

The primers have been previously described (G/A^{-455} by Thomas et al.[13], *Bcl*1 and *Taq*1 by Thomas et al[22]). 10 μl of the PCR product was digested with the appropriate restriction enzyme. These digestion products were separated by electrophoresis through a 2% agarose gel in 44 mmol/L tris-borate 1 mmol/L EDTA containing 0.5 μg/ml ethidium bromide and visualized under UV-light. The alleles with the restriction site and the non-cleavable alleles were designated B1 and B2 for the *Bcl*1 polymorphism, G^{-455} and A^{-455} for the G/A^{-455} polymorphism and T1 and T2 for the *Taq*1 polymorphism, respectively.

Plasma protein measurements

Fibrinogen activity levels were measured with the modified Clauss assay[23]. The within-day and between-day coefficients of variation (CV) were 3.2% and 4.9%, respectively. Fibrinogen antigen levels were measured nephelometrically, using rabbit polyclonal anti-human fibrinogen (Dako, Denmark) antibodies. The within-day and between-day coefficients of variation (CV) were 1.7% and 4.2%, respectively. Normal plasma (Nycomed Pharma, Oslo, Norway) was used as calibrator for the fibrinogen assays. The ratio of the two fibrinogen assays and its 99% confidence interval were calculated. Seven samples were outside this range and omitted. Levels of C-reactive protein were measured with an EIA using rabbit antibodies against human C-reactive protein (Dako, Denmark) as catching and tagging antibodies. The

within-day and between-day coefficients of variation (CV) were 2.9% and 6.2%, respectively. CRP-standard serum (Behringwerke, Marburg, Germany) was used as calibrator.

Statistical analysis

Deviations of the genotype distributions in the Inuit samples from that expected for a population in Hardy-Weinberg equilibrium were analyzed using the χ^2-test. Genotype frequencies in the Inuit and published frequencies were compared with a χ^2-test. Standardised disequilibrium statistics were calculated as described by Chakravarti[24]. Allele frequencies in the Inuit were determined by gene counting, 95% confidence

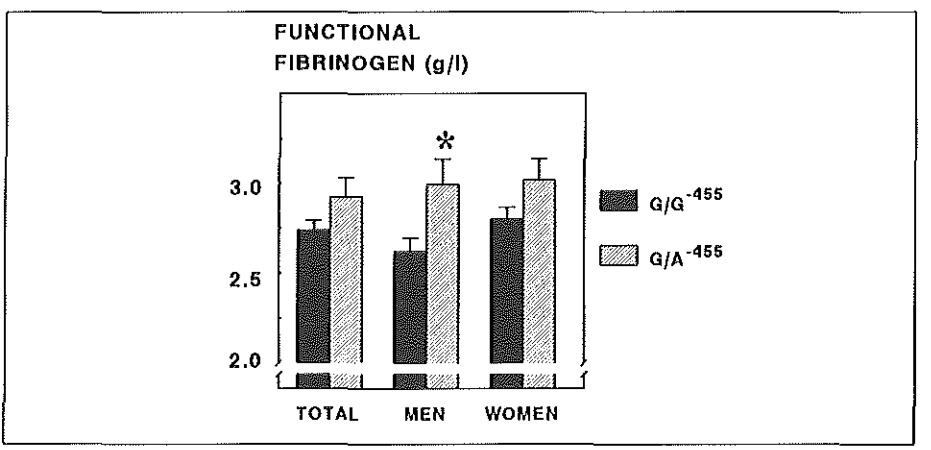

Figure 2. The mean (SEM) of the functional fibrinogen levels in Inuit with the G/G^{-455} ( ) and G/A^{-455} (/////) genotypes of the G/A^{-455} restriction fragment length polymorphism (RFLP) of the total group, men and women. ⋆ indicates a significant difference from the levels in the G/G^{-455} group.

intervals (95% CI) of the allele frequencies were calculated from sample allele frequencies[25], based on the approximation of the binomial and normal distributions when n is large.

With analysis of covariance (ANCOVA) the adjusted fibrinogen levels for each genotype were estimated and the significance of genotypes in determining plasma fibrinogen levels was estimated, with BMI, waist-hip ratio and CRP levels as covariables. A multiple linear regression model was used to assess the amount of variance in plasma fibrinogen levels, explained by BMI, CRP, smoking status and genotype in men and women, separately.

Statistical analysis was performed using the "SOLO" and "Lotus123" computer programmes. Statistical significance was taken as $p < 0.05$.

Table 1. Frequency (and 95% confidence intervals) of the rare allele of fibrinogen polymorphisms

	*Bcl*1			G/A^{-455}			*Taq*1		
	n*	freq.†	95% CI‡	n*	freq.†	95% CI‡	n*	freq.†	95% CI‡
Study:									
London[12]	91	0.25§	0.19-0.32				91	0.28§	0.22-0.35
Norway[27]	118	0.17§	0.13-0.23				118	0.27§	0.22-0.33
London[28]	53	0.16§	0.10-0.23				53	0.26§	0.18-0.36
UK all[13]				292	0.19§	0.16-0.22			
UK smokers[13]				120	0.20§	0.15-0.26			
UK non-smokers[13]				172	0.19§	0.15-0.24			
Edinburgh[29]	126	0.10	0.07-0.15				126	0.25§	0.14-0.23
Sweden all[14]				86	0.25§	0.20-0.31			
Sweden smokers[14]				57	0.20§	0.13-0.27			
Sweden non-smokers[14]				29	0.34§	0.22-0.46			
UK and France[17]				648	0.21§	0.19-0.23			
UK[22]	293	0.15	0.12-0.18				276	0.27§	0.24-0.31
Inuit (This study)	126	0.12	0.09-0.17	131	0.11	0.08-0.15	121	0.47	0.41-0.53

n* number of individuals evaluated
† frequency of the rare allele
‡ 95% confidence interval of the rare allele frequency
§ frequency is significantly different from the Inuit ($p<0.05$ in χ^2-test)

RESULTS

Genetic distribution compared to Caucasian populations

In the Inuit population the allele distributions of the *Bcl*1, G/A^{-455} and *Taq*1 polymorphisms were in Hardy Weinberg equilibrium, as expected in a general population. A different genetic background was indicated for the Inuit when compared to Caucasian populations, since the allele frequencies of the rare alleles of the G/A^{-455} and *Bcl*1 polymorphisms and the frequency of the common allele of the *Taq*1 polymorphism in the Inuit were significantly lower when compared to most, but not all, of the Caucasian populations (table I[12,13,22,27-29].

Allelic associations

In Caucasian populations[13,14] there was a strong linkage between the two polymorphisms of the fibrinogen Bß chain giving a strong association between the B1 and the G^{-455} allele. However, the linkage was weak between the polymorphism of the Aα chain and those of the Bß chain, since there was no significant linkage between the T2 allele on the one side and the B1 and the G^{-455} alleles on the other (table 2)[13,14,30,31]. In the Inuit, the B1 allele was in strong allelic association with the G^{-455} allele as in other populations, but, in contrast, there was also a significant association with the T1 allele (Table 2).

Table 2 The allelic association between the *Taq*1 polymorphism of the α-fibrinogen gene and the G/A^{-455} and *Bcl*1 polymorphisms of the β-fibrinogen gene.

	Inuit	Caucasian	
Bcl1 - G/A^{-455}	Δ= 0.91, χ^2=222, p<0.001	Δ= 0.85, χ^2=322, p<0.001	(22)
G/A^{-455} - Taq1	Δ=-0.32, χ^2= 24, p<0.001	Δ=-0.09, χ^2= 3, p>0.1	(22)
Bcl1 - Taq1	Δ=-0.29, χ^2= 22, p<0.001	Δ= 0.01, χ^2= 1, p>0.1	(22)
		Δ=-0.07, p>0.1	(31)
		Δ= 0.01, p>0.1	(30)

Δ linkage coefficient

Genetic contribution to the plasma fibrinogen levels

The acute phase markers fibrinogen and CRP were both higher (2.81 g/L in the functional assay, 2.81 g/L for fibrinogen antigen and 2.9 mg/L for CRP) in the Inuit than in the Danish control group (2.30 g/L in the functional assay, 2.19 g/L for fibrinogen antigen and below 1.5 mg/L for CRP). Adjustment for the acute phase

state by adding CRP in ANCOVA still gave a comparable difference between these populations (results not shown).

If all Inuit are studied as a single group, there is no significant association in the ANCOVA between either of the three fibrinogen polymorphisms and plasma fibrinogen level. However, when the group is divided into men and women, we observed only in the men a significant significantly higher plasma fibrinogen levels with the B1B2 or the G/A^{-455} genotype. In women, there was a similar trend, but this was not significant (figure 2, table 3). Stratification by combined α- and ß-fibrinogen genotype did not reveal any more informative genotype combination (data not shown).

In the Inuit, BMI, CRP, smoking status and genotypes together accounted in the men for 25% and in the women for 35% of the variation in plasma fibrinogen levels. Removal of the genotype from the regression model reduced the amount of variance explained to 16% in men and to 26% in women, suggesting that the fibrinogen polymorphism genotypes accounted in both men and women for 9% of the variation in fibrinogen levels after adjustment for covariables. Removal of other covariates from the regression equations in men and women respectively, suggested that CRP levels accounted for 7% and 19% of the variation in plasma fibrinogen levels, BMI accounted for 9% and 2% in men and women, respectively.

DISCUSSION

The polymorphisms of α- and ß-fibrinogen were studied in the Inuit, a population with a low incidence of ischaemic heart disease (20). We observed different allele frequencies of the polymorphisms of the fibrinogen chain, with lower frequencies of rare A^{-455} and B2 alleles of the ß-fibrinogen polymorphisms and higher frequencies of the rare T2 allele of the α-fibrinogen polymorphism when the Inuit were compared with Caucasian populations. In this study and other studies, the A^{-455}, B2 and T1 alleles are associated with higher fibrinogen levels suggesting that their lower frequency among the Inuits may explain in part their lower incidence of ischaemic heart disease.

The Inuit are a population with very few genetic influences from other populations. This isolation has resulted in some genetic differences in blood groups (ABO, rhesus and MN blood group system), HLA system and red cell enzymes (review: 32). It might therefore also be possible that the fibrinogen gene locus has developed differently from that in Caucasians and has another allelic distribution. To study a possible genetic difference of the fibrinogen genes in the Inuit, we assessed the linkage disequilibrium between the polymorphisms. The association between *Taq*I and the polymorphisms of the β-fibrinogen gene was different in the Inuit. In the Caucasian populations there was no correlation between the α- and β-fibrinogen polymorphisms. In the Inuit, however, we found linkage between these

Table 3 Mean (SEM) of plasma fibrinogen levels (g/L)(functional and immunological methods) in Inuit with different genotypes of G/A^{-455}, *Bcl*1 and *Taq*1 fibrinogen polymorphisms.

A. Functional assay

		WHOLE GROUP			MEN			WOMEN		
		n*	mean	SEM**	n*	mean	SEM**	n*	mean	SEM**
G/A^{-455}	G/G^{-455}	93	2.69	0.05	44	2.58	0.07	49	2.75	0.06
	G/A^{-455}	26	2.86	0.10	12	2.93$	0.13	14	2.95	0.11
	A/A^{-455}	0			0			0		
*Bcl*1	B1B1	101	2.70	0.05	46	2.63	0.06	55	2.70	0.05
	B1B2	22	2.85	0.11	10	2.94	0.14	12	2.96	0.12
	B2B2	0			0			0		
*Taq*1	T1T1	25	2.79	0.10	13	2.77	0.14	12	2.77	0.12
	T1T2	71	2.72	0.06	27	2.66	0.10	44	2.78	0.06
	T2T2	17	2.59	0.13	8	2.45	0.17	9	2.78	0.14

B. Immunological assay

		WHOLE GROUP			MEN			WOMEN		
		n*	mean	SEM**	n*	mean	SEM**	n*	mean	SEM**
G/A^{-455}	G/G^{-455}	93	2.78	0.06	44	2.63	0.08	49	2.86	0.08
	G/A^{-455}	26	2.86	0.12	12	2.98$	0.14	14	2.92	0.15
	A/A^{-455}	0			0			0		
*Bcl*1	B1B1	101	2.79	0.06	46	2.69	0.08	55	2.84	0.08
	B1B2	22	2.86	0.13	10	2.99	0.18	12	2.92	0.16
	B2B2	0			0			0		
*Taq*1	T1T1	25	2.79	0.13	13	2.77	0.17	12	2.79	0.16
	T1T2	71	2.76	0.08	27	2.71	0.12	44	2.80	0.09
	T2T2	17	2.90	0.15	8	2.69	0.21	9	3.15	0.19

n* numbers of individuals evaluated
** standard error of the mean
§ levels are significantly different from G/G^{-455} ($p<0.05$ in ANOVA)

polymorphisms.

In the total Inuit group no significant relation could be found between fibrinogen levels and genotypes of the β-fibrinogen genes. However, we observed increased fibrinogen levels in Inuit men with the B1B2 and G/A^{-455} genotypes, when we compare them with the men with genotypes B1B1 and G/G^{-455}. In women, no significant associations were found, although we found a similar trend. As the percentage of smokers in the Inuit was ±97%, our observation that there is a correlation between genotype and fibrinogen level in the Inuit might corroborate the theory of Green et al[14] that a low grade stimulation of the fibrinogen synthesis, like smoking, is expressed more strongly in men with an A^{-455} allele. The direct involvement of β-fibrinogen polymorphisms in regulation of fibrinogen expression is suggested by a differential binding of nuclear proteins to DNA with G^{-455} or A^{-455} (Green, unpublished data). This study also suggests that the fibrinogen levels are regulated differently in men and women.

Our results may explain the inconsistency that is found in the literature describing the relation between fibrinogen levels and fibrinogen polymorphisms[12-14,17,27,28]. The reported studies vary in a number of factors that are known to have an effect on plasma fibrinogen levels, like the men/women ratio and the fraction and definition of smokers. Furthermore these studies are also inconsistent in the adjustment for gender, BMI, smoking status, acute phase status and age. The importance of controlling for such factors that affect the plasma fibrinogen levels is clearly illustrated by the present study, and thereby we could show a gender difference in the association between genetic polymorphisms and plasma levels of fibrinogen. It has been stressed before that men and women should not be studied together in atherosclerosis research[33], a statement which seems to be supported by the results of our study.

The role of the B2 and A^{-455} alleles of the *Bcl*1 and G/A^{-455} ß-fibrinogen polymorphisms in the regulation of the fibrinogen level under conditions that induce a low grade inflammation merits further investigation.

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CHAPTER 12

DNA-POLYMORPHISMS OF FIBRINOGEN IN MEN WITH SYMPTOMATIC CORONARY HEART DISEASE.

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ABSTRACT

Increased plasma fibrinogen levels have been identified as a risk indicator for myocardial infarction, stroke and thrombosis. Healthy individuals with the rare allele of the G/A^{-455} polymorphism of the fibrinogen Bß-chain have higher plasma fibrinogen levels than in individuals without this allele. The frequency of this allele has been reported to be higher in patients with peripheral arterial disease than in healthy individuals. We determined the distribution of the G/A^{-455} genotypes in a group of 492 men with symptomatic stable coronary heart disease (CHD) and a reference group of 214 healthy men and also we evaluated whether the allele frequencies were different in smoking and non-smoking patients or in patients with and without a positive family history of coronary artery disease affected The G/A^{-455} polymorphism of the fibrinogen Bß chain and the *Taq*I polymorphism of the Aα chain were determined and the plasma levels of fibrinogen were measured with an enzyme immuno assay.

In our study, we observed no difference in the allele frequencies or linkage disequilibrium of the G/A^{-455} and *Taq*I RFLP in the CHD patients and healthy individuals. The allele frequencies were comparable in smoking and non-smoking patients, and in patients with and without positive family history of coronary artery disease. Plasma fibrinogen levels were similar in patients with the G/G^{-455} and G/A^{-455} genotype, while patients with the A/A^{-455} genotype had significantly higher fibrinogen levels. *Taq*I polymorphism was not associated with the fibrinogen levels. The results of this study suggest that the G/A^{-455} and *Taq*I fibrinogen polymorphisms do not predispose to coronary artery disease.

INTRODUCTION

There is growing interest in fibrinogen since several epidemiological studies have reported on a clear association between elevated plasma levels of fibrinogen and increased risk for myocardial infarction[1-9], stroke[10-12], thrombotic risk in patients with venous thrombosis[13] and mortality in stable claudicants[14]. A direct association between fibrinogen and thrombotic events is suggested by several mechanisms by which elevated fibrinogen might be deleterious, such as by the effect of fibrinogen levels on plasma viscosity[15], the growth[16] and migration[17] of smooth muscle cells, platelet aggregation[18], thrombus size[19] and formation and growth of atherosclerotic lesions[20].

The three chains of fibrinogen are encoded by three different genes that are located on the long arm of chromosome 4 in a 50 kb cluster[21]. Several DNA polymorphisms of the three genes have been described[23-28] and the restriction fragment length polymorphisms (RFLP) of the Bß-chain (*Bcl*I and G/A^{-455}) are associated with differences in the plasma levels of fibrinogen[23,28]. Healthy individuals who are homozygous for the rare allele of polymorphisms of the Bß-chain have the

highest fibrinogen levels; individuals who are homozygous for the common allele have the lowest plasma fibrinogen levels while the fibrinogen levels in heterozygotes are intermediate[23,28-32].

The G/A^{-455} polymorphism is located close to the interleukin-6 responsive element in the promoter of the Bß-chain. This might suggest an effect of the RFLP on the acute phase induced increase of the fibrinogen synthesis. Indications for this mechanism come from studies in survivors of a myocardial infarction[31,34] where the association between genotype of the G/A^{-455} polymorphism and plasma fibrinogen levels is much stronger in smokers than in non-smokers. In smokers a low level increase of a chonic inflammatory status has been suggested by increased levels of fibrinogen[overview; 35] and other acute phase proteins[36-38].

Fowkes et al[33] reported that they observed higher frequencies of the rare allele of the G/A^{-455} RFLP in patients with peripheral arterial disease than in a healthy reference group (frequencies of 0.197 and 0.097, respectively, $p<0.005$). This might suggest that individuals with the rare A^{-455} allele have a greater tendency to develop peripheral arterial disease. A similar predisposition might exist for other arterial diseases, like coronary heart disease.

To assess a possible contribution of the fibrinogen DNA polymorphisms to the prevalence of coronary heart disease we studied the frequency and linkage disequilibrium of the G/A^{-455} and *Taq*I genotypes in men with coronary heart disease and in healthy volunteers; frequencies were also compared in smoking and non-smoking patients, and in patients with and without a positive family history of CHD. We also documented the association between the fibrinogen polymorphism and the plasma fibrinogen levels in the CHD patients, taking into account smoking habits and the acute phase state.

PATIENTS AND METHODS

Study design

REGRESS is a double blind, placebo controlled, multicentre study to assess the effect of a two year treatment with the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor pravastatin, on progression and regression of angiographically documented coronary atherosclerosis in 885 male patients undergoing coronary cine-arteriography to assess anginal complaints. The patients were below 70 years of age, had normal to moderately raised serum cholesterol levels, i.e. between 4.0 and 8.0 mmol/L, and at least one coronary stenosis $\geq$ 50% (visually assessed). Baseline and follow-up coronary arteriograms were analyzed by quantitative computer analysis. A number of substudies were performed in addition to the angiographic main study. Substudies include: B-mode ultrasound studies of the carotic and femoral arteries, ambulatory electro-cardiographic monitoring, specialized lipid research and DNA studies. The study was conducted under the auspices of the Interuniversity

Table 1. Baseline characteristics of the patients with coronary heart disease (CHD) and the reference group.

	CHD patients	reference group
number	679	214
age (years)(mean (SD))	55.6 (8.0)	50.0 (7.6)
BMI (kg/m²)(mean (SD))	26.1 (2.6)	25.0 (2.4)
non-smokers (%)	27%	43%
ex-smokers (%)	61%	33%
smokers (%)	12%	24%
< 10 cigarettes/day (%)	12%	34%
10-20 cigarettes/day (%)	60%	25%
> 20 cigarettes/day (%)	27%	40%
positive family history of MI (%)	48%	38%

Cardiology Institute of the Netherlands (ICIN), Utrecht, the Netherlands. Written consent was obtained from the patients and the study was performed in accordance with the Declaration of Helsinki.

Subjects

In the substudy reported here the baseline data of the above-mentioned patients was evaluated. DNA was available from 679 patients and from 492 of these patients also plasma were available. Information about smoking and family history of coronary heart disease was obtained through a questionnaire. The information about smoking consisted of the current smoking status and the number of sigarettes smoked per day. The family history was considered positive when one of the parents had had a myocardial infarction before the age of 60. As a reference a group of 214 healthy Dutch men was selected from the general population of Amsterdam, Doetinchem and Maastricht, three of the cities that participated in the multicentre patients study. The men in the reference group were without history of coronary artery disease and comparable to the patient group with respect to age, BMI, smoking habits and lipid levels. The baseline characteristics of the patients and the reference group are given in table 1.

Assays

Plasma fibrinogen levels were determined with an enzyme immuno assay that uses a monoclonal antibody against the carboxyl terminal end of the fibrinogen Aα-chain as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain (Y18) as the tagging antibody[39].

C-reactive protein levels were determined with an enzyme immune assay, using

rabbit anti-human C-reactive protein (Dako, Denmark) as the catching and the tagging antibody.

The **G/A^{-455} polymorphism** of the ß-fibrinogen gene is assessed as described by Thomas et al.[24]. Briefly, genomic DNA was amplified by polymerase chain reaction (PCR) and incubated with HaeIII restriction enzyme. The DNA fragments were then visualized under UV light after separation on 2% agarose gels with ethidium bromide. The rare allele was called the A^{-455} allele.

Statistical evaluation. The frequencies of the different alleles was assessed by gene-counting. Linkage disequilibrium between the Bß and Aα-RFLP was calculated as described by Chakravarti[40]. The CRP levels were logarithmically transformed because they were positively skewed (skewness 2.3). The fibrinogen levels were also a little skewed (skewness 1.03); but because similar results were obtained with untransformed and logarithmically transformed fibrinogen data, the results of the untransformed calculations are given. The effect of genetic polymorphisms on fibrinogen levels was studied using analysis of covariance with age, BMI and CRP as covariables.

RESULTS

Genetic polymorphisms

In the patient group the frequency of the A^{-455} allele of the G/A^{-455} RFLP was 0.21, with a 95% confidence interval of 0.19-0.23. The frequency of the T2 allele of the *Taq*1 RFLP was 0.31, with a 95% confidence interval of 0.28-0.33. There was no linkage between the two polymorphisms (Δ=0.02, χ^2=0.51, ns). The frequency of the A^{-455} and T2 allele was similar in the non-, ex- and current smokers. The frequency of the rare alleles of the G/A^{-455} and the *Taq*1 RFLP did not differ in the patients with and without a family history of ischaemic heart disease.

In the reference group the frequency of the A^{-455} allele of the G/A^{-455} RFLP was 0.22, with a 95% confidence interval of 0.18-0.27. The frequency of the T2 allele of the *Taq*1 RFLP was 0.25, with a 95% confidence interval of 0.21-0.30. No linkage the two polymorphisms (Δ=0.06, χ^2=1.44, ns) was observed. In the reference group also no differences in the allele frequencies were observed in non-smoking and smoking men, and men with and without a positive family history of ischaemic heart disease.

Effects of genetic polymorphisms on the plasma fibrinogen levels

The individuals who were homozygous for the common G^{-455} allele and the heterozygotes had comparable plasma fibrinogen levels, while the homozygotes for the rare A^{-455} allele had significantly higher fibrinogen levels (table 2). When the smokers and non-smokers were studied separately, an effect of the polymorphism on the plasma fibrinogen level, adjusted for age and BMI, was observed only in

Table 2. Geometrical mean (95% CI) of fibrinogen levels in the three genotypes of the G/A^{-455} (a) and *Taq*1 (b) fibrinogen polymorphisms. Fibrinogen levels are adjusted for age and BMI or for age, BMI and CRP.

	G/A^{-455} RFLP Bß chain						*Taq*1 RFLP Aα chain			
		n	adjusted for age, BMI		adjusted for age, BMI, CRP			n	adjusted for age, BMI	
total group	G/G^{-455}	288	3.15	(3.00-3.30)	3.14	(2.99-3.29)	T1T1	209	3.13	(2.65-3.70)
	G/A^{-455}	154	3.12	(2.92-3.33)	3.14	(2.94-3.35)	T1T2	179	3.24	(3.05-3.44)
	A/A^{-455}	16	3.90*	(3.18-4.78)	3.89	(3.11-4.85)	T2T2	48	2.97	(2.64-3.35)
never- plus ex-smokers	G/G^{-455}	217	3.05	(2.89-3.22)	3.05	(2.89-3.22)	T1T1	154	3.07	(2.88-3.27)
	G/A^{-455}	111	3.14	(3.01-3.28)	3.15	(2.92-3.40)	T1T2	130	3.18	(2.97-3.41)
	A/A^{-455}	11	3.63	(2.85-4.61)	3.87	(2.97-5.04)	T2T2	37	2.87	(2.52-3.27)
current smokers	G/G^{-455}	71	3.45	(3.12-3.80)	3.40	(3.08-3.75)	T1T1	55	3.35	(2.97-3.77)
	G/A^{-455}	43	3.07	(2.70-3.49)	3.12	(2.75-3.53)	T1T2	49	3.42	(2.98-3.84)
	A/A^{-455}	5	4.65*	(3.20-6.76)	4.08	(2.72-6.13)	T2T2	11	3.30	(2.50-4.25)

n gives the number of individuals in the groups

* the fibrinogen level in this group is significantly higher than in the G/G^{-455} group

smokers (table 2). When the fibrinogen levels were also adjusted for CRP, the effect of the polymorphism was no longer significant. The *Taq*1 RFLP had no effect on the fibrinogen level, although there was a trend towards lower levels in the individuals who were homozygous for the rare allele.

DISCUSSION

In our study the frequencies of the rare alleles of the fibrinogen DNA polymorphisms (G/A^{-455} of the Bß chain and *Taq*1 of the Aα chain) were comparable in the patient group and in the healthy reference group. The frequencies in our study (0.21 for G/A^{-455} and 0.31 for *Taq*1 in the patients and 0.22 for G/A^{-455} and 0.25 for *Taq*1 in the reference group) were also similar to frequencies that were reported for most healthy Caucasian populations (between 0.19 and 0.25 for G/A^{-455} and between 0.25 and 0.28 for *Taq*1)[23-26]. Fowkes et al[33] reported a higher frequency of the *Bcl*1 polymorphism of the Bß gene in patients with peripheral arterial disease (PAD) than in a healthy control group (0.197 and 0.097, respectively). In view of our results with coronary artery patients, this might be typical for PAD. Also the ECTIM Study[34] did not find different allele frequencies of Bß-chain polymorphisms in myocardial infarction patients compared to healthy controls.

If individuals with the rare allele have a greater risk of vascular diseases, they may die at an earlier age and therefore relatively less frequently included in studies in older individuals. The age distribution in our group was comparable to that in the study of Fowkes[33], which reduces a selection bias by age, however age-related mortality differences in the two diseases remain.

Subgroup analysis on non-smokers, ex-smokers or current smokers showed no differences in the frequencies of the rare alleles of both the G/A^{-455} and the *Taq*1 polymorphism, indicating that there is no increased predisposition to a cardiovascular disease in smokers with the A^{-455} allele. Such a predisposition has been suggested in a study in young survivors of myocardial infarction from Sweden where the frequency of the rare allele of the G/A^{-455} RFLP was somewhat lower in the non-smokers[31]. Subgroup analysis on patients with or without a positive family history of CHD also did not reveal different allele frequencies.

The linkage disequilibrium between the RFLP on the Bß and Aα chains is comparable in our CHD patients and the reference group. The linkage is also comparable to that described for healthy Caucasian populations[23-26].

The relation between the G/A^{-455} RFLP and the plasma fibrinogen levels is different in this patient group from the relationship that has been described for healthy volunteers, where the individuals homozygous for G^{-455} have the lowest, those homozygous for A^{-455} the highest and heterozygotes intermediate plasma fibrinogen levels[23,28-32]. In our patients with CHD the homozygotes for the rare allele had significantly higher plasma fibrinogen levels, but the heterozygotes had levels

that were comparable with those in homozygotes for the common allele. This observation has recently also been made in the LETS study[13] in patients with thrombotic disease. In the ECTIM study[34] a similar pattern appeared, with comparable levels in individuals homozygous for the common allele and heterozygotes, while only homozygotes for the rare allele had higher levels. Fowkes[33] found no correlation between *Bcl*I RFLP and plasma fibrinogen levels in patients with peripheral arterial disease in Scotland, in accordance with Wiseman[41] studying a comparable group in England.

Patients with CHD consistently have higher plasma fibrinogen levels than healthy individuals[36,42] and in several studies associations were observed between the plasma level of fibrinogen and the severity of the vascular disease[7,43,44]. In CHD patients the effects of smoking on the plasma fibrinogen levels were smaller than in healthy individuals[36,42], suggesting a lower reaction to stimuli when the fibrinogen level is already increased. The association between G/A^{-455} RFLP and the plasma fibrinogen levels in the patients is different from that in healthy individuals, which further illustrates that associations that were found in healthy individuals can not be simply transferred to patients.

When the fibrinogen levels were adjusted for CRP levels the relation between the G/A^{-455} RFLP and the plasma fibrinogen levels was weaker and no longer significant. There is also a stronger relation between the genotype and the plasma fibrinogen levels in smokers than in non-smokers. Since the location of the G/A^{-455} RFLP is close to the promoter of the Bß-gene it is therefore suggested that the induction of the fibrinogen synthesis by the acute phase reaction is, at least partly, associated with the G/A^{-455} RFLP.

In summary, we observed no differences in the frequencies or the linkage disequilibrium of the G/A^{-455} or *Taq*I RFLP in the CHD patients and healthy reference group when the patient group was studied as a whole or when the patients were studied in subgroups according to their smoking state and family history. The association between G/A^{-455} genotypes and plasma fibrinogen levels in the patient group was different from the association reported in healthy reference groups but not different from the association in other patient groups with vascular disease.

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SUMMARY AND GENERAL DISCUSSION

Several aspects that are related to the role of fibrinogen as a cardiovascular risk indicator were studied for this thesis.

Measurement of fibrinogen

First of all, the currently available fibrinogen assays were reviewed with special attention to their possible contribution to identifying prethrombotic states. The *clotting rate assays* are the most frequently used methods in routine laboratories; they specifically measure the functional levels of fibrinogen and they are sensitive to some factors that influence the time that is needed for clotting, such as levels of fibrin(ogen) degradation products and proteolytic degradation of the fibrinogen molecule. The *clottable protein assays* are independent of the clotting rate but spuriously high levels can be obtained in the presence of fibrin monomers or early degradation products of fibrinogen, that are still clottable. Measurement of clottable protein levels can be based on assessment of the amount of protein that can clot or can be based on the change in turbidity as the result of clot formation. The first type of method is not frequently used but the turbidity methods are gaining interest because they can be automated and performed in combination with a prothrombin time. The immunological methods that use polyclonal antibodies are mainly performed when a dysfunctional fibrinogen is suspected. New immunological methods that use monoclonal antibodies may be valuable in measuring specific fractions of fibrinogen, for example the fraction with the shortest clotting time. All available assays have their own characteristics and their value in risk assessment has to be compared in future in clinical and epidemiological studies (CHAPTER 1).

In Chapter 2 we evaluated five methods (clotting rate assays with thrombin, reptilase and peptidase, immunological assays with polyclonal and monoclonal antibodies) to what extent the heterogeneity of the fibrinogen molecule in plasma influenced the results and contributed to the notion of different specificity of the methods. Several molecular weight (MW) forms of fibrinogen can be detected in plasma: the high MW (HMW) form has both carboxyl-terminal parts of the Aα-chains intact, the low MW (LMW) form has one Aα-chain intact and the other degraded while in the LMW' form both Aα-chains have been degraded. We observed that the three forms were detected equally well in an enzyme immuno assay (EIA) with polyclonal antibodies, indicating that this EIA gives an appropriate representation of the molar concentration of fibrinogen. The characteristics of the three fibrinogen forms were found to differ in clotting rate assays, with the shortest clotting time for the HMW form, independent of whether the clotting was initiated by thrombin, reptilase or peptidase. The clotting time of LMW was slightly longer, and LMW' needed again somewhat longer to clot. The concentration curves of the three fibrinogen forms in the three clotting rate assays were not parallel. In monoclonal antibody based EIA for HMW+LMW fibrinogen the sensitivity for the

HMW forms of fibrinogen was three times higher than the sensitivity for the LMW form while the very low molecular weight (LMW') form was scarcely detected. In conclusion, the EIA using polyclonal antibodies gave a good illustration of the molar fibrinogen concentration, in the functional fibrinogen assays a complex image was observed of the different MW forms of fibrinogen and the HMW+LMW EIA was most specific for the HMW forms (CHAPTER 2).

The diagnostic value of each type of assay should eventually be assessed in clinical and epidemiological studies. Comparative studies in epidemiological studies are outside the scope of this thesis; we did, however, undertake a comparison of methods for application in patients with liver cirrhosis. Three methods for measuring plasma fibrinogen levels, namely a clotting rate method (Clauss-method), a total clottable protein method and an EIA for HMW+LMW fibrinogen, were evaluated in patients with mild, moderate and severe cirrhosis of the liver and in healthy controls. With each of the three methods, the fibrinogen levels were normal or slightly increased in patients with mild or moderate cirrhosis, whereas the levels in severe cirrhotics were decreased. Although the levels of the fibrin(ogen) degradation products were increased in the patients with moderate and severe cirrhosis, the levels were not that high that they might have disturbed the Clauss assay. We advise the use of the Clauss assay to determine the fibrinogen levels in cirrhotic patients, because with the three assays comparable plasma fibrinogen levels were observed and because the Clauss assay is very reproducible, quick, cheap and easy to perform (CHAPTER 3).

The acute phase reaction and fibrinogen

Fibrinogen is an acute phase protein and in several studies the level of fibrinogen has been associated with the degree of atherosclerosis. It has further been postulated that atherosclerosis is an inflammatory process of the vascular wall. Taken together, it might be suggested that the inflammatory state of the vascular wall is reflected by increased levels of fibrinogen in patients with coronary heart disease (CHD). Other acute phase proteins, like C-reactive protein (CRP) would then also be expected to be increased.

In chapter 4 cultured hepatocytes were used to study the acute phase reaction in vitro. The study focused on HRG suggested to be a negative acute phase protein since decreased HRG plasma levels have been observed in patients with an acute phase reaction. Decreased HRG levels have been observed in patients with an acute phase reaction which suggests that HRG is a negative acute phase protein. We could confirm this in a study in cultured hepatocytes from cynomolgus monkeys. HRG secretion was decreased dose-dependently by conditioned medium of LPS-stimulated monocytes (CM), tumour necrosis factor α (TNFα) and interleukin 1ß (IL1ß). The increase of the HRG mRNA levels by TNFα and IL1ß was higher than the increase of the secreted HRG. The HRG secretion and the HRG mRNA levels were not

affected by interleukin 6 (IL6). Evaluation of the positive acute phase proteins fibrinogen and CRP in the same model showed that the secretion of both proteins was markedly increased by CM. We further observed a down regulation of fibrinogen and CRP by TNFα, while fibrinogen was also decreased by IL1ß. Both positive acute phase reactants showed a marked increase upon addition of IL6. These studies suggest the evaluation of IL6 as an important marker for acute phase reactions and they warn for complexity due to differences in the regulation of the acute phase proteins (CHAPTER 4).

This difference in regulation of the acute phase proteins was also observed *in vivo*. We observed increased fibrinogen levels in patients with CHD, but CRP levels were comparable in the patient group and healthy controls, rendering the acute phase hypothesis less likely. Smoking is known to increase the plasma levels of both fibrinogen and CRP in healthy volunteers. We could confirm this in our study, but to our surprise smoking increased fibrinogen only in the healthy controls, while CRP was increased by smoking both in the patients and the controls. In conclusion, it is suggested that the increased fibrinogen levels in CHD might not be the result of an acute phase reaction or that the regulation of the acute phase proteins fibrinogen and CRP is not parallel (CHAPTER 5).

In most cross-sectional and prospective epidemiological studies, the parameters are measured only in one blood sample. Since fibrinogen, PAI, CRP and histidine rich glycoprotein (HRG) are all acute phase proteins, it might be expected that their plasma levels show variation due to acute phase reactions, which might affect the required sample size in epidemiological studies or which might require multiple sampling in the assessment of habitual levels of individuals. In a longitudinal study the relations between intra- and inter-individual variation of these variables were investigated for young healthy volunteers. From those data it was determined that in epidemiological studies multiple sampling is not required since a relatively small increase in the number of participants is necessary to compensate for the biological variation. For the assessment of a habitual level single sampling is sufficient for HRG and PAI, but fibrinogen and CRP will need duplicate sampling to get the within-individual variation below an acceptable 10% of the total variation (CHAPTER 6). A small pilot study of the placebo period in the healthy volunteers and the CHD patients in the ticlopidine study (described in CHAPTER 9) suggests a comparable relation between the intra- and interindividual variation (results not shown).

Modulation of plasma fibrinogen levels by medication

It has been suggested that N-3 fatty acids, when they are administered in combination with the antioxidant vitamin E, affect the synthesis of fibrinogen, CRP and PAI-1. In our study on healthy young volunteers, however, no effects of either fish oil containing vitamin E or vitamin E alone were observed. However, it might

be possible that vitamin E and N-3 fatty acids affect the plasma levels of fibrinogen, CRP and PAI activity in patient groups, in older individuals or in smokers, who might for instance have a low grade acute phase reaction or higher lipid levels and increased fibrinogen levels. We conclude that in healthy young volunteers fish oil and vitamin E do not affect fibrinogen, CRP and PAI activity (CHAPTER 7).

Several drugs have been described that can, in addition to their originally intended medical effects, decrease the plasma fibrinogen levels. One example is ticlopidine, a drug that inhibits the ADP-induced aggregation of blood platelets. Surgical procedures induce an increase of the fibrinogen levels, probably by an acute phase reaction. If the effect of ticlopidine on fibrinogen is mediated through modulation of the acute phase reaction, it might be expected that the acute post surgical fibrinogen levels are lower in the patients that are treated with ticlopidine, compared with the patients that received placebo. However, there was no difference in post surgical fibrinogen levels, indicating that ticlopidine does not influence the post surgical increase of the plasma fibrinogen levels (CHAPTER 8).

It has been reported several times that ticlopidine can decrease the plasma fibrinogen levels in patients with vascular diseases, but no studies into the mechanism have yet been performed. The decrease might be a direct effect on the fibrinogen synthesis, in which case effects on fibrinogen levels in healthy volunteers would be expected. If, however, the action of ticlopidine is through regulation of the chronically stimulated low-grade acute phase or through the fibrin(ogen) degradation products induced fibrinogen synthesis, then the effect would be much more pronounced in patients than in volunteers or only observed in the patients. In this study a 0.20 g/L (9%) decrease of the fibrinogen levels in patients and a 0.39 g/L (11%) decrease in healthy volunteers was observed, which makes a clear distinction of the suggested mechanisms impossible. However, our results suggest the occurrence of a disease independent effect on fibrinogen synthesis. A remarkable finding was that the effect was only observed in the clotting rate assay and not in the HMW+LMW fibrinogen EIA, suggesting that the effect involves a change in the quality rather than quantity of fibrinogen. Further analysis is required to elucidate the mechanism by which ticlopidine lowers fibrinogen. Also, a genetic contribution had been suggested, but no effects could be observed of the genetic polymorphism of the fibrinogen Bß-chain (CHAPTER 9).

Another group of drugs that has been reported to be able to decrease the plasma fibrinogen levels are the fibrates. In patients that were treated with these drugs for high lipid levels, an additional decrease of the functional fibrinogen levels was observed. In an attempt to elucidate the mechanism of this decrease, we evaluated the effects of gemfibrozil and ciprofibrate on fibrinogen levels, and studied the association of the fibrinogen decrease with the lipid lowering effect, effects on the acute phase and a genetic contribution. After 12 weeks of treatment, we observed a decrease of the functional fibrinogen levels with gemfibrozil and a stronger decrease

with ciprofibrate. However, no associations with changes in lipids, CRP or genetic polymorphisms were observed which suggests that there must be an additional mechanism for the regulation of fibrinogen levels (CHAPTER 10.

Genetic polymorphisms and fibrinogen

In patients with peripheral arterial disease a higher frequency was observed of the rare B2 allele of the *Bcl*I RFLP of the Bß-chain of fibrinogen. Furthermore, in healthy individuals higher plasma fibrinogen levels are associated with the occurrence of the rare allele of the fibrinogen Bß-chain polymorphisms. Since Greenland Inuit are a population with a low incidence of myocardial infarctions, despite a "normal" atherosclerosis level, it might be interesting to study if there might be population differences in the characteristics of fibrinogen RFLP. Therefore, we studied the fibrinogen polymorphisms of the Aα- and Bß-genes, the linkage disequilibrium between them and their associations with the plasma fibrinogen levels. We observed that the frequencies of RFLP of genes for the Aα and Bß chains of fibrinogen and the linkage disequilibrium between them were different in the Inuit group from what has been described for Caucasian populations. In the Inuit the relation between polymorphisms of the fibrinogen Bß-chain and the plasma fibrinogen levels was comparable to the relations that are known for Caucasian populations, with the highest levels in the individuals that are homozygous for the rare allele of the Bß-chain RFLP. The relation was stronger in men than in women. The higher frequencies in Inuit of the alleles that are associated with lower fibrinogen when compared to the Caucasian might suggest that they play a role in differences in CHD in the Inuit (CHAPTER 11).

The higher frequency of the B2 allele in peripheral arterial disease might suggest a possible role for the RFLP in the gene of the fibrinogen Bß-chain in other vascular diseases. Therefore, the allele frequencies and linkage disequilibrium of fibrinogen polymorphisms were studied in men with symptomatic CHD in relation to the frequencies in healthy individuals. We found no differences in the frequency of alleles or the linkage between RFLP of the Aα and Bß-chains of fibrinogen. Subgroup analysis according to smoking habits or family history of CHD also showed no frequency differences. The plasma fibrinogen levels were comparable in patients that were heterozygote or homozygote for the common allele of the Bß-chain RFLP, while patients homozygous for the rare allele had higher fibrinogen levels. This effect was only significant in smokers. Therefore we concluded that in this study there is no indication for a relation between Bß-fibrinogen RFLP and CHD (CHAPTER 12).

The overall objective of this thesis has been to investigate different aspects of fibrinogen in its role as risk indicator for vascular events. At present, not sufficient information is available to definitively decide whether fibrinogen is a risk indicator for vascular diseases and vascular events and/or whether it plays a pathogenetic/etiological role in the development of disease. It is also conceivable that initial elevations in fibrinogen mark the existence of a continued tissue repair process and inflammation and that persistence of elevated fibrinogen contributes to progression of the disease. It is also possible that the role of elevated fibrinogen is different for the different forms of vascular disease such as peripheral arterial disease, atherosclerosis and acute thromboembolic events such as myocardial infarction. Based, among others, on the results of the ECAT Angina Pectoris Study, it has been argued by Haverkate that inflammatory factors, such as fibrinogen, might be a marker for the occurrence of atherosclerotic disease and may at the same time play a causal role in thromboembolic complications of such diseases.

Assays of fibrinogen: discussion and conclusions

The first aspect that was considered was the measurement of the plasma fibrinogen level. In the last decade, experts have had difficulties in defining what fibrinogen is since there is a large heterogeneity in the circulating fibrinogen molecules, with estimates of 10^6 different fibrinogen molecules. This heterogeneity creates a problem for the assay methodology. The uncertainty mentioned above about the role of fibrinogen in vascular diseases makes it at present impossible to select those aspects of fibrinogen that should be assayed for optimal prediction of risk. It is a fact that different types of assays have been used in the various epidemiological studies and that all showed a predictive value. Until now, only one direct comparison of different methods in one study has been reported: in the Caerphilly and Speedwell Studies the predictive value of a nephelometric assay was significantly larger than the Clauss assay (2nd International Symposium on Fibrinogen and Cardiovascular disease, November 1994, Edinburgh). Further comparative studies need to be performed and such study will inform us firstly about the aspect of fibrinogen that is most important and secondly, it will indicate which assay should be used for optimal prediction. For such a comparison a selection of a set of assays that each represent different aspects of fibrinogen can be proposed. Our analysis of existing methods, including a recent assay that uses monoclonal antibodies, revealed more than expected differences between assays in the specificity for fractions of fibrinogen.

If fibrinogen is a risk factor because more fibrinogen gives more substrate to form a clot, it is expected that the clottable protein assays will give the data that directly represents the cardiac risk. If the clotting rate of fibrinogen is important for cardiac risk clotting rate assays are indicated but might also result in choosing the HMW+LMW EIA for the assessment of the fibrinogen levels, since this EIA mainly detects the HMW form, which has the highest clotting rate. If, however, the

plasma fibrinogen level is a risk marker that reflects the chronic inflammatory state of the vascular wall, it could be a choice measure the molar fibrinogen concentration, for example by using polyclonal antibody-based immunological methods or to measure the HMW fraction, which is most probably the form in whcih fibrinogen is newly synthesized. The fibrinogen that is synthesized as a result of the acute phase is HMW fibrinogen, with a short clotting time. Therefore, using functional assays might also provide good marking in inflammatory states. Furthermore, also the effect of fibrinogen on the blood viscosity, its stimulating effect on smooth muscle cells, its association with platelet aggregation or its role in plaque formation might be the mechanism behind the relation between the fibrinogen levels and the risk for vascular events. These possible mechanisms would each be best represented by a different assay (CHAPTER 1,2,3).

Next to assays for the molar concentration of total fibrinogen, no assays specific for intact or degraded forms of fibrinogen could be evaluated as yet, but are desired. It was a surprise that the functional property, the clotting rate, was a complex function of the various molecular forms when in mixture and thus should be evaluated as such. The importance of assay choice was illustrated further in this thesis when effects of ticlopidine on fibrinogen were only observed with a clotting rate assay and not with an enzyme immuno assay for HMW plus LMW fibrinogen.

It is a conclusion from our studies that further attention should be paid to definition of assay specificity and that additional assays for specific fractions and specific functions of fibrinogen are required for research. For each vascular disease and clinical question a rationally designed comparison of assays will provide information about pathogenetic mechanisms and which assay is the best for predictive purposes. Future research in the field of fibrinogen assays might therefore concentrate on a) comparison of the different fibrinogen assays in epidemiological studies to compare their capacity to predict (venous or arterial) thrombotic risk and b) the identification of the mechanism of the association between fibrinogen and thrombotic risk will probably make it necessary to develop a new fibrinogen assay, since the available methods have a number of restrictions

Mechanisms of elevation of fibrinogen

Only the acute phase reaction and induction of fibrinogen synthesis by fibrin(ogen) degradation products and hormones are biochemically defined regulatory mechanisms which might be involved in the elevation of fibrinogen.

To evaluate the role of these mechanisms we determined in various studies the acute phase condition extensively (IL6, IL1ß, TNFα, CRP) or more limited (CRP, fibrin(ogen) degradation products) in relation to the effects on the synthesis or condition of fibrinogen. We performed cross-sectional patient studies, analyzed effects of medication and followed the acute phase reaction during surgery. A

general conclusion is that the acute phase reaction and fibrin(ogen) degradation products do not seem to explain the changes in the plasma fibrinogen levels or to correlate with it, suggesting strongly the occurrence of another (as yet unknown and speculative) mechanism for elevation of fibrinogen. Observations in cultured primary hepatocytes showed a difference in regulation of CRP and fibrinogen by cytokines, which reduces the power of conclusions on the role of the acute phase reaction concluded on levels and changes in CRP. Also in cross-sectional analysis of patients awaiting a PTCA it was observed that differences occurred in relations between fibrinogen, CRP and cytokines when non-smoking and smoking patients were compared with non-smoking and smoking healthy individuals. Therefore as a first approach it is required to further underscore the above conclusion about another mechanism, with analysis of cytokines and other acute phase proteins in a number of the studies that we have performed and in other patient groups. Next a thorough study into the putative new mechanisms that influence the fibrinogen synthesis is indicated. From our studies it can be concluded that it is not related to lipid metabolism since lipid lowering and fibrinogen changes were clearly dissociated in two of our studies. We observed no difference in the postoperative increase of the plasma fibrinogen levels when we compared patients who received placebo with those who were treated with ticlopidine, which makes it unlikely that ticlopidine regulates the acute phase induced fibrinogen synthesis (CHAPTER 7). Treatment of hyperlipemic patients with fibrates resulted in a decrease of the fibrinogen levels. The C-reactive protein levels were normal in these patients, not affected by the fibrate treatment and changes in fibrinogen were not related to changes in CRP (CHAPTER 10).

We also observed effects of ticlopidine in healthy volunteers which suggests that the baseline plasma fibrinogen levels (or function) were affected. A clue might be found in the fact that the clotting rate of fibrinogen can be changed independently from levels of HMW and LMW fibrinogen (see chapter 9) suggesting the possibility that an important qualitative/functional change in the fibrinogen moiety is possible. Further studies should certainly take the aspect of molecular heterogeneity of fibrinogen and related assay methodology into account. Further studies on the mechanism of regulation of fibrinogen synthesis needs to concern a) analysis of the complexity of the relation between fibrinogen decrease by medication and the acute phase reaction or FDP, for example by the measurement of the cytokines IL6, IL1ß and TNFα and b) the other, yet unidentified mechanisms that are involved in the regulation of the fibrinogen decrease by medication, for example a direct effect on hepatocytes, possible via effects on signal transduction.

Genetic studies on fibrinogen

When elevated fibrinogen levels plays an etiological role in vascular diseases it can be expected that also genetically determined elevations might contribute. In this

respect, the report of Fowkes et al about a higher frequency in British patients with peripheral arterial disease of the rare allele of the G/A^{-455} fibrinogen Bß-chain polymorphism is of considerable interest, especially since this A^{-455} allele is associated with higher plasma fibrinogen levels. Our analysis of Dutch men with anginal complaints and documented coronary artery disease showed no such difference at all. This either indicates a difference between populations or a difference between the type of vascular disease. It strongly suggests to repeat the analysis reported by Fowkes et al on Dutch patients with peripheral arterial disease, and/or to focus analysis on well-defined patient groups in order to obtain information about the vascular complications/diseases that are "sensitive" of genetically increased fibrinogen levels. We confirmed a relationship between genotypes of Bß-chain fibrinogen polymorphisms and plasma fibrinogen levels in healthy volunteers (chapter 6,11). However, relationships between genetic background and fibrinogen levels depended upon other fibrinogen determinants, such as gender and smoking habits. Apparently in patients who as a result of their disease have increased fibrinogen levels, the effect of the fibrinogen polymorphisms on the plasma fibrinogen levels are weakened or even overruled.

In conclusion, the characteristics of fibrinogen polymorphisms are different in healthy populations with different cardiovascular risks and in healthy volunteers and CHD patients, which indicates a desire for further research. Since in the Inuit the genetics of the fibrinogen genes are in a direction that might suggest a contribution of fibrinogen polymorphisms in the reduced risk of cardiac events, research into different populations with different cardiac risks might give further information. In CHD patients no effect of genetics is observed, and therefore future research might concentrate on other disease populations, e.g. patients with unstable angina that either do or do not have complications.

SAMENVATTING

Voor dit proefschrift werden verschillende aspecten van de rol van fibrinogeen als risicofactor voor hart- en vaatziekten bestudeerd.

Meting van fibrinogeen

In **HOOFDSTUK 1** worden de momenteel beschikbare fibrinogeenbepalingen besproken, waarbij speciaal de aandacht uitging naar de bijdrage van de respectievelijke bepalingen bij het vaststellen van thrombofilie. De *stolsnelheids-bepalingen* worden het meest frequent gebruikt in routine laboratoria; deze bepalingen meten specifiek het functionele fibrinogeen gehalte en zijn gevoelig voor factoren die de stolsnelheid beïnvloeden, zoals daar zijn de fibrin(ogeen) afbraak produkten en enzymatische afbraak van het fibrinogeen molekuul. De *stolbaar eiwit bepalingen* zijn onafhankelijk van de snelheid van stolselvorming, maar te hoge gehaltes worden gevonden in de aanwezigheid van fibrine monomeren of vroege afbraakprodukten van fibrine. De meting van stolbaar eiwit kan geschieden via het bepalen van de hoeveelheid eiwit die stolt of gebruikt de verandering in turbiditeit die optreedt als gevolg van de stolling. Deze eerste methode wordt nauwelijks gebruikt maar er komt steeds meer aandacht voor de turbiditeitsmetingen omdat deze kunnen worden geautomatiseerd en omdat ze kunnen worden gecombineerd met de prothrombine-tijd. De *immunologische* methoden die gebruik maken van polyclonale antilichamen worden voornamelijk gebruikt als er een disfunctioneel fibrinogeen wordt verwacht. De immunologische methoden die specifieke monoclonale antilichamen gebruiken kunnen waardevol zijn in het identificeren van specifieke fracties van fibrinogeen, bijvoorbeeld de fractie die het snelst stolt. De verschillende methoden hebben ieder hun eigen karakteristieken en waarde in risico-vaststelling en de waarde van de verschillende methoden moet worden bestudeerd in epidemiologische studies.

In **HOOFDSTUK 2** werden van vijf methoden (stolsnelheids-bepalingen met thrombine, reptilase en peptidase, immunologische bepalingen met poly- en monoclonale antilichamen) geëvalueerd in welke mate de heterogeniteit van het fibrinogeenmolecuul bijdroeg aan de specificiteit van de verschillende bepalingen. In plasma kunnen fibrinogeen moleculen met verschillend moleculair gewicht worden waargenomen: de hoog moleculair gewicht (HMW) vorm met beide carboxyl einden van de Aα-keten intact, de laag MW (LMW) vorm met een Aα-keten intact en de andere afgebroken terwijl bij de LMW' vorm beide Aα-ketens zijn gedegradeerd. We constateerden dat de drie vormen even goed werden gemeten met een enzyme immuno assay (EIA) die gebruik maakte van polyclonale antilichamen, hetgeen doet vermoeden dat deze EIA een goede weerspiegeling vormt van de molaire fibrinogeen concentratie. De drie fibrinogeen vormen hadden verschillende stoltijden, waarbij de HMW vorm de kortste stoltijd gaf, onafhankelijk van het enzym dat de stolling in gang zette: thrombine, reptilase of peptidase. De stoltijd van LMW wat iets langer terwijl LMW' weer wat meer tijd nodig had om te stollen. De concentratie curven

van de drie fibrinogeen vormen in de drie stolsnelheids-bepalingen waren niet parallel.

In de EIA met monoclonale antilichamen voor de HMW en LMW vormen van fibrinogeen was de gevoeligheid waarmee HMW werd gemeten drie keer zo hoog als die waarmee LMW werd gemeten, terwijl de test nauwelijks gevoelig was voor LMW'. We concludeerden dat de EIA met polyclonale antilichamen een goede afspiegeling gaf van de molaire fibrinogeen concentratie, dat 'de functionele bepalingen met de verschillende MW vormen een complex beeld te zien gaven en dat de HMW + LMW EIA het meest specifiek was voor de HMW vorm.

De diagnostische betekenis van elk type bepaling dient uiteindelijk te worden vastgesteld in klinische en epidemiologische studies. Vergelijkende studies binnen epidemiologische onderzoeken liggen buiten het bereik van dit proefschrift; we hebben wel fibrinogeen methoden vergeleken in patiënten met milde, gematigde of ernstige cirrhose van de lever. In **HOOFDSTUK 3** worden drie methoden voor het meten van fibrinogeen geëvalueerd, namelijk een stolsnelheids bepaling (Clauss), meting van de hoeveelheid stolbaar eiwit en een EIA voor HMW + LMW fibrinogeen. Met elk van de drie methoden was het fibrinogeen gehalte normaal of licht verhoogd in patiënten met milde of gematigde cirrhose, terwijl de gehaltes in ernstige cirrhose verlaagd waren. Hoewel de gehaltes van fibrin(ogeen) afbraak produkten verhoogd waren in patiënten met matige en ernstige cirrhose, was deze verhoging niet zodanig, dat het de Clauss bepaling zou beïnvloeden. Wij adviseerden om de Clauss methode te gebruiken bij het meten van fibrinogeen gehaltes in patiënten met cirrhose, omdat de drie methoden vergelijkbare fibrinogeen levels gaf en omdat de Clauss methode reproduceerbaar, snel, goedkoop en makkelijk uit te voeren is.

De acute fase reactie en fibrinogeen

Fibrinogeen is een acute fase eiwit en in verschillende studies is het gehalte van fibrinogeen in verband gebracht met de mate van atherosclerose. Verder is gesuggereerd (Ross) dat atherosclerose een ontstekingsproces van de vaatwand is. Hieruit zou kunnen volgen dat de verhoogde fibrinogeen gehaltes by patiënten met coronaire hartziekten (CHD) een weerspiegeling vormen van de ontstekingstoestand van de vaatwand. Als dat zo zou zijn, dan is het ook de verwachting dat andere acute fase eiwitten, zoals C-reactief eiwit (CRP) verhoogd zal zijn.

In **HOOFDSTUK 4** werden hepatocyten in kweek gebracht om de acute fase reactie *in vitro* te bestuderen. De studie was gericht op het bestuderen van het negatieve acute fase karakter van histidine-rijk glycoproteïne (HRG), dat in de literatuur werd gesuggereerd door verlaagde plasma HRG gehaltes by patiënten met een acute fase reactie. Wij konden dit negatieve acute fase gedrag bevestigen in gekweekte hepatocyten van makaak apen. De uitscheiding van HRG in het kweekmedium werd dosis afhankelijk verlaagd door geconditioneerd medium van

LPS-gestimuleerde monocyten (CM), tumor necrosis factor-α (TNFα) en interleukine-1ß (IL1ß). De verlaging van de hoeveelheid HRG mRNA was sterker dan de verlaging van het uitgescheiden eiwit. De uitscheiding van HRG en de hoeveelheid HRG mRNA werden niet beïnvloed door interleukine 6 (IL6). Bij evaluatie van de positieve acute fase eiwitten fibrinogeen en CRP in hetzelfde model bleek dat de uitscheiding van beide eiwitten duidelijk werd verhoogd door CM. Verder werd bemerkt dat fibrinogeen en CRP werden verlaagd door TNFα, waarbij fibrinogeen ook werd verlaagd door IL1ß. De secretie van beide positieve acute fase reactanten werden sterk verhoogd door IL6. Deze studies doen vermoeden dat IL6 een belangrijke marker is voor acute fase reacties en ze waarschuwen voor de complexiciteit in de verschillen van de regulatie van acute fase eiwitten.

Bij patiënten met CHD werden verhoogde fibrinogeen gehaltes gevonden, maar de CRP gehaltes waren vergelijkbaar in de patiëntengroep en de gezonde vrijwilligers, hetgeen een verschil in acute fase tussen deze groepen onwaarschijnlijk maakt. Het is bekend dat roken in gezonde vrijwilligers het plasma gehalte van zowel fibrinogeen als CRP verhoogd. Wij konden dit bevestigen in deze studie, maar tot onze verrassing verhoogde roken het fibrinogeen gehalte alleen in de vrijwilligers, terwijl CRP in beide groepen werd verhoogd. Wij concludeerden daarom dat waarschijnlijk de verhoogde fibrinogeen gehaltes bij CHD niet het resultaat zijn van een acute fase reactie of dat de regulatie van de acute fase eiwitten fibrinogeen en CRP verschillend is (HOOFDSTUK 5).

In een groot deel van de cross-sectionele en prospectieve epidemiologische studies worden de parameters in slechts een monster gemeten. Omdat fibrinogeen, PAI, CRP en HRG acute fase eiwitten zijn zou het kunnen worden verwacht dat hun plasma gehaltes variatie vertonen als gevolg van acute fase reacties. Dit zou dan weer de grootte van de studiepopulatie in epidemiologische studies beïnvloeden of het zou meervoudige bloedafname nodig maken bij het vaststellen van het basale gehalte van een individu. In een longitudinale studie werden de relaties tussen intra- en interindividuele variatie van deze variabelen bestudeerd in gezonde, jonge vrijwilligers. Uit de verkregen gegevens werd geconcludeerd dat in epidemiologische studies meervoudige afname geen duidelijke voordelen biedt daar een relatief kleine vergroting van de studiegroep nodig is om voor de biologische variatie te compenseren. Voor de bepaling van de basale level van een individu is eenmalige afname voldoende voor de bepaling van HRG en PAI, maar voor fibrinogeen en CRP zal meervoudige afname nodig zijn om de intra-individuele variatie binnen een acceptabele 10% van de totale variatie te houden (HOOFDSTUK 6). Een kleine pilot studie van de placebo periode in de gezonde vrijwilligers en de CHD patiënten in de ticlopidine studie suggereert een vergelijkbare relatie tussen de intra- en interindividuele variatie (resultaten worden niet gegeven).

Modulatie van plasma fibrinogeen gehaltes door medicatie

Het is beschreven dat N-3 vetzuren, wanneer ze worden toegediend in combinatie met het antioxidant vitamine-E, de synthese van fibrinogeen, CRP en PAI-1 kunnen beïnvloeden. In onze studie bij gezonde, jonge vrijwilligers werd geen effect van vitamine E bevattende visolie of vitamine E geconstateerd. Dit zou kunnen worden verklaard door de keuze van de studie-populatie. Het effect zou mogelijk kunnen verwacht in individuen met een laag niveau ontstekingsreactie, verhoogde lipid-gehaltes en verhoogde fibrinogeen gehaltes, zoals patiënten, oudere mensen of rokers. Wij concluderen dat in gezonde, jonge vrijwilligers visolie en vitamine E geen invloed hebben op fibrinogeen en CRP (HOOFDSTUK 7).

Van verschillende stoffen is het beschreven dat ze, naast hun belangrijkste effecten, ook de plasma fibrinogeen gehaltes verlagen. Een voorbeeld is ticlopidine, een medicijn dat de ADP-geïnduceerde aggregatie van bloedplaatjes remt. Operaties zorgen voor een verhoging van de fibrinogeen gehaltes, mogelijk door een acute fase reactie. Indien aangenomen wordt dat het effect van ticlopidine op fibrinogeen wordt geschiedt door modulatie van de acute fase reactie, zou worden verwacht dat de post-operationele fibrinogeen gehaltes lager zouden zijn in patiënten die worden vergeleken met ticlopidine dan in patiënten die placebo krijgen. In onze studie was er geen verschil in post-operationele fibrinogeen gehaltes, hetgeen suggereert dat ticlopidine geen invloed heeft op de post-operationele stijging van fibrinogeen (CHAPTER 8).

In verschillende studies is gebleken dat ticlopidine de plasma fibrinogeen gehaltes verlaagd in patiënten met vaatziekten, maar er zijn nog geen studies verricht naar het mechanisme. Het is mogelijk dat de verlaging een direct effect is op de fibrinogeen synthese, hetgeen ertoe zal leiden dat ticlopidine de fibrinogeen gehaltes van patiënten en ook van gezonde vrijwilligers zou verlagen. Indien ticlopidine de regulatie van fibrinogeen synthese door de acute fase of de TDP-produkten beïnvloed, zou het effect sterker zijn in patiënten dan in vrijwilligers. In onze studie werd fibrinogeen 0.20 g/L (9%) verlaagd in de gezonde vrijwilligers en 0.39 g/L (11%) in gezonde vrijwilligers, hetgeen een duidelijk onderscheid van de gesuggereerde mechanismen onmogelijk maakt. Onze resultaten suggereren een ziekte-onafhankelijk effect op de fibrinogeen-synthese. Een opmerkelijke bevinding was dat het effect alleen werd waargenomen met de Clauss-bepaling en niet met de HMW+LMW fibrinogeen EIA, hetgeen suggereert dat het effect een verandering in de kwaliteit in plaats van de hoeveelheid zou kunnen zijn. Verdere analyse is nodig om dit te bestuderen. Verder zou een genetische bijdrage een rol kunnen spelen, maar in deze studie werden geen effecten van het genetische polymorfisme van de fibrinogeen Bß-keten (HOOFDSTUK 9).

De fibraten vormen andere groep medicijnen waarvan bekend is dat ze fibrinogeen verlagen. In patiënten die werden behandeld met deze medicijnen omdat ze hoge lipid gehaltes hadden, werd een verlaging van de functionele fibrinogeen gehaltes

waargenomen. In een poging om het mechanisme van deze verlaging op te helderen, werd het effect van gemfibrozil en ciprofibraat of fibrinogeen gehaltes bestudeerd. De associatie tussen de fibrinogeen verlaging en de lipid verlaging, het effect op de acute fase en een genetische bijdrage werden geëvalueerd. Na een behandelingsperiode van 12 weken was het functionele fibrinogeen gehalte gedaald met beide medicijnen, maar het was sterker gedaald met ciprofibraat dan met gemfibrozil. Geen associatie werd gevonden met veranderingen in lipiden, CRP of genetische polymorfismen, hetgeen suggereert dat een ander mechanisme het fibrinogeen reguleert (HOOFDSTUK 10).

Genetische polymorfismen en fibrinogeen

In patiënten met perifeer vaatlijden werd een hogere frequentie waargenomen van het zeldzame B2 allel van het *Bcl*1 polymorfisme van de Bß keten van fibrinogeen. Ook werden in gezonde vrijwilligers hogere plasma fibrinogeen gehaltes waargenomen in individuen met het zeldzame allel van de fibrinogeen Bß keten polymorfismen. Daar Groenlandse Inuit een lage incidentie hebben van myocard infarcten, ondanks een "normaal" atherosclerose gehalte, waren wij geïnteresseerd naar de populatie verschillen van de fibrinogeen polymorfismen. Daartoe bestudeerden wij de frequenties en de relatie met het fibrinogeen gehalte van de polymorfismen van de Aα en de Bß ketens van fibrinogeen. In de Inuit waren er verschillen in de frequentie van de RFLP en de linkage wanneer ze werden vergeleken met Caucasische populaties. De relatie tussen de RFLP en de plasma gehaltes was hetzelfde in de Inuit en in de Caucasiers, met de hoogste gehaltes in degenen die het zeldzame allel van de Bß-keten polymorfismen bezitten. Deze relatie was sterker in mannen dan in vrouwen. De hogere frequentie in de Inuit an de allelen die worden geassocieerd met een lager fibrinogeen zou kunnen suggereren dat ze een rol spelen in de verschillen in CHD in de Inuit (CHAPTER 11).

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Dit proefschrift is tot stand gekomen met de hulp van velen. Ik wil hier iedereen bedanken en met name

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CURRICULUM VITAE

De schrijfster van dit proefschrift is geboren op 14 mei 1960 te Rosmalen. Na het doorlopen van het Atheneum-B te Vlaardingen werd de HBO-B opleiding tot chemisch medisch analist gevolgd van 1978 tot 1981 aan het Van 't Hoff Instituut te Rotterdam. In juni 1981 werd een aanstelling als research analist verkregen bij de afdeling Hematologie van het Dijkzigt Ziekenhuis te Rotterdam (Hoofd: Prof. J. Abels). In 1986 werd overgestapt naar de afdeling Inwendige Geneeskunde II (Hoofd: Prof. J.H.P. Wilson). Van 1987 tot 1991 werd de deeltijdopleiding Chemie gevolgd aan de Rijksuniversiteit te Utrecht. Het hoofdvak werd verricht bij de vakgroep Enzymologie en Protein Engineering (Prof. G.H. de Haas) en werd praktisch uitgevoerd op het Gaubius Instituut TNO, te Leiden. Na het behalen van het doctoraalexamen volgde een aanstelling als wetenschappelijk medewerking bij de afdeling Inwendige Geneeskunde II van de Erasmus Universiteit Rotterdam. Tijdens de studie chemie werd aangevangen met het onderzoek dat in dit proefschrift is beschreven en dat voor het grootste deel werd uitgevoerd in het Gaubius Laboratorium TNO-PG. Vanaf 1 januari 1994 is de schrijfster tevens verbonden aan het Instituut for Thrombosis Research van het South Jutland University Center in Esbjerg, Denemarken.