THE EFFECTS OF VITAMIN C ON THE HAEMOSTATIC SYSTEM

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OPSOMMING

Motivering:

Kardiovaskulêre siektes (KVS) is een van die hoofoorsake van sterftes in Suid-Afrika, asook wêreldwyd. Dislipidaemia en 'n verhoogde stollingstoestand dra by tot die ontwikkeling van KVS. Die kwaliteit van die fibriennetwerkstruktuur (FNS) kan ook tot die risiko vir KVS en trombose bydra. Veranderinge in die fibrinogeenkonsentrasie beïnvloed die FNS direk. Die behandeling van hierdie risikofaktore is noodsaaklik en dieetintervensie vorm 'n essensiële deel hiervan. Verhoogde vitamien C-inname kan 'n afname in die vatbaarheid vir infeksie te weeg bring, en daardeur moontlik verlaagde vlakke van hemostatiese faktore (wat tot 'n antitrombotiese toestand kan lei) tot gevolg hê. Dit kan dus tot 'n afname in KVS asook mortaliteit bydra. Vitamin C kan moontlik ook voordelig wees deurdat dit profibrinolitiese aktiwiteite van FNS kan verhoog (vorming van dik fibrienvesels en klonte wat maklik afgebreek kan word) en sodoende moontlik tot 'n afname in arteriosklerose en daaropvolgende KVS kan lei.

Doel:

Om die effek van supplementasie met "FoodState Vitamin C complex®" op hemostatiese faktore, die FNS, serum lipiede asook lipoproteïen (a) (Lp(a)) in hiperlipidemiese pasiënte te ondersoek.

Metodes:

Dertig hiperlipidemiese vrywilligers uit die Lipiedkliniek, Potchefstroomse Universiteit vir Christelike Hoër Onderwys, het aan hierdie ewekansige plasebo gekontroleerde dubbelblinde oorkruisstudie deelgeneem. Proefpersone is ewekansig in twee groepe (A of B) verdeel. Na 'n inloopfase van 4 weke, waartydens geen vitamiensupplemente ingeneem is nie, het Groep A 2 tablette/dag "FoodState Vitamin C complex®" (500mg vitamien C, 600mg magnesiumvoedselkompleks, 900mg vitamien B kompleks en 160mg bioflavonoïedes) en Groep B 2 tablette/dag van die plasebo, vir ten minstens 8 weke ontvang. 'n Uitwasperiode van 8 weke het gevolg waarna die behandelings omgeruil is vir 'n verdere 8 weke. Bloedmonsters tydens vasting is 8 keer (twee bloedmonsters, 1 week uitmekaar aan die begin en einde van elke fase) gedurende die studie geneem.

Resultate:

"FoodState Vitamin C complex®" supplementasie het nie betekenisvolle verskille in plasma-fibrinogeen, plasminogeenaktiveerderinhibeerder-1-aktiwiteit ($PAI\text{-}1_{akt}$), weefselplasminogeen-

aktiveerderantigeen (tPA_{ag}) of d-dimeer veroorsaak nie. Serumlipiede en Lp(a) was ook nie betekenisvol beïnvloed nie. Die mediaanplasmien-antiplasmienkompleks (PAP) en trombien-antitrombienkompleks (TAT), wat onderskeidelik merkers van plasmien - (inisïeer fibrinolise) en trombien - (inisïeer koagulasie) vorming is, was albei betekenisvol verlaag in vergelyking met plasebo (PAP: -4.05[–23.39, -0.23]% teenoor 1.81[-8.95, 8.09]%; TAT: -5.81[-18,47, 0.39]% teenoor 0.12[-8.03, 13.5]%). FNS is ook betekenisvol deur "FoodState Vitamin C complex®" beïnvloed, deurdat dit die kompaksie verhoog het (49.95[47.55, 53.70]% na 51.85[48.55, 56.65]%).

Gevolgtrekking:

Die verlagings in TAT en PAP is moontlik 'n aanduiding daarvan dat "FoodState Vitamin C complex®" die inisiëring van hemostase verlaag het, wat weer 'n kompensatoriese verlaging in fibrinolise tot gevolg gehad het. "FoodState Vitamin C complex®" mag dus dalk teen KVS beskerm, deur 'n verlaging van die ewewigstoestand van die hemostatiese balans en die vorming van meer afbreekbare klonte (verhoogde kompaksie).

Sleutelwoorde:

Hemostase; Hemostatiese faktore; Fibriennetwerkstruktuur; Kardiovaskulêre siekte; Arteriosklerose; Inflammasie; Vitamien C; Anti-oksidante

ABSTRACT

Motivation:

Cardiovascular disease (CVD) is one of the leading causes of mortality and morbidity in South Africa and worldwide. Dyslipidaemia and an increased coagulation state contribute to the development of CVD. The quality of fibrin network structure (FNS) may also contribute to the risk for CVD and thrombosis. Changes in fibrinogen concentration directly affect FNS. Management of these risk factors is important and dietary intervention forms an essential part of this management program. An increased intake of vitamin C can lead to a decreased susceptibility to infection and subsequently to decreased levels of haemostatic factors (that give rise to an anti-thrombotic state) and thus reduction in CVD and mortality. Furthermore, vitamin C may prove to be beneficial by increasing the pro-fibrinolytic activities of FNS (formation of thick fibrin fibers and more lysable clots) that could result in a reduction in atherosclerosis and subsequent CVD.

Objective:

To investigate the effects of FoodState Vitamin C complex® supplementation on haemostatic factors, FNS, serum lipids and lipoprotein (a) (Lp(a)) in hyperlipideamic adults.

Methods:

Thirty free-living hiperlipidaemic volunteers from the Lipid Clinic, Potchefstroom University for Christian Higher Education (CHE), participated in this randomised placebo controlled double blind crossover study. The subjects were randomly divided into two groups (A or B). After a run-in period of 4 weeks during which the subjects excluded all vitamin supplements, Group A received 2 tablets/day of FoodState Vitamin C complex® (500mg vitamin C, 600mg magnesium food complex, 900mg vitamin B complex and 160mg bioflavonoids) and Group B received 2 tablets/day of placebo, for at least 8 weeks. A washout period of 8 weeks followed after which the treatments were crossed-over for a further 8 weeks. Fasting blood samples were drawn 8 times (two samples, one week apart at the beginning and end of each treatment).

Results:

FoodState Vitamin C complex® supplementation did not significantly influence the levels of plasma fibrinogen, plasminogen activator inhibitor 1 activity ($PAI\text{-}1_{act}$), tissue plasminogen activator antigen (tPA_{ag}) or d-dimer. Serum lipids and Lp(a) were also not affected. Median

plasmin-antiplasmin complex (PAP) and thrombin-antithrombin complex (TAT) levels, which are markers of plasmin (initiate fibrinolysis) and thrombin (initiate coagulation) generation respectively, were both significantly decreased compared to placebo (PAP: -4.05[–23.39, -0.23]% *vs* 1.81[-8.95, 8.09]%; TAT: -5.81[-18,47, 0.39]% *vs* 0.12[-8.03, 13.5]%). FoodState Vitamin C complex® beneficially affected FNS by significantly increasing compaction (49.95[47.55, 53.70]% to 51.85[48.55, 56.65]%).

Conclusion:

The decreases in TAT and PAP are possibly an indication that the FoodState Vitamin C complex® decreased the initiation of haemostasis, which in turn led to a compensatory reduction in fibrinolysis. FoodState Vitamin C complex® may, therefore be protective of cardiovascular disease by causing a new reduced steady state of haemostatic balance and more lysable clots (increased compaction).

Keywords:

Haemostasis; Haemostatic factors; Fibrin network structure; Cardiovascular disease; Atherosclerosis; Inflammation; Vitamin C; Antioxidants

LIST OF ABREVIATIONS

A

a	Activated
ADP	Adenosine diphosphate
APC	Activated protein C
aPTT	Activated partial thromboplastine time
AT	Antithrombin
ATP	Adenosine triphosphate

B

BMI	Body mass index
BP	Blood pressure

C

CAD	Coronary artery disease
CAM	Cellular adhesion molecules
CHD	Coronary heart disease
CI	Confidence intervals
CRP	C reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
CHE	Christian Higher Education

D

DIC	Disseminated intra-vascular coagulation
DNA	Deoxyribonucleic acid
DRI	Dietary reference intakes

E

e.g.	exempli gratia (for example)
EAR	Estimated average requirement
EC	Endothelial cells

EDTA	Ethylenediaminetetra-actetic acid
ELISA	Enzyme-linked-immunosorbent assay
EPCR	Endothelial cell protein C receptor

F

F	Factor
FC	Fibrin content
FDP	Fibrinogen degradation product
FIX	Factor IX
FIXa	Activated factor IX
FNS	Fibrin network structure
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII

H

HDL-C	High density lipoprotein cholesterol

I

ICAM-1	Inter cellular adhesion molecule 1
IHD	Ischaemic heart disease
IL-1	Interleukin 1
IL-6	Interleukin 6

IL-8	Interleukin 8

K

kJ	Kilojoule (Energy intake)
K_s	Permeability coefficient

L

LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
Lp(a)	Lipoprotein (a)
LPS	Lipopolysaccharides

M

MI	Myocardial infarction
MLR	Mass length ratio
MPC-1	Monocyte chemoattractant protein 1

P

PAF	Platelet activating factor
$PAI\text{-}1_{act}$	Plasminogen activator inhibitor 1 activity
$PAI\text{-}1_{ag}$	Plasminogen activator inhibitor 1 antigen
PAI-2	Plasminogen activator inhibitor 2
PAP	Plasmin-antiplasmin complex
PAR-1	Protease activated receptor 1
PC	Protein C
PCI	Protein C inhibitor
PGI_2	Prostacycline
PL	Phospholipid
PS	Protein S
PT	Prothrombin time

R

ROS	Reactive oxygen species

S

s	Soluble
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophosresis
SEM	Scanning electron microscopy

T

TAFI	Thrombin-activatable fibrinolysis inhibitor
TAFIa	Activated thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TC	Total cholesterol
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
$TFPI_a$	Activated tissue factor pathway inhibitor activity
TG	Triglyceride
TM	Thrombomodulin
TNF-α	Tumor necrosis factor α
tPA_{act}	Tissue type plasminogen activator activity
tPA_{ag}	Tissue type plasminogen activator antigen
TRAPs	Thrombin activating peptides
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2

U

UL	Tolerable upper intake levels
uPA	Urokinase type plasminogen activator

V

VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipoprotein
vWF	Von Willebrand factor

SYMBOLS

α	Alpha
β	Beta
$\downarrow$	Decrease
γ	Gamma
$\uparrow$	Increase
λ	Lambda (wavelength)
$\leftrightarrow$	No effect
μ_T	Mass-length ratio determined from turbidimetry

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PREFACE

AIM AND OBECTIVES

Main aim

The main aim of this dissertation is to investigate the effects of FoodState Vitamin C complex® supplementation on haemostatic factors and fibrin network structures (FNS) in hyperlipidaemic adults in a randomised, placebo controlled, double blind, crossover study.

Objectives

To investigate the effects of FoodState Vitamin C complex® supplementation on:

- **Haemostatic factors:** Plasma fibrinogen, d-dimer, plasminogen activator inhibitor 1 activity ($PAI\text{-}1_{act}$), tissue plasminogen activator antigen (tPA_{ag}), thrombin-antithrombin complex (TAT), plasmin-antiplasmin complex (PAP).
- **Fibrin network structure:** Mass length ratio from turbidity, compaction of fibrin networks, and network fibrin content.

In this study the effects of FoodState Vitamin C complex® supplementation on serum lipids and lipoprotein (a) (Lp(a)) were also examined and are reported in the dissertation of my fellow M.Sc student Ms. C. Spies (Spies, 2001).

HYPOTHESIS

Background

Cardiovascular disease (CVD) is one of the leading causes of mortality and morbidity in South Africa (Bradshaw *et al.*, 1995) and worldwide (Murray & Lopez, 1996). Dyslipidaemia and an increased coagulation or decreased fibrinolytic state contribute to the development of CVD (Vorster *et al.*, 1997a). In addition, hypercholesterolaemia is associated with atherosclerosis, a process that is looked upon as chronic inflammation in the vessel wall, accompanied by endothelium dysfunction (Berliner *et al.*, 1995; Ridker, 1997a; Ross, 1999; Seljeflot *et al.*, 1998).

There is a known interrelationship between atherosclerosis, coagulation, fibrinolysis and inflammation (Berliner *et al.*, 1995; Cicala & Cirino, 1998; Mezzano *et al.*, 2001; Ridker, 1997a; Ross, 1999). An understanding of the interaction between these systems and the role of dietary intervention can prove to be useful in the management and prevention of resultant subsequent clinical events, such as CVD. The quality of FNS (the result of fibrinogen that forms fibrin monomers which in turn are polymerised to form fibrin threads: the end product of coagulation) may also contribute to the risk for CVD and thrombosis (Blombäck *et al.*, 1992). Changes in fibrinogen concentration or any other constituents of the plasma may directly affect the FNS (Blombäck *et al.*, 1992; Nair *et al.*, 1991).

Dietary vitamin C has been implicated in protection against CVD (Gaziano, 1999). Due to it's antioxidant effects, an increased intake of vitamin C may lead to a decreased susceptibility to infection and free radical damage to the endothelium which may result in decreased activation of the coagulation system (Bordia & Verma, 1985; Gaziano, 1999; Horning *et al.*, 1997; Khaw & Woodhouse, 1995; Woodhouse *et al.*, 1997; Woodward *et al.*, 1997). It may further be speculated that the expected changes in FNS, haemostatic variables or any of the other plasma constituents with intake of vitamin C may result in a reduced steady state with a reduction in atherosclerosis and subsequent CVD. It may furthermore be speculated that a reduction in the coagulation system, with the addition of vitamin C, resulted in changes in the FNS.

STRUCTURE OF THIS DISSERTATION

This dissertation is in article format. The empirical work consists of one clinical study. This randomised, placebo controlled, double blind, crossover study, investigated the effects of FoodState Vitamin C complex® supplementation on haemostatic factors, FNS, serum lipids (Spies, 2001) and Lp(a) (Spies, 2001) in hyperlipidaemic adults. The focus of this dissertation falls upon haemostatic factors and FNS.

Following this Preface, Chapter 1 provides background information necessary for the interpretation of the data in the article. An overview of the haemostatic system and FNS is given. The interaction between coagulation, inflammation and atherosclerosis is discussed. Furthermore, the effects of vitamin C on CVD, haemostatic variables and vascular function are discussed. Lastly, recommendations for vitamin C intake are given.

Chapter 2 consists of the submitted manuscript, containing the results of both this author and Spies (2001), on the effects of FoodState Vitamin C complex® supplementation on haemostatic factors, FNS, serum lipids and Lp(a) in hyperlipideamic adults (The manuscript was submitted for publication in Atherosclerosis).

The relevant references of Chapter 2 are provided at the end of the chapter according to the authors' instruction of Atherosclerosis. The references used in the unpublished Chapters (Preface and Chapter 1) are provided according to the mandatory style stipulated by the Potchefstroom University for Christian Higher Education (CHE) at the end the dissertation.

AUTHORS' CONTRIBUTION

The study reported in this dissertation was planned and executed by a team of researchers. The contribution of each of the researchers is depicted in the table hereafter. Also included in this section is a statement from the co-authors confirming their individual roles in the study and giving their permission that the article may form part of this dissertation.

NAME	ROLE IN THE STUDY
Ms. D. Loots B.Sc. Hons. (Nutritionist)	Together with W. Oosthuizen and C. Spies were responsible for the execution of the total study, dietary intakes, laboratory analyses, data management and statistical analyses. Main author of the paper.
Prof. W. Oosthuizen Ph.D. (Nutritionist)	Project co-ordinator and scientist; responsible for all aspects of the study. Significant contribution towards writing of the paper. Study leader of D. Loots and C. Spies
Dr. M. Pieters Ph.D. (Dietitian, Nutritionist)	Assistant supervisor of D. Loots with regard to FNS. Contribution toward writing of paper.
Prof. J.C. Jerling Ph.D. (Nutritionist)	Together with W. Oosthuizen and D. Loots responsible for laboratory analyses of haemostatic factors and statistical analyses. Significant contribution toward writing of paper.
Ms. C. Spies B.Sc-Hons. (Nutritionist)	Together with W. Oosthuizen and D. Loots responsible for the execution of the total study, dietary intakes, laboratory analyses (except for FNS), data management and statistical analyses. Fellow M.Sc student.
Prof. H.H. Vorster Ph.D.(Physiologist, Nutritionist)	Design, planning and approval of final protocol.

I declare that I have approved the above mentioned article and that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that it may be published as part of the M.Sc. dissertation of Deirdré Loots.

Prof. W. Oosthuizen

Dr. M. Pieters

Prof. J.C. Jerling

Ms. C. Spies

Prof. H.H. Vorster

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CHAPTER 1 LITERATURE: REVIEW

A. INTRODUCTION

In this chapter a concise review of the literature that will assist in the understanding and interpretation of the article presented in this dissertation will be given. Firstly, the background for the formulation of the hypothesis is summarised, followed by an overview of the haemostatic system in order to understand the roles of the different variables measured in this study. The interaction between coagulation and inflammation and atherosclerosis as an inflammatory disease will be discussed, followed by an overview of the fibrin network structure (FNS). This will be followed by a discussion on the protective effect of antioxidants, in particular that of vitamin C, on cardiovascular disease, platelet aggregation and adhesion, endothelium and other haemostatic variables. Lastly, recommendations for vitamin C intake are given.

B. BACKGROUND: FORMULATION OF HYPOTHESIS

Cardiovascular disease (CVD) is one of the leading causes of mortality and morbidity in South Africa (Bradshaw *et al.* 1995) and worldwide (Murray & Lopez, 1996). An increased coagulation state (e.g. increased coagulation, decreased fibrinolytic activity) contributes to the development of CVD (Vorster *et al.*, 1997a). The quality of FNS may also contribute to the risk for CVD and thrombosis (Blombäck *et al.*, 1992). Changes in fibrinogen concentration and other constituents of plasma directly affect FNS (Blombäck *et al.*, 1992; Nair *et al.*, 1991). Management of these risk factors is very important and dietary intervention may form an essential part of this management program. The effect of vitamin C on FNS, has however, not been investigated before, and furthermore, very little is known about the effects of diet, foods or nutrients on FNS. Due to the potential anti-thrombotic effects that might be exerted by vitamin C, it could be hypothesised that FNS might also be positively affected. Therefore, the effects of dietary intervention, in particular that of vitamin C, on haemostatic factors as well as FNS could well prove to be an important risk management factor through the antioxidant properties exerted by this specific dietary intervention with vitamin C.

According to Blombäck & Okada (1983), the FNS forms the "hinge" of the haemostatic balance. The balance of the haemostatic system, between clot formation and clot degradation, depends upon this very integral and lattice network of fibrin fibres. Moreover, the initiation of

coagulation results in fibrinogen forming fibrin monomers. These monomers polymerise to form fibrin threads leading to the formation of the FNS. The FNS consists of a lattice-work of fibrin threads. This lattice-work forms the basis in which red blood cells and platelets are trapped, resulting in the formation of blood clots. These blood clots are known as thrombi when they become dislodged. FNS with either long and thin threads, or short and wide threads are formed (Blombäck & Okada, 1983). A proneness to formation of tight, rigid and space-filling FNS with small pores (thin fibres) appears to be associated with premature CVD, including coronary heart disease (CHD) and stroke (Fatah *et al.*, 1992). These tight and rigid FNS are not lysed easily, raising possibilities of re-infarction. According to Blombäck *et al.* (1990), this may be associated with an increased fibrinogen concentration and increased clotting potential. Furthermore, Shats *et al.* (1997) demonstrated the possible role of FNS in atherogenesis (the type of fibrin deposited may influence different outcomes). Thick fibres may influence initial disorganisation of endothelium cells (EC), promotion of a more pro-fibrinolytic/anti-thrombotic environment, fibrinolysis and finally the EC are re-organised. However, thin fiber promotes a more anti-fibrinolytic/pro-thrombotic environment and may induce a further exposure of sub-endothelium, resulting in increased platelet adhesion, migration of monocytes and growth of atherosclerotic plaque.

It could, therefore, be hypothesised that the formation of more porous, less rigid and more lysable clots (thus thicker fibrin fibres) may have a positive effect on the risk for CVD of individuals as these FNS create a pro-fibrinolytic/anti-thrombotic environment.

Normal haemostasis is a balance between the formation and breakdown of blood clots in the circulation. Due to the fact that both these systems are subjected to the same intricate control mechanisms, they cannot be studied separately. On the one side of the haemostatic balance is blood clotting which consists of three individual but complementary systems namely platelets, vascular endothelium and the plasma protein clotting system (Vorster *et al.*, 1997a). This plasma protein clotting system has always been believed to be controlled by a cascade of protein coagulation factors. But according to Hoffman and Monroe (2001), coagulation does not merely occur as a cascade but can actually be divided into three overlapping stages (1) initiation, (2) amplification and (3) propagation, in which the cell surfaces themselves have important regulatory properties. Blood clotting is an autocatalytic, self-limiting process in which the formation of thrombin plays a central role. On the other side of the haemostatic balance is fibrinolysis, which is primarily responsible for the breakdown of fibrin clots. The end-product of

the clotting system is the stable FNS, which is also the target of the fibrinolytic enzymes (Vorster *et al.*, 1997a; Vorster & Venter, 1994).

The main cause of CVD is atherosclerosis that causes the development of a fatty plaque within the intima and media of medium and large arteries resulting in thickening of the arterial wall and obstruction of blood flow to the heart and other tissues (Berliner *et al.*, 1995; Haverkate, 2002; Ross, 1999). Atherosclerosis is a process that is looked upon as a chronic inflammation in the vessel wall, accompanied by endothelial dysfunction (Berliner *et al.*, 1995; Ross, 1999; Seljeflot *et al.*, 1998). Hypercholesterolaemia and the haemostatic system play an important role in the development of atherosclerosis. In addition, it has become clear that the relationship between coagulation and inflammation is not unidirectional, but instead, an interrelationship, or an interaction as such between the systems occurs by which activation of coagulation will also affect inflammatory activity. Abnormality of the coagulation system is a frequent occurrence in patients with sepsis, an inflammatory state. The abnormalities consist of an increased pro-coagulant activity, a decreased anti-coagulation activity and impaired fibrinolysis. Parameters that have been shown to be elevated during the derangement of the coagulation system include: antigen levels of both tissue plasminogen activator (tPA_{ag}) and its primary inhibitor, plasminogen activator inhibitor 1 ($PAI\text{-}1_{ag}$) as well as PAI-1 activity ($PAI\text{-}1_{act}$), fibrin degradation products (such as d-dimer) and clot lysis time (Lee *et al.*, 1995; Pearson *et al.*, 1997; Ridker, 1997a; Vorster *et al.*, 1997a). In particular, vascular EC seem to play a pivotal mediatory role in the coagulation response to systemic inflammation and the interaction between them (Berliner *et al.*, 1995; Cicala & Cirino, 1998; Levi *et al.*, 2002). EC respond to haemodynamic forces with the expression of different phenotypes with disparate functional properties. At arterial bends and flow dividers, cells are relatively deprived of fluid-shear-stress-induced cell differentiation and exhibit phenotypes with increased mitotic rate, decreased inter-cellular contact, increased permeability for macromolecules and the expression of molecules favouring constriction, adhesion and thrombosis. Arterial sites covered by such cells are vulnerable to atherogenic effects of hypercholesterolaemia. A certifiable characteristic of EC exposed to hypercholesterolaemia is a reduced capacity to release endothelium-derived relaxing factors. Hyperlipidaemic states exhibit systemic signs of an inflammatory response, including leukocytosis, lymphocytosis, activation of the complement and kinin system and elevation in acute phase reactants, such as fibrinogen (Henry *et al.*, 1995).

Ascorbate (or vitamin C) is a primary antioxidant against photo-oxidation in plasma and it may prevent oxidative damage to coagulation factors, other proteins and EC effectively (Parkkinen *et al.*, 1996). An improvement in endothelium dependent vaso-activity has been demonstrated after antioxidant supplementation in combination with cholesterol lowering therapy among patients with atherosclerosis. Furthermore, dietary antioxidants may protect against oxidation mediated inflammation and tissue damage by scavenging free radicals and by inhibiting the activation of oxidant sensitive transcription factors, such as the nuclear factor-$_{k}$B, resulting in an attenuated inflammatory response (Berliner *et al.*, 1995). Indeed in studies in patients with inflammatory diseases, antioxidant nutrients reduced the inflammatory symptoms. The amount and the relation of pro- and anti-oxidative nutrients had an impact on inflammation in rheumatic disease (Adam, 1995; Berliner *et al.*, 1995). Supplementation with vitamin C also showed a reduction in platelet aggregation and platelet adhesiveness (Bordia & Verma, 1985; Bordia, 1980).

Although research is inconsistent, epidemiological studies have shown an inverse relationship between vitamin C intake or serum vitamin C levels and CVD (summarised by Simon & Hudes, 1998). This relationship may, in part, be mediated through effects on serum lipid levels (Jacques, 1992; Ness *et al.* 1996; Simon & Hudes, 1998) and coagulation factors (Woodhouse *et al.* 1997; Woodward *et al.* 1997). Moreover, in the Third Glasgow MONICA Survey, increases in factors VII, VIII and IX and decreases in protein C (PC) were accompanied by increases in thrombin-antithrombin complex (TAT) and pro-thrombin fragment 1 + 2. In this study, serum vitamin C levels showed a significant negative correlation with TAT (Woodward *et al.*, 1997). Very few randomised placebo controlled trials have been conducted to confirm a causal relationship between vitamin C intake and the haemostatic system. The effect of vitamin C intake on FNS is also unknown.

In inflammatory diseases, antioxidants have also shown an improvement in the inflammatory symptoms. Seeing that atherosclerosis is considered a chronic inflammatory disease where interaction between inflammation and coagulation exists, it is hypothesised that by decreasing the inflammatory response, one could also decrease coagulation and subsequently may also reduce cardiovascular risk.

The study conducted for this dissertation in part, aspired to prove the hypothesis that the antioxidant properties exerted by vitamin C may improve haemostasis and FNS in

hyperlipidaemic patients. This research may give clues as to possible biological mechanisms involving antioxidants, haemostasis and FNS.

Due to the expected anti-thrombotic effect exerted by vitamin C, it could be hypothesised that FNS could also be positively affected (due to changes in the plasma environment as well as changes in the kinetic factors responsible for fibrin formation). This hypothesis may possibly, in part, be supported by the study of Nappo *et al.* (1999) who showed that after a methionine load, vitamin C and vitamin E reduced *inter alia*: fibrinopeptide A (FPA) (that reflects thrombin activity) and d-dimer (a marker of atheroslerosis), which may be associated with a decrease in the cross-linking of fibrin fibres. It is important to note that the above mentioned study was based on surrogate end points (cardiovascular risk factors e.g. endothelial dysfunction, atherosclerosis) and not *per se* on the structure and/formation of fibrin networks, but it did, however, measure the mentioned variables involved in FNS formation.

As depicted in the above mentioned discussion, it becomes clear that an understanding of the interaction between atherosclerosis, coagulation, fibrinolysis and inflammation is imperative to the development of risk management programs. As a good understanding of these interactions can prove to be useful in the management and prevention of resultant subsequent clinical events, such as CVD, in which vitamin C intake proves to play an integral role. Since vitamin C, atherosclerosis, inflammation and haemostasis as well as FNS have all been implicated in CVD, the very crux of the hypothesis of this dissertation may prove to be of considerable health importance in patients at risk for CVD, of which, an increased intake of vitamin C (through supplementation) will lead to decreased levels of haemostatic factors (that give rise to an anti-thrombotic state) and increases in the pro-fibrinolytic activities of FNS (formation of thick fibrin fibres and more lysable clots), forms the basis. Variables measured in this study included for the haemostatic system are: plasma fibrinogen, d-dimer, PAI-1_{act}, tPA_{ag}, TAT and PAP. The mass-length-ratio from turbidity, compaction of fibrin networks and network fibrin content were used to measure the effect of vitamin C on FNS.

C. THE HAEMOSTATIC SYSTEM

The haemostatic system is described in several review articles. This section is a summary of these review articles.

The term 'haemostasis' describes the combined processes of coagulation, platelet aggregation, fibrinolysis and secretion of substances by the vascular endothelium aimed at preventing bleeding from injured smaller blood vessels. Abnormal haemostasis, characterised by an imbalance in pro-coagulant and anti-coagulant activities, is now accepted as a major risk factor for atherosclerosis, thrombosis and resultant CVD (Vorster *et al.*, 1997a). The haemostatic system contributes to atherogenesis and controls intra-vascular thrombus formation and thus plays an important role in the development of CHD, notably acute ischaemic events (myocardial infarction {MI}, unstable agina pectoris, and sudden death) and ischaemic stroke. These diseases are dominant causes of death in industrial countries. Accordingly, it is of utmost interest to gain insight into the haemostatic system and its possible modification by internal and external factors. Such knowledge could pave the way for rational prevention of cardio and cerebrovascular disease (Marckmann *et al.*, 1998).

The haemostatic system is essential for the preservation of life. The haemostatic system protects man from exsanguinations after wound injuries and also forms the basis for the numerous tissue repair processes that are believed to be constantly ongoing within the vascular bed as initially proposed by Tage Astrup more than four decades ago (summarised by Marckmann *et al.*,1998).

The review articles further revealed that the haemostatic system or network consists of four closely related systems:

- the vessel endothelium
- blood platelets/platelet aggregation
- coagulation factors and inhibitors
- fibrinolytic promoters and inhibitors.

Failure of any one of the above can result in either haemorrhagic or thrombotic tendency. Moreover, these systems normally function together in complicated, orderly, co-coordinated and tightly controlled processes to prevent thrombus formation and thus to ensure the fluidity of blood, to arrest bleeding and to assist in wound healing. The interaction of the molecules from the

different components of the system also ensures that when the system is triggered by injury, the coagulant activities and subsequent fibrinolysis are localized within the area of injury. The balance between bleeding (pro-coagulant activity) and thrombus formation (anti-coagulant activity) in the system is accomplished through positive and negative feedback mechanisms:

- ❖ through the secretion of multifunctional molecules such as thrombin which influence all parts of the network (such as fibrinolytic, platelet and endothelial cell components)
- ● through a system of activators and inhibitors of key enzymes/factors (Cicala & Cirino, 1998; Levi *et al.*, 2002; Marckmann *et al.*, 1998; Oosthuizen, 1999; Vorster *et al.*, 1997a; Vorster *et al.*, 1997b).

Figure 2 gives an illustration of the haemostatic system and the balance between clot formation and dissolution. Figure 3 shows the "cascade" model of coagulation illustrating the "intrinsic" and "extrinsic" pathways as well as a simplified fibrinolytic diagram. Figure 4 illustrates the cell-based model of haemostasis (coagulation) proposed by Hoffman & Monroe (2001).

It is of interest to look briefly at the four components involved in haemostasis. The endothelium participates in haemostasis *inter alia* by expressing and secreting a large number of substances that influence the formation and dissolution of blood clots. The secretion of several of these substances changes in the damaged endothelium and may serve as markers of the damage. Damage to the endothelium of the vessel results in: activation of platelets and coagulation, as well as the release of serotonin and thromboxane A_2 (TXA_2) from activated platelets (this contributes to vasoconstriction). The anti-thrombotic properties of the endothelium are maintained by the synthesis and secretion of anti-coagulant and pro-fibrinolytic substances such as prostacyclin, PC and protein S (PS), thrombomodulin (TM), tissue factor pathway inhibitor (TFPI) and tissue type plasminogen activators (tPA).

Blood platelets react to vascular injury, become activated, spread, adhere, aggregate and secrete substances, interacting with other parts of the haemostatic network in order to form a platelet plug to arrest bleeding. Injury to a vessel disrupts the endothelium and exposes the underlying connective-tissue collagen molecules. Platelets adhere to collagen *via* an intermediatory called von Willebrand factor (vWF), a plasma protein secreted by EC and platelets. This protein forms the bridge between the damaged vessel and the platelets in that vWF binds to collagen and then platelets bind to vWF. These platelet characteristics and functions, therefore, reflect vascular injury. Central to normal platelet function is platelet prostaglandin synthesis, which is induced by

platelet activation and leads to the formation of TXA_2 in platelets. TXA_2 is a powerful vasoconstrictor and activates platelets to aggregate. There is also good evidence that in addition to participating in thrombus formation, activated platelets and platelet products also participate in atherosclerosis development (Ridker, 1997a; Ridker, 1997b; Ross, 1999).

Blood coagulation is an autocatalytic, self-limiting process which is triggered by tissue injury (Vorster *et al.*, 1997a). Many coagulation factors are zymogens of serine proteinases, becoming activated during the overall process. The coagulation factors or enzymes are responsible for the formation of the blood clot of fibrin network. A clot is formed when thrombin, generated in the cascade, removes FPA + fibrinopeptide B (FPB) from fibrinogen. The concentrations, activation, inhibition and coagulant activities of factors in the coagulation cascade ensures that clot formation is limited to sites where needed. Hypercoagulability that contributes to a pro-thrombotic state may develop when the balance between activation and inhibition in the clotting cascade is disturbed. There is convincing evidence that the concentration and/or activity of several coagulation factors and inhibitors are related to atherothrombosis and CVD.

The fibrinolytic system is responsible for the continuous dissolution of fibrin clots. The system also plays a role in cell migration, extra-cellular matrix degradation, tissue repair and pathological processes such as atherothrombosis, tumor invasion and metastasis (Vorster *et al.*, 1994). The system is tightly regulated under normal circumstances by activators and inhibitors of plasmin, the key enzyme that degrades fibrin. The levels and activities of these activators and inhibitors, as well as products formed when fibrin is digested, may serve as markers of the function of the system and its relationship with atherothrombosis.

A summary of the proteins involved in haemostasis is given in Figure 1 for quick reference. For simplicity, each protein is placed under one of three categories: (1) factors that promote thrombin generation, (2) factors that participate in clot formation, and (3) factors inhibiting thrombin formation.

Another important point of interest to be taken into consideration when one talks about the haemostatic system, is that of inflammation. This is necessary because inflammation can lead to activation of the coagulation system and this relationship is not unidirectional, but instead a significant inter reaction between the systems occurs by which activation of coagulation will also affect inflammation activity. In particular, vascular EC seem to play a pivotal mediator role in the

coagulation response to systemic inflammation and the interrelationship between them. Furthermore, coagulation represents a double edged sword necessary for haemostasis and the acute containment of an infectious focus; it also amplifies the inflammatory response, decreases bacterial clearance and in the critically ill patient, contributes to end-organ damage and death.

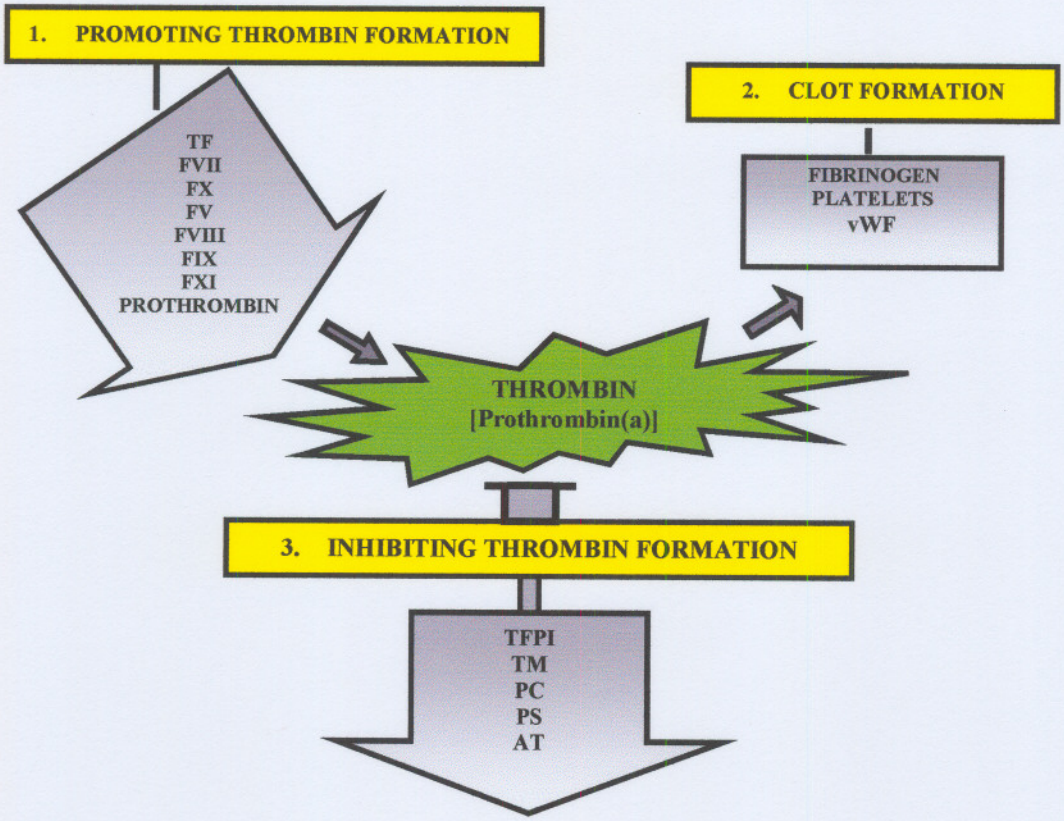

Figure 1 **Summary of the proteins involved in haemostasis**
TF: tissue factor; **F:** factor; **vWF:** von Willebrand factor; **TFPI:** tissue factor pathway inhibitor; **TM:** thrombomodulin; **PC:** protein C; **PS:** protein S; **AT:** antithrombin

Atherosclerosis is looked upon as a chronic inflammatory state and lies at the center of the development of CHD and ischaemic strokes (Berliner *et al.*, 1995; Cicala & Cirino, 1998; De Maat *et al.*, 2000; Esmon, 2000; Haverkate, 2002; Levi *et al.*, 2002; Ridker, 1997a; Ridker, 1997b; Ross, 1999; Tracy, 1999). It is, therefore, foreseeable that the existence of an interaction between inflammation, haemostasis (coagulation, fibrinolysis) and atherosclerosis are undeniable. In light of the above, the interaction between inflammation and coagulation, some of the

connecting points between them, as well as the process of atherosclerosis and the influences of haemostasis and inflammation on this chronic inflammatory state, will be discussed in more detail later on in this dissertation.

As mentioned before, there is normally a balance between fibrin network formation (coagulation) and dissolution (fibrinolysis), known as the "haemostatic balance". In many circumstances an increase in coagulability is accompanied by a compensatory increase in fibrinolytic activity (Takada *et al.*, 1994; Vorster *et al.*, 1997a). The haemostatic balance can be measured by comparing thrombin and plasmin generation (of which TAT and PAP are markers of thrombin and plasmin generation, respectively). Recently, a new inhibitor of fibrinolysis was described, which downregulates fibrinolysis after it was activated by thrombin and was, therefore, named thrombin-activatable fibrinolysis inhibitor (TAFI) (Figure 2). TAFI provides an important link between the coagulation and fibrinolytic cascade and hence more insight into the haemostatic system (Bajzar, 2000; Bouma *et al.*, 2001). A disturbance in this balance, often because of an inability of the fibrinolytic system to adjust to hypercoagulability, is known to be related to atherothrombosis and may also serve as a marker of disease and risk of adverse events.

C.1 Coagulation

As mentioned before, blood coagulation or clotting is the transformation of blood into a solid gel termed a clot or thrombus and consisting mainly of a protein polymer known as fibrin. Clotting occurs locally around the original platelet plug and is the dominant haemostatic defense. Its function is to support and reinforce the platelet plug and to solidify blood that remains in the wound channel (Haemostatic system is given in Figure 2).

There seems to be a paradigm shift regarding the coagulation process – from a concept that views the coagulation process as a "cascade" of reactions ("cascade" model) to one that considers the process to be controlled by cellular components (cell-based model). The prevailing view of haemostasis with regards to the "cascade" model remains that protein coagulation factors direct and control the process with cells serving primarily to provide a phosphatidylserine containing surface on which the pro-coagulant complexes are assembled. In contrast, Hoffman & Monroe (2001) propose a model in which coagulation is regulated by proteins of cell surfaces. This model emphasises the importance of specific cellular receptors for the coagulation proteins.

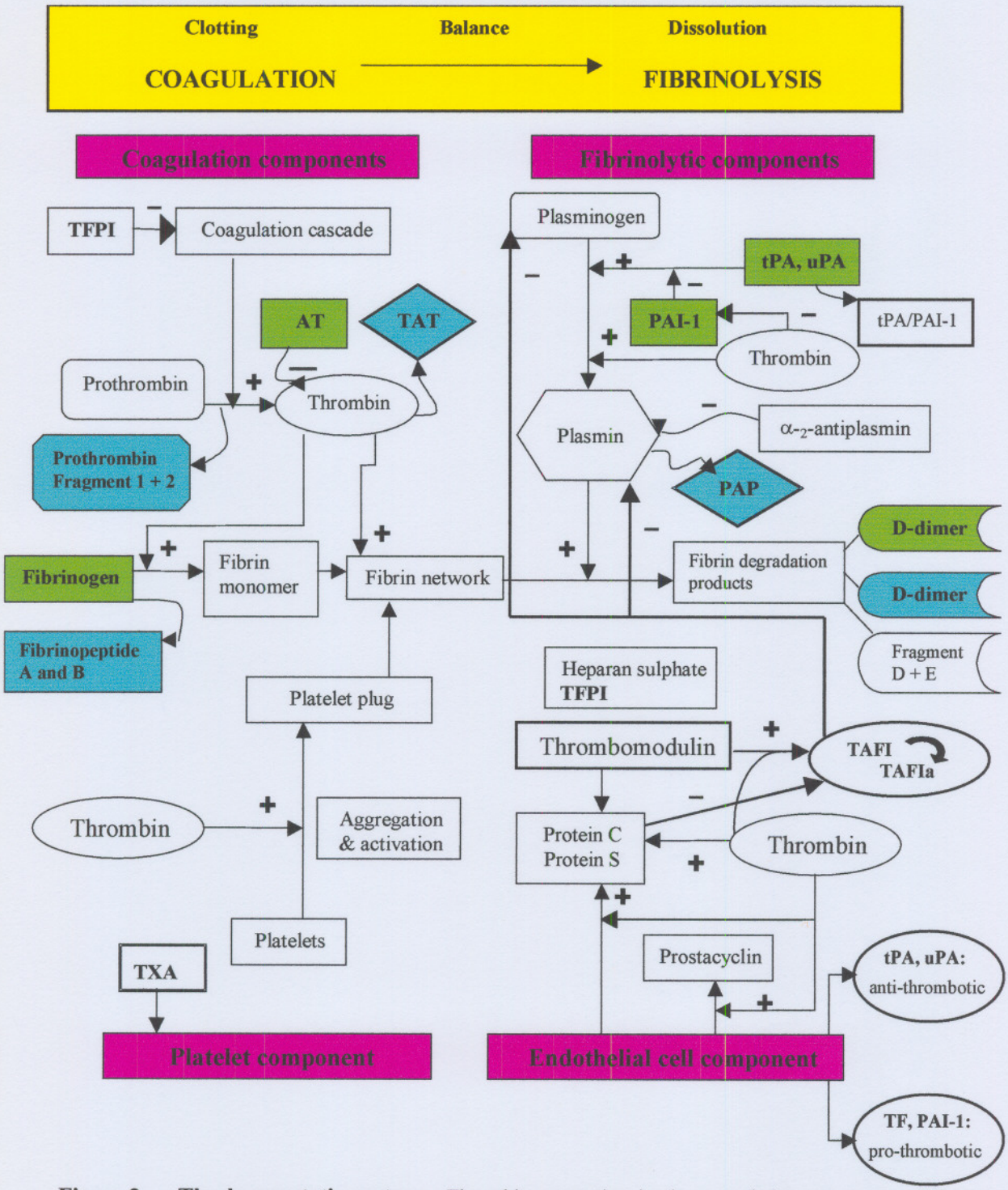

Figure 2 **The haemostatic system.** Thrombin generation in the coagulation components influences events in other components. **AT:** Antithrombin; **PAI-1:** Plasminogen activator inhibitor 1; **PAP:** Plasmin-antiplamin complex (marker of plasmin generation);**TAT:**Thrombin-antithrombin complex (marker of thrombin generation); **tPA:** Tissue type plasminogen activator; **uPA:** Urikinase type plasminogen activator; **TFPI:** Tissue factor pathway inhibitor; **TXA:** Thromboxane; **TF:** Tissue factor; **+:** Activation; **-:** Inhibition; **TAFI**: Thrombin-activatable fibrinolysis inhibitor; **a:** activated; ■: Marker of the activity of the haemostatic system; ■: Coronary heart disease risk factor (adapted from Bajzar, 2000; Jerling 1998; Takada *et al.*, 1994; Vorster *et al.*, 1997a).

Thus, cells with similar phosphatidylserine content can play very different roles in haemostasis depending on their complement of surface receptors. Hoffman & Monroe (2001) propose that coagulation occurs not as a "cascade" as described by many review papers (Vorster *et al.*, 1997a; Vorster & Venter, 1994; Takada *et al.*, 1994; Marckmann *et al.*, 1998; Levi *et al.*, 2002; Luther & Mackman, 2001; Oosthuizen, 1999), but as three overlapping stages: (1) initiation, which occurs on a tissue factor (TF) bearing cell (2) amplification, in which platelets and cofactors are activated to set the stage for large scale thrombin generation and (3) propagation, in which large amount of thrombin are generated on the platelet surface (Figure 4). This cell-based model explains some aspects of haemostasis that a protein-centric model does not. Therefore, both models (a way of conceptualising and understanding a complicated system) will be discussed.

"Cascade"/"waterfall"-model

The activation, inhibition and coagulant activities of factors in the coagulation system ensuring that clot formation is limited to sites where needed, are illustrated in Figure 2. Hereafter follows an overview of the coagulation "cascade" leading to the formation of thrombin.

Coagulation is mainly initiated when TF is exposed to blood. This can occur as the result of either monocyte activation or exposure of the blood to extra-vascular cells. Factor from here on will be referred to as only F. The complex of TF and activated factor VII (FVIIa) (TF:FVIIa) can then activate either FX or FIX. These in turn form complexes with activated factor V (FVa) and activated FVIII (FVIIIa) respectively, probably on the surface of activated platelets (Luther & Mackman, 2001). The activated FX (FXa) and FVa complex (FXa:FVa) leads to explosive thrombin formation. Thrombin cleaves fibrinogen to form fibrin monomers, FPA and FPB. The monomers polymerise to form the fibrin network or clot in which activated factor XIII promotes the formation of cross-links (Blombäck *et al.*, 1978). The single chain glycoprotein antithrombin (AT) plays an important role in regulating and targeting blood coagulation and preventing intra-vascular clotting by inhibiting FIXa, FXa, FXIa and FXIIa as well as FVIIa in the presence of heparan. When the serpin (serine protease inhibitor) ATIII binds with and inhibits thrombin, the TAT is formed. TAT levels should, therefore, reflect thrombin generation and activation of the coagulation cascade (Hoffman & Monroe, 2000; Vorster & Venter, 1994). Unchecked, the thrombin would cause platelet activation and fibrin deposition and initiate an inflammatory cascade. All steps to this point occur better on negatively charged phospholipids. These can be made available by complement activation of the cells and by other agents that mobilise intra-

cellular calcium (Levi *et al.*, 2002; Marckmann *et al.*, 1998; Oosthuizen, 1999; Vorster & Venter, 1994; Vorster *et al.*, 1997a).

Several potent natural anti-coagulant factors exist, including the heparin-AT mechanism responsible for inhibition of FXa and thrombin and the TFPI mechanism, responsible for control of the TF:FVIIa complex. Because the impact of inflammation on these pathways is less characterised than that on the PC pathway, emphasis is placed on the latter pathway.

The PC anti-coagulant pathway is triggered when thrombin binds to TM on the surface of the endothelium. This complex (thrombin:TM), does not appear to need negatively charged phospholipids, especially when the endothelial cell PC receptor (EPCR) is present. Once activated PC (APC) is generated, it can either remain bound to the EPCR or dissociated to PS. The APC- PS complex can then inactivate FVa or FVIIIa. In the case of FVIIIa, the reaction is stimulated by FV.

In addition to playing a role in the regulation of the coagulation cascade *per se*, TM serves other functions as well. TM accelerates thrombin inhibition by AT and PC inhibitor (PCI), therefore, providing a mechanism for clearance of thrombin from the circulation. Therefore, when inflammatory mediators down regulate TM, proteolysis and oxidation, thrombin inhibition is compromised. In addition, TM can accelerate the activation of TAFI. In its active form (TAFIa) this pro-carboxypeptidase B down regulates fibrinolysis after it is activated by TM in complex with thrombin by the removal of carboxyl-terminal lysines from fibrin and thereby limiting plasmin formation. These carboxy-terminal lysines are exposed upon limited proteolysis of fibrin by plasmin act as ligand for the lysine-binding sites of plasminogen and tPA. Elimination of these lysines by TAFIa abrogates the fibrin cofactor function of tPA-mediated plasminogen activation, resulting in a decreased rate of plasmin generation and therefore down regulation of fibrinolysis. The activation of TAFI by thrombin implies that the coagulation system plays a role in the regulation of fibrinolysis and that any disturbance in the generation of thrombin will result in an increased rate of clot lysis. As mentioned before, TM stimulates the activation of both TAFI and PC. Whereas APC inhibits the activation of TAFI by down regulation of thrombin formation, a process in which PS acts as cofactor. Moreover, PS inhibits TAFI in two ways: on the one hand, (1) PS functions as a cofactor for APC which results in a reduction of the maximum induced TAFI activity; and (2) on the other hand, PS inhibits the initial thrombin formation independently of APC which results in a decreased rate of TAFI activation. The effect of the

APC-independent anti-coagulation activity of PS on the activation of TAFI provides a new mechanism for the regulation of fibrinolysis in the early stages of clot formation (Bajzar, 2000; Bouma & Meijers, 2003; Bouma *et al.*, 2001; Mosnier *et al.*, 2001). Furthermore, this loss of the fibrin stabilisation resulting from TM down regulation may compensate in part for the loss of anti-coagulant functions of TM. The APC is then cleared from the circulation by α_2-macroglobulin, 1-antitrypsin and PCI. Of importance to inflammation, 1-antitrypsin behaves as an acute-phase reactant and with respect to the haemostatic balance, functions primarily as an inhibitor of APC. It should therefore shift the balance slightly in favour of clot formation (Cicala & Cirino, 1998; Dahlbäck, 1994; Esmon, 2000; Golino, 2002; Nesheim *et al.*, 1997; Takada *et al.*, 1994; Vorster & Venter, 1994 & Vorster *et al.*, 1997a).

"Imperfections" in the "cascade" model according to Hoffman & Monroe (2001)

The "cascade"/ "waterfall" model was subsequently refined to the scheme shown in Figure 3, as more was learned about the biochemistry of coagulation factors. This model resulted from the work that was aimed at elucidating the identity, function and interactions of the individual pro-coagulant proteins. It accurately represents the overall structure of the coagulation process as a series of proteolytic reactions, each protease cleaves and activates the subsequent protease in the series. It also included the recognition that anionic phospholipid, especially phosphatidylserine was acquired for the assembly and optimal function of most coagulation complexes.

However, the viewpoint that is implicit in this concept of coagulation is that the role of cells, especially platelets, is primarily to provide anionic phospholipids for coagulation complex assembly (Vorster *et al.*, 1997a; Vorster *et al.*, 1994). The coagulation "cascade" models very well the screening coagulation laboratory tests, the prothrombin time (PT) and activated partial thrombin time (aPTT) which corresponds to the extrinsic and intrinsic pathways (Takada *et al.*, 1994; Vorster *et al.*, 1997a).

However, according Hoffman & Monroe (2001), it is clearly inadequate to explain the pathways leading to haemostasis *in vivo* as this model currently exists. They found it to be inconsistent with clinical observations in several key aspects (focusing on hemophilia). Hoffman & Monroe (2001) concluded that there are very likely not separate "intrinsic" and "extrinsic" pathways operating under normal conditions *in vivo* and the overall model of coagulation, therefore, required rethinking. Hence they proposed their "cell-based" model of coagulation. Hoffman &

Monroe (2001), further stated that different cell surfaces have very different properties as related to the coagulation process, even if the cells have similar membrane lipid compositions.

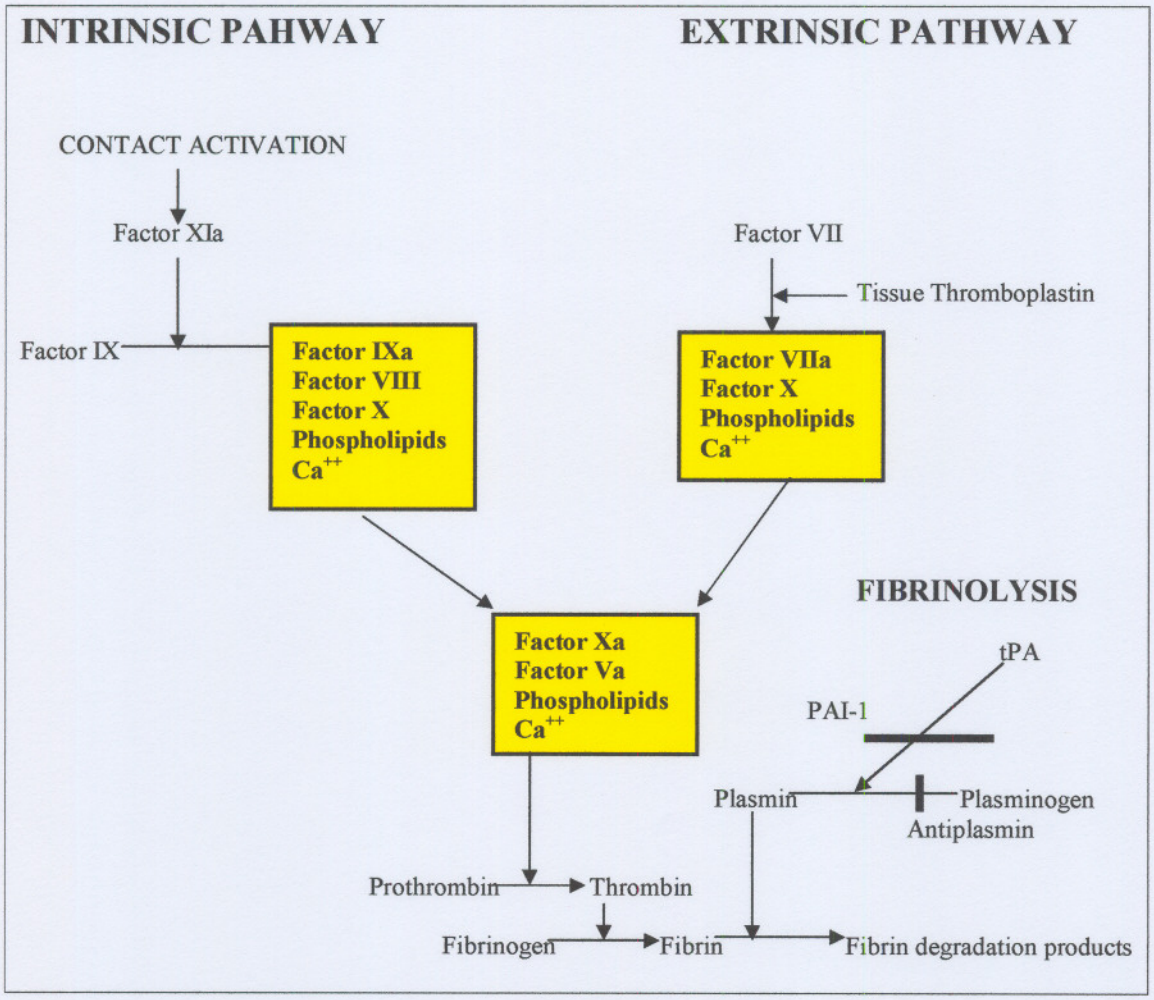

Figure 3 **The "cascade" model of coagulation and a simplified model of fibrinolysis.** The "intrinsic" and "extrinsic" pathways of coagulation are reflected in the cinical laboratory tests, aPTT and PT, respectively (Hoffman & Monroe, 2001). **F:** Factor; **a:** activated; **PAI-1:** Plasminogen activator inhibitor 1; **tPA:** Tissue plasminogen activator (adapted from Marckmann *et al.*, 1998; Vorster *et al.*, 1994).

Coagulation properties result from expression of a variety of cell features including protein receptors that localise components of the coagulation system to specific cell surfaces.

Hoffman & Monroe's (2001) work led them to focus on how the localisation of the coagulation reactions to different cell surfaces serves to control the coagulation process and hence the development of their "cell-based" model that reflects the pathways of haemostasis *in vivo*.

Hoffman & Monroe's cell-based model of haemostasis (coagulation)

As mentioned before, Hoffman & Monroe (2001) view haemostasis as three overlapping phases, illustrated in Figure 4, and summerised hereafter:

(1) The initiation of coagulation takes place on TF-bearing cells, such as fibroblasts. If the pro-coagulant stimulus is sufficiently strong, enough FXa, FIXa and thrombin are formed to successfully initiate the coagulation process

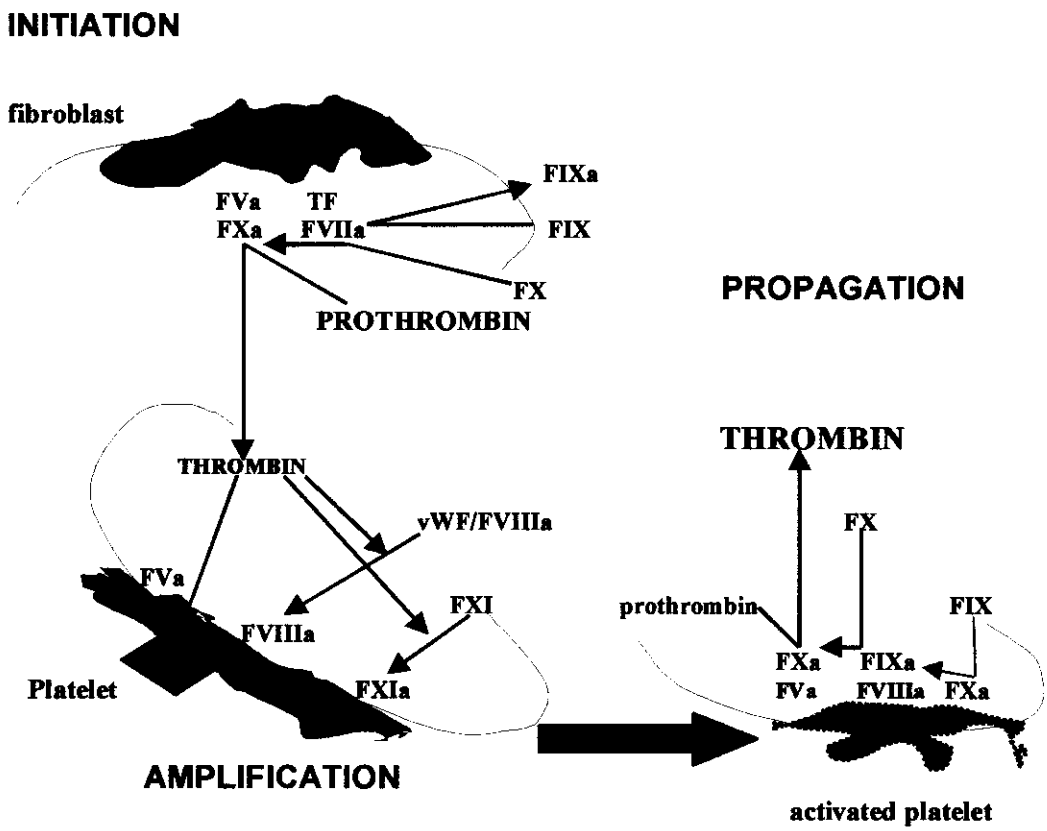

Figure 4 **A cell-based model of coagulation.** The three phases of coagulation occur on different cell surfaces: *Initiation* on the tissue factor (TF) bearing cell; *Amplification* on the platelet as it becomes activated; and *Propagation* on the activated platelet surface. **F**: factor; **a**: activated; **vWF/FVIIa**: von Willebrand factor-activated factor VII-complex (adapted from Hoffman & Monroe, 2001).

(2) Amplification of the coagulant response occurs as the "action" moves from the TF-bearing cell to the platelet surface. The pro-coagulant stimulus is amplified as platelets adhere, are activated and accumulated activated cofactors on their surfaces

(3) Finally, in the propagation phase, the active proteases combine with their cofactors on the platelet surface, the site best adapted to generate haemostatic amounts of thrombin. The activity of the pro-coagulant complexes produce the burst of thrombin generation that results in fibrin plymerisation.

Inappropriate coagulation is prevented by several mechanisms. The activation and pro-coagulation steps are localised on different cell surfaces. The plasma protease inhibitors localise the reaction to cell surfaces by inhibiting active protease that diffuses into the fluid phase. Finally, endothelial cells express active AT features that prevent coagulation from being initiated in the intact endothelium (Hoffman & Monroe, 2001).

C.2 Fibrinolysis

Fibrinolysis is the enzymatic degradation of fibrin clots (through the proteolytic action of a blood component, plasmin). The fibrinolytic system is also involved with cell migration and wound healing and it plays a role in metastasis and tumor invasion, atheroslcerosis, thrombosis, embolism and bleeding. This makes the modulation of fibrinolytic function an approach in the prevention of many diseases (Ridker, 1997b; Takada, 1994; Vorster *et al.*, 1997a).

A simplified diagram of the fibrinolytic system is depicted in Figure 3. The fibrinolytic system consists of the inactive zymogen, plaminogen, which is activated by activators such as tPA and urokinase plasminogen activator (uPA) to active plasmin. Plasmin lysis both, cross-linked and non-cross-linked networks, producing d-dimer and to a lesser extent fibrinogen, producing fibrinogen degradation products (FDPs). Plasmin is inactive when bound to antiplasmin, forming the PAP. PAP values in plasma should therefore reflect plasmin generation, and the TAT/PAP ratio, the balance between thrombin and plasmin generation (and thus the haemostatic balance) (Cicala & Cirino, 1998; Vorster *et al.*, 1997a; Vorster & Venter, 1994).

TAFIa inhibits the activation of plasminogen as discussed in section C.1. As mentioned before, fibrinolysis is initiated when plasminogen is converted to plasmin by tPA. Plasmin then degrades

the fibrin clot into soluble FDPs. The formation of plasmin is enhanced by a positive feedback loop. The carboxyl-terminal lysine residues of fibrin, generated after limited plasmin cleavage, act as template onto which both tPA and plaminogen bind. Whereas the binding of tPA to fibrin is primarily mediated by the finger-like domain and secondarily by the second kringle of tPA, the binding of plasminogen to fibrin is entirely kringle-dependant. TAFIa cleaves off the carboxyl terminal lysine residues from partially degraded fibrin and thereby abrogates the fibrin cofactor function in the tPA-mediated catalysis of plasminogen. Elimination of the carboxy-terminal lysine residues prevents the formation of the tPA/plasminogen/fibrin complex and thereby inhibits the formation of plasmin and the degradation of the fibrin clot (Bajzar, 2000; Bouma & Meijers, 2003; Bouma *et al.*, 2001). The two activators of plasminogen conversion, tPA and uPA, are inhibited by plasminogen activation inhibitors PAI-1 and PAI-2. Increased concentration and/or activity of PAI-1 are probably the main cause of impaired fibrinolytic capacity (Cicala & Cirino, 1998; Dahlbäck, 1994; Mosnier *et al.*, 2001; Vorster & Venter, 1994).

C.3 Markers and risk factors of the haemostatic system

Haemostatic factors that have been implicated in the literature to be risk factors for CVD include: increased plasma fibrinogen, FVII coagulant activity, decreased fibrinolytic activity, increased platelet aggregability and impaired fibrinolysis because of decreased tPA and increased PAI-1 (Vorster *et al.*, 1997a).

There is now convincing epidemiological and clinical evidence that the pre-thrombotic state is an important predictor of CVD. Two distinct mechanisms have been invoked to describe possible roles for haemostatic factors in CVD: involvement of thrombotic factors in the development of atherosclerotic plaques and involvement of thrombotic factors in the thrombotic occlusion, embolisation, or both at sites of the destabilised atherosclerotic plaques. Indeed, these mechanisms are not mutually exclusive and may both be active in the causation of clinical events (Pearson *et al.*, 1997).

For the purpose of this dissertation, only the haemostatic factors that were measured will be described.

The following markers and risk factors of the haemostatic system are composed and summarised from the following references: Degen, 1999; De Maat *et al.*, 2000; Emeis *et al.*, 1997; Haverkate,

2002; Held *et al*, 2000; Jerling, 1998; Lee *et al.*, 1995; Mezzano *et al.*, 2001; Marckmann *et al.*, 1998; Pearson *et al.*, 1997; Ridker, 1997a; Ridker 1997b; Takada *et al.*, 1994; Ten Cate *et al.*, 1997; Tracy, 1999; Vorster *et al*, 1997a; Vorster & Venter, 1994.

C.3.1 Fibrinogen

Of all the proteins in the haemostatic system, plasma fibrinogen has been studied most extensively. As mentioned before, fibrinogen is the complex, multifunctional soluble protein that polymerises to form the fibrin network or clot after thrombin has removed FPA and FPB. Fibrinogen is a major determinant of blood viscosity but also an acute phase-reactant, and is positively related to other risk factors of CVD and known to be increased in CVD patients. Plausible biological mechanisms and evidence from a variety of studies support these relationships and a causative role of fibrinogen in both atherosclerosis and thrombosis (or atherothrombosis). The predictive value of fibrinogen for primary and secondary CVD event are almost identical (Ridker 1997b; Takada *et al.*, 1994; Ten Cate *et al.*, 1997).

Furthermore, fibrinogen is a general indicator of inflammation and the independent relationship to prognosis indicate that it may be causally related, but that it could also merely be a marker of underlying atherothrombotic disease. Moreover, local fibrin/fibrinogen deposition and dissolution appears to be features of both early and advanced lesions, a finding that is consistent with the fact that the majority of the cell types participating in lesion formation (e.g. platelets, monocyte/macrophages, EC, and smooth muscle cells) are known to express a variety of pro-coagulant and/or fibrinolytic factors (Degen, 1999; De Maat *et al.*, 2000; Held *et al*, 2000; Jerling, 1998).

C.3.2 Plasminogen activator inhibitor 1 activity (PAI-1_{act})

As mentioned before, PAI-1 inhibits tPA and uPA, contributing to the tight regulation of the fibrinolytic system. Results of various studies showed that PAI-1_{act} is higher in patients with CHD and confirmed relationships between elevated PAI-1_{act} and atherosclerosis (therefore, also implicated in inflammation) (Esmon, 2000; Haverkate, 2002; Jerling, 1998; Ridker, 1997a; Vorster *et al*, 1997a).

Furthermore, increased PAI-1 expression has been reported in atherosclerotic plaques. With increased PAI-1 levels, the ability to generate plasmin is lost and lesions can grow relatively unimpeded. In part, the function of the intrinsic fibrinolytic system reflects the balance between

2 serine proteins secreted in the vascular endothelium and adjacent smooth muscle (as mentioned before), tPA and its primary inhibitor PAI-1. Impairment of the balance between tPA and PAI-1 appears to be a major risk factor for future MI and stroke (Marckmann *et al.*, 1998; Mezzano *et al.*, 2001; Pearson *et al.*, 1997; Ridker, 1997b).

C.3.3 Tissue plasminogen activator antigen (tPA_{ag})

As mentioned before, tPA is the physiological activator that converts plasminogen to plasmin. Furthermore, tPA is synthesised, stored and secreted by EC and forms an inactive complex with PAI-1. It can be expected that because of the complex formation with PAI-1, tPA_{ag} should have a positive relationship with CVD and a negative one with free active tPA. Furthermore, it has been suggested that tPA_{ag} is a stronger predictor of thrombosis than of atherosclerosis. Increased risk for MI or sudden death is associated with higher baseline tPA_{ag} (Esmon *et al*, 1991; Haverkate, 2002; Marckmann *et al.*, 1997; Pearson *et al.*, 1997).

Both increased levels of PAI-1 as well as tPA_{ag} have been implicated as risk factors for the development of CVD, especially CHD. This is remarkable as the two proteins are supposed to have opposite effects on atherothrombosis. In considering the relative importance of tPA and PAI-1 respectively, one should remember that these proteins interact in the circulation, resulting in the formation of a tPA-PAI-1 complex. Consequently, an increase in plasma $PAI\text{-}1_{act}$ will result in an increase in plasma tPA_{ag}, since tPA-PAI-1 complex is cleared slower than free tPA (the reverse is not true, since free PAI-1 is supposedly cleared more slowly than the tPA-PAI-1 complex) (Marckmann *et al.*, 1998; Ten Cate *et al.*, 1997; Vorster & Venter, 1994).

The mechanism by which increased tPA_{ag} may contribute to CVD events is not well understood. Moreover, circulating tPA_{ag} reflects the balance between tPA released from the endothelium (possibly in response to increased coagulation) and the availability of PAI-1 to complex free tPA. The tPA_{ag} relationship with CVD events may, therefore, also reflect the relationship of PAI-1 with CVD events (Jerling, 1998; Pearson *et al.*, 1997; Tracy, 1999).

C.3.4 Plasmin-antiplasmin complex (PAP)

As mentioned before, the PAP complex forms when α_2-AP binds and inactivates free plasmin. Theoretically PAP values in plasma should, therefore, reflect plasmin generation and the TAT/PAP ratio the balance between thrombin and plasmin generation (Cicala & Carino, 1999; Jerling, 1998; Vorster *et al.*, 1997a).

Therefore, increased PAP reflects increased *in vivo* plasmin generation (hyperfibrinolysis) with fibrin and fibrinogen breakdown (Jerling, 1998; Mezzano *et al.*, 2001; Takada *et al.*, 1994). Very little is known about the relationship of PAP or the TAT/PAP ratio with CVD and judgement on PAP's value as a marker of future CVD is not possible at this stage

C.3.5 Thrombin-antithrombin complex (TAT)

As mentioned before, the TAT complex is formed when ATIII binds with and inhibits thrombin. TAT values should, therefore, reflect thrombin generation and activation of the coagulation cascade. In the Third Glasgow MONICA Survey increases in FVII, FVIII and FIX and decreases in PC were accompanied by increases in TAT. In that study, serum vitamin C levels also showed a significant negative correlation with TAT (Woodward *et al.*, 1997).

There is evidence that TAT levels are raised, reflecting coagulation activation (or a pro-thrombotic environment) in patients with CAD/ischaemic heart disease (IHD), acute MI and directly after stroke. However, in a population-based, cross-sectional study (Woodward *et al.*, 1997), the relationship between TAT and prevalent CVD was not significant. Moreover, not many studies have examined the relationship of TAT with CVD events, but the available data does suggest that it is not a good marker.

Clearly, more information is needed for a judgement on the suitability of the TAT complex as a marker for future CVD.

C.3.6 D-dimer

D-dimer (marker of fibrin turnover) is produced when plasmin digests cross-linked fibrin. There is evidence that d-dimer is increased in subjects with atherosclerotic and CVD events and that it correlates with plasma fibrinogen concentrations. Increased d-dimer levels would indicate both thrombin generation (because thrombin is necessary for fibrin formation – catalising fibrinogen to fibrin monomers and also for activating FXIII for cross-linking of fibrin fibres) and plasmin generation (probably in response to increased fibrin formation and because digestion of the cross-linked fibrin releases d-dimers) (Jerling, 1998; Lee *et al.*, 1995; Mosesson, 1997; Takada, 1994).

C.4 Interaction between inflammation and coagulation

It has long been known that inflammation can lead to activation of the coagulation system. Therefore, consideration must be given to concomitant events occurring in the coagulation cascade when one studies the inflammatory response. This response is a multifactorial defensive process of an organism to injury such as infection/noxious stimuli (Cicala & Cirino, 1998; Degen, 1999).

The interaction between the inflammatory and coagulation pathways is critical to the development of acute and chronic inflammatory diseases. Atherosclerosis is looked upon as a chronic inflammatory state. Acute inflammation, as a response to severe infection and trauma, results in systemic activation of the coagulation system, termed disseminated intra-vascular coagulation (DIC). In the 1990s, it became apparent that the principal initiator of inflammation-induced thrombin generation is TF (Berliner *et al.*, 1999; Cicala & Cirino, 1998; De Maat *et al.*, 2000; Held *et al.*, 2000; Ross, 1999). Furthermore, it has become apparent that cytokines mediate many of the responses triggered by severe inflammation, thereby placing cells, other than circulating mononuclear cells, in the spotlight. The derangement of coagulation and fibrinolysis in sepsis is mediated by several pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin 1 and 6 (IL-1 and IL-6), of which IL-6 seems to be the principle mediator of coagulation activation in sepsis. TNF-α indirectly influences the activation of coagulation because of it's effect on IL-6 and it is the pivotal mediator of the dysregulation of the physiologic anti-coagulant pathway and fibrinolytic defect (Levi *et al.*, 2002). Evidence has accumulated to suggest that more complex mechanisms might be involved in the relationship between inflammation and activation of coagulation. In addition, it has become clear that this relationship is not unidirectional, but instead, an interaction between the systems occurs by which activation of coagulation will also affect inflammatory activity. In particular, vascular EC seem to play a pivotal mediatory role in the coagulative response to systemic inflammation and the interaction between coagulation and inflammation. Therefore, inflammation and coagulation cannot be considered as two separate processes since there are several connecting points making them part of a unique defensive host response (Cicala & Cirino, 1998; Degen, 1999; Esmon, 2000; Levi *et al.*, 2002; Tracy, 1999).

The most important mediators and relationship among them, according to the proposed mechanism of interaction between inflammation and coagulation can be seen in Figure 5.

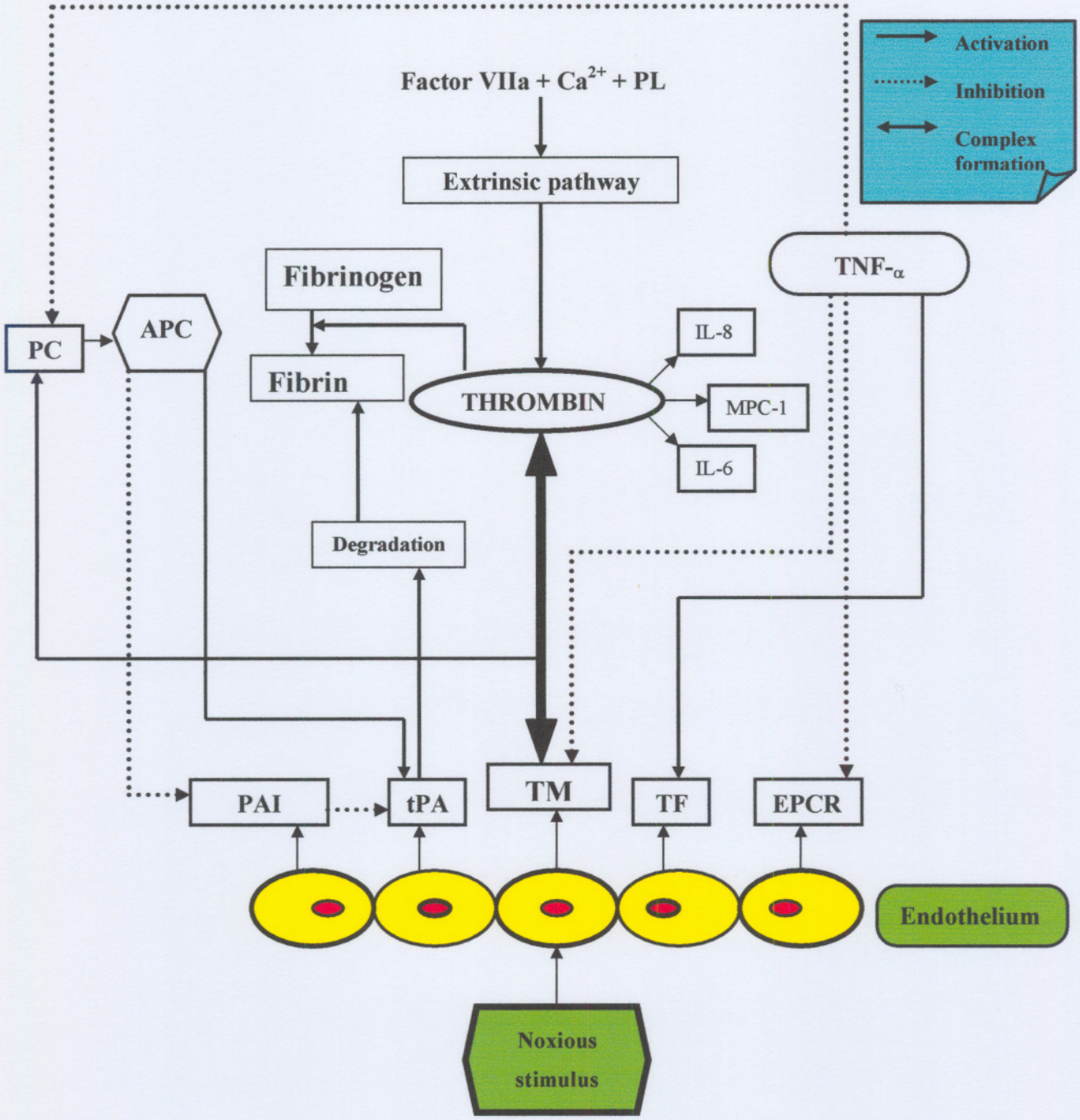

Figure 5 **Inflammation and coagulation interaction.**
The most important mediators and relationship among them, according to the proposed mechanism of interaction: **PL:** phospholipids; **PC:** Protein C; **APC:** Activated protein C; **IL-6:** Interleukin 6; **IL-8**: Interleukin 8; **MPC 1:** Monocyte chemoattractant protein 1; **TNF-$_\alpha$:** Tumor necrosis factor α; **PAI:** Plasminogen activator inhibitor; **tPA:** Plasminogen activator; **TM**: thrombomodulin; **TF:** Tissue factor; **EPCR:** Endothelial protein C receptor (adapted from Cicala & Cirino, 1998; Luther & Mackman, 2001; Ridker,1997a; Tracy, 1999).

Coagulation yields protease that not only interacts with coagulation zymogens, but also with specific cell receptors to induce signaling pathways. The activation of coagulation during systemic infection proceeds through the extrinsic TF mediated pathway and activation of the contact system does not contribute to DIC but is involved in the development of shock (Cicala & Cirino, 1998; Levi, 2000). The extrinsic pathway not only contributes to coagulation activation, but also has likely effects on other inflammatory systems yet to be defined. Similarly, the PC-PS system probably influences other inflammatory cascades besides its function as an important anti-coagulant mechanism (Ten Cate *et al.*, 1997). During inflammation cytokines modulate the coagulation system by down regulating the expression of TM and activation of PC pattern, but at the same time they induce the expression of TF modifying in this way the balance between pro-coagulant and anti-coagulant activities. At the same time, at the site of tissue injury, platelets become activated and release several mediators that modify tissue integrity. Thrombin, formed following activation of the coagulation cascade, is essential to promote haemostasis but also stimulates several cell functions, including chemotaxis and mitogenesis which are responsible for spreading of lesions and tissue repair process (Cicala & Cirino, 1998; Levi *et al.*, 2002). FXa, thrombin, and TF:FVIIa have each been shown to elicit pro-inflammatory activities. Fibrinogen is important to the host defense mechanism and probably has an additional role that is not directly related to clotting *per se* (Degen, 1999). Thrombin has been shown to induce a variety of non-coagulant effects, some of which may influence DIC by affecting cytokine levels in blood. It induces production of monocyte chemotattractant protein 1 (MPC-1) and IL-6 in fibroblasts, epithelial cells and mononuclear cells *in vitro*. Endotoxin-stimulated whole blood produces significant interleukin 8 (IL-8) levels, which has a pro-coagulant effect that is TF and thrombin dependant. Thrombin also induces IL-6 and IL-8 production in EC. These effects of thrombin on cell activation are probably mediated by protease-activated receptors, which are cellular receptors for activated protease that may contribute to intracellular signaling. TF:FVIIa complex also activates cells by binding to protease-activated receptors. Binding of the catalytic TF:FVIIa complex elicits a variety of inflammatory effects in mononuclear cells that influences their contribution to coagulation activity. The PC system forms another link between inflammation and coagulation. APC has anti-inflammatory effects on mononuclear cells and granulocytes, which may be distinct from its anti-coagulant activity (Esmon, 2000; Levi *et al.*, 2002).

An overview of the importance of some of the connecting points between inflammation and coagulation will now be outlined.

C.4.1 Inflammation and endothelial damage

EC are now considered as a dynamic, heterogeneous tissue that plays key roles in haemostatic balance and vessel tone regulation (Catelli *et al.*, 2001; Liao, 1998). EC respond to the cytokines expressed and released by activated leukocytes but can also release a wide panel of pro-inflammatory cytokines and chemokines themselves. Furthermore, EC are able to express adhesion molecules and growth factors that may not only promote the inflammatory response further but also effect the coagulation response (Catelli *et al.*, 2001; Levi *et al.*, 2002; Liao, 1998). Inflammatory responses are, therefore, also associated with increased levels of the circulating adhesion molecules, such as E-selectin and vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecules (ICAM) (Seljeflot *et al.*, 1998; Tracy, 1999). The CAMs mediate the slowing down of leukocytes from the axial blood stream to roll along and attach to the endothelium. In addition to these mostly indirect effects of the endothelium, EC interfere directly with the initiation and regulation of fibrin formation and removal during severe infection. In fact, EC play a prominent role in all three major pathogenic pathways associated with coagulopathy in sepsis: TF-mediated thrombin generation, dysfunctional anti-coagulant pathways and blocked fibrinolysis (Levi *et al.*, 2002). EC also possesses both pro-coagulant and anti-coagulant properties. Its anti-thrombotic properties include synthesis and secretion of anti-coagulant and fibrinolytic substances. However, when it is injured or stimulated by substances such as thrombin or inflammatory and pathological agents, its anti-thrombotic properties are lost, therefore acquiring pro-thrombotic properties which also initiates atherosclerosis (Vorster *et al.*, 1997a).

During an inflammatory state such as sepsis, the endothelium represents a target organ for the action of cytokines. Following endothelial damage, TF is expressed and the extrinsic pathway of coagulation is activated. EC also release t-PA, a serine protease enzyme able to convert plasminogen to plasmin, whose function is to degrade fibrin. The action of t-PA is modulated by PAI-1, also released by EC. As mentioned before, the balance between t-PA and t-PAI-1 is essential for the modulation of the fibrinolytic pathway (Cicala & Cirino, 1998; Liao, 1998; Luther & Mackman, 2001; Vorster *et al.*, 1997a).

TM, a high affinity receptor protein for thrombin, is expressed on the endothelial surface (Sadler, 1997; Vorster *et al.*, 1997a). In addition to its role in accelerating PC activation, TM accelerates the inactivation of thrombin by AT and PC inhibitors. Thus, down regulation of TM would be anticipated to reduce the rate at which thrombin was cleared from the circulation, presumably

promoting fibrin formation and platelet activation, leading to thrombus formation. Therefore, reduced levels could point to the reduced anti-coagulant potential at the cell surface and an increase in TM as a marker of endothelial injury that is closely associated with atherosclerosis, could possibly be secondary to an increased generation of thrombin. Moreover, an anti-fibrinolytic property of TM has recently been demonstrated as well. In complex with thrombin (thrombin-TM complex) it is able to activate the plasmaprotein designated TAFI to TAFIa, which inhibits the conversion of plasminogen to plasmin, the main fibrinolytic enzyme (Cicala & Cirino, 1998; Esmon, 2000; Nesheim *et al.*, 1997; Seljeflot *et al.*, 1998). Moreover, the existence of TAFI provides the explanation for the apparent pro-fibrinolytic effect of APC and implies the existence of an explicit molecular connection between the blood coagulation of fibrinolytic cascades that is expressed through the thrombin-TM dependent activation of TAFI. Therefore, thrombin generation can, in principle, result in the suppression of fibrinolysis. The thrombin-TM complex activates PC, a vitamin K dependent glycoprotein that circulates in plasma as a zymogen. Once PC is activated, it forms a complex with vitamin K dependent cofactor, PS, on the endothelium. APC plays a major role in regulating the heamostatic process by inactivating FVa and FVIIIa and at the same time, stimulating the fibrinolytic pathway, by inactivating PAI-1. Therefore, APC inhibits coagulation and activates the fibrinolytic pathway (Nesheim *et al.*, 1997). In the plasma APC is slowly inhibited by complex formation with PCI or α_1-antitrypsin. On the other hand, thrombin, by activating the PC pathway, acts as a feedback inhibitor of its own production. Control of fibrin generated during activation of the coagulation cascade is dependent upon a balance between pro-coagulant and anti-coagulant factors. The presence of TM on the endothelial surface and the integrity of the PC pathway are fundamental requirements for a correct balance between coagulation and fibrinolysis. This balance between pro- and anti-coagulant factors is strongly impaired during inflammation. Cytokines, and in particular TNF-α, are mainly responsible for the down regulation of TM and PC expression. At the same time, cytokines (IL-1 and TNF-α) induce the expression of TF. In this way, thrombin does not bind TM and the PC pathway is not activated, thereby causing an impaired modulation of the coagulation cascade and shifting the balance towards a pro-coagulatory state which may lead to critical thrombosis. APC in low doses act as an anti-coagulant and by inhibiting PAI, facilitates fibrin degradation (Cicala & Cirino, 1998; Levi *et al.*, 2002; Tracy 1995).

An EPCR has been identified and it seems to participate in APC regulation by binding to an exo-site of APC and blocking the ability of APC to inhibit the FVa. EPCR is down regulated by

TNF-α. Its function has not completely been clarified, however, it seems to modulate APC in a manner that resembles the action of TM on thrombin.

According to Cicala & Cirino (1998), another important aspect to be considered when the link between coagulation and inflammation is examined is the contribution of platelets to both processes. According to Meyer *et al.* (1976) platelets have especially two important functions. Firstly they protect the body against foreign materials by adhering to the material isolating it temporarily, promoting phagocytosis. Secondly, they promote haemostasis. Platelets have a role as effector and mediator cells in inflammation, because platelets themselves also contain an array of potent pro-inflammatory substances (reviewed by Grey & Meyer, 1976). Therefore, platelets participate actively in inflammation (Klinger, 1997). When the endothelium is damaged, such as during inflammation, collagen is exposed. Platelets adhere to collagen fibres of the sub-endothelium, thereby becoming activated. This platelet aggregation and the formation of a platelet plug are part of the haemostatic mechanism to prevent blood loss from injured vessels. Aggregating platelets also participate in fibrin network formation, clot retraction and tissue repair (Vorster *et al.*, 1997a). Platelet adhesion to sub-endothelium is mediated by adhesion molecules (such as E-selectin, VCAM-1 and ICAM-1), expressed on both EC and platelets following cytokine stimulation and by the expression of vWF on the platelet surface. Activated platelets release from their granules a number of substances that modify tissue integrity. More specifically, platelets release serotonin which contributes to increased vascular permeability; adenosine diphoshate (ADP), which contributes to recruitment of other platelets into the site of inflammation; platelet activating factor (PAF), which has been shown to be involved in several inflammatory disorders such as sepsis; the growth factors (platelet derived growth factor and transforming growth factor beta) which are potent mitogens and chemotactic agents. Furthermore, cationic proteins and proteolytic enzymes (collagenase and elastase) contained in platelet granules are all substances that modify tissue integrity (Cicala & Cirino, 1998; Klinger, 1997; Vorster *et al.*, 1997a).

Platelet adhesion to endothelium is an early event during inflammation. Thus, platelet activation represents an event contributing to the spreading of inflammation and to the recruitment of other inflammatory cells in the early stage of the inflammatory response. Moreover, the finding that, following stimulation, platelets express IL-1 on their surface suggests that platelets provide an early source of this cytokine at the site of injured endothelium. In addition, platelet-granulocyte interaction has been shown to increase endotoxin lipopolysaccharide (LPS) induced TF activity in

monocytes. The expression of TF activity on monocytes is critical to the promotion of thrombosis during inflammation. P-selectin is an adhesion receptor, expressed on the surface of activated platelets. Expression of P-selectin on activated platelets mediates interactions among platelets, leukocytes and EC (also in the absence of inflammation) and is a key event for the expression of TF on monocytes. Moreover, P-selectin is up regulated on endothelium stimulated with inflammation mediators and serves as a tethering site for neutrophils. Inhibition of P-selection interaction also blocks the platelet leukocyte interaction that can facilitate thrombus formation. Not only does the inflammatory response permit the leukocyte-platelet thrombus formation to be enhanced, but inflammatory cytokines, IL-6, in particular, up regulate platelet sensitivity to thrombin. These responses lead to an increase in both pro-coagulant surface (pro-thrombinase) and the ability to form a thrombus (Esmon, 2000; Klinger, 1997).

Thus, the endothelium represents the interface between inflammation and coagulation: it is a surface where coagulation is activated but, at the same time, under cytokine stimulation, it provides a site of attachment for inflammatory effector cells. Evidence that platelets, whose main function is in haemostasis, play a role in inflammation further implies an important link between inflammation and coagulation.

C.4.2 Thrombin in inflammation

Thrombin is a serine protease, which plays a major role in haemostasis and thrombosis by cleaving fibrinogen to form fibrinogen monomeres and FPA and FPB. The monomeres polymerise to form the fibrin network or clot in which activated coagulation FXIII promotes the formation of cross-links (Cicala & Cirino, 1998). Thrombin plays a central role in haemostasis because it also promotes the expression of pro-thrombotic endothelial factors and platelet aggregation. As mentioned before, when bound to TM, thrombin activates PC (which inhibits thrombin formation) as well as TAFI, which probably prevents premature lysis of haemostatic plugs by inhibiting fibrinolysis (Nesheim *et al.*, 1997).

AT, the PC system and TFPI limit thrombin generation. During severe infection, all three regulatory systems are defective, primarily as a result of endothelial dysfunction (Levi *et al.*, 2002). TFPI is a protease inhibitor and it is regarded as the major physiologic inhibitor of the extrinsic pathway because it inhibits, in the presence of FXa, the TF-FVII/VIIa complex (thus acts as an anti-coagulant) (Levi *et al.*, 2002; Vorster *et al.*, 1997a). TFPI may also inhibit the

induction of TF by inflammatory and EC by blocking the interaction of endotoxin LPS with its receptor (Luther & Mackman, 2001).

During inflammation as described above, the coagulation pathway is activated following endothelial damage and the expression of TF and thrombin is formed from its precursor, pro-thrombin. Apart from its pivotal role in haemostasis, thrombin has been shown to induce several cell responses, which are involved in inflammation. Thrombin is chemotactic for monocytes and neutrophils (mediated by high affinity receptors, present on both macrophages and neutrophils). Moreover, thrombin increases IL-1- and TNF-α-induced neutrophil chemotaxis, induces mitogenesis of several different cell types and stimulates EC to prostacycline (PGI_2), a powerful inhibitor of platelet aggregation (Cicala & Cirino, 1998; Vorster *et al.*, 1997a). Furthermore, thrombin has been shown to exhibit vaso-active properties. Depending on the vascular target tissue, it can cause either endothelium dependent vaso-relaxation or endothelium dependent contraction. All these features suggest a role for thrombin in normal tissue repair processes and in pathological proliferative responses. It is, therefore, clear that thrombin generated in the coagulation components of the haemostatic system also influences events in the fibrinolytic, platelet and EC components (Vorster *et al.*, 1997a).

Powerful molecular biology tools have made it possible to better understand and clarify the mechanisms involved in thrombin-induced cell activation. The thrombin receptor, protease activated receptor 1 (PAR-1) on the cell surface has been identified, characterised and cloned. It has furthermore been demonstrated that thrombin cleaves its receptor between $Arginine_{41}$ and $Serine_{42}$ residues, unmasking a new N-terminus peptide (which consists of 14 amino acids and functions as a tethered ligand for the receptor itself, causing activation). This has led to the identification of a new role of thrombin receptor activation in cellular responses. It is now accepted that several cellular events mediated by thrombin are independent of its catalytic activity and may be mimicked by peptides called thrombin activating peptides (TRAPs), which are devoid of the catalytic activity of thrombin. It is this discovery that has led to a large body of research focusing on the interaction between the coagulation cascade and inflammation. Both TRAPs and thrombin are able to induce contraction or relaxation of vascular or parenchymal smooth muscles (Cicala & Cirino, 1998).

Furthermore, both are able to induce cytokine expression in several cellular types. Thrombin induces MCP-1 expression in vascular smooth muscle cells, in monocytes and in EC. MCP-1 is a

potent chemoattractant for monocytes which facilitates monocytes migration to sub-endothelium, an important event in the pathogenesis of atherosclerosis. Therefore, thrombin generated at the site of injury through multiple actions on cells participates in the spreading of inflammation, to atherogenesis (a chronic inflammatory state) and vascular remolding.

To conclude, the above mentioned events can be summarised as follows. During inflammation, thrombin is formed from its zymogen, pro-thrombin as a consequence of endothelial damage and activation of the coagulation cascade. Thrombin acts on fibrinogen to promote fibrin formation and on platelets to promote aggregation and degranulation. In addition, by binding the PAR-1 receptor on the cell surface, thrombin affects a series of endothelial functions and stimulates cells involved in inflammation. Most of thrombin cellular actions may be ascribed to the new N-terminus peptide generated following proteolysis of PAR-1 receptor. Thus, thrombin present at a site of injury stimulates cell proliferation, chemotaxis and other events involved in inflammation and tissue repairing processes, all events that occur in parallel but independently of coagulation. Most importantly, these cellular effects of thrombin may be recapitulated by TRAPs, suggesting that TRAP antagonists could be useful agents able to modify inflammatory and atherosclerotic disorders without affecting physiological haemostatic processes (Cicala & Cirino, 1998; Levi *et al.*, 2002; Nesheim *et al.*, 1997).

C.4.3 Atherosclerosis – an inflammatory disease

Markers of inflammation such as acute phase reactants, CRP, amyloid A, plasma fibrinogen levels, as well as cellular adhesion molecules and interleukins are potential haemostatic and thrombotic markers for arterial thrombosis (Held *et al.*, 2000; Ridker, 1997a; Tracy, 1999).

More specifically, the other potential haemostatic and thrombotic markers for arterial thrombosis are the fibrinolytic markers: tPA, PAI-1, colt lysis time, fibrin degradation products and d-dimer. The coagulation, platelet and process markers are FV, FVII & FVII activity, vWF antigen, homocysteine, pro-thrombin fragment 1 + 2, TAT and PAP (adapted from Haverkate, 2002; Held *et al.*, 2000, Ridker, 1997a; Tracy, 1999). In addition to the inflammatory, fibrinolytic and coagulation markers mentioned above, several platelet related parameters have also proven to have predictive value in determining arterial thrombotic risk, such as total platelet count, volume, and aggregability (Ridker, 1997a).

Recent evidence from a wide variety of sources implicates inflammation in the process of atherosclerosis and ultimately, clinical CVD (Berliner *et al.*, 199; Held *et al.*, 2000; Ridker, 1997a; Ridker, 1997b; Ross, 1999; Tracy, 1999). As summarised recently by Ross (1999), the biochemical and cell biological evidence clearly supports the position that inflammation is involved in all stages of atherosclerotic development including, but not limited to, oxidative damage, cell proliferation and plaque development and destabilisation. The interaction between atherosclerosis, coagulation, fibrinolysis and inflammation can be seen in Figure 6. The interactions depicted in Figure 6 can be summarised as follows: The generally accepted model of atherosclerotic progression is depicted on the left side of the figure. This results in thrombosis and fibrinolysis and the elaboration of FDP's. In turn, this causes an increase in monocyte IL-6 production. In fact, IL-6 production (likely along with the other pro-inflammatory cytokines) is generally increased in atherosclerosis, most likely through associations with other risk factors, such as obesity and through general tissue damage accompanying the disease. IL-6 increases, whatever the cause (including co-morbidities, such as infection and diabetes) will result in low-level changes in all the liver proteins generally considered acute phase reactants. The increased IL-6, itself, may make the atherosclerotic progression worse, since it can function as a potent growth regulator. In turn, the liver protein may or may not have direct effects on various stages of the process (Berliner *et al.*, 199; Held *et al.*, 2000; Ridker, 1997a; Ridker, 1997b; Tracy, 1999).

As mentioned before, inflammatory responses are mediated through the cytokine pathway at least initially with the major pro-inflammatory cytokines being IL-1, IL-6 and TNF-α. As an example, the generation of pro-inflammatory cytokines in the setting of sepsis is a powerful pro-coagulant and several coagulation factors such as fibrinogen and FVIII, have been known to be acute phase reactants for some time. However, coagulation and fibrinolysis themselves are inflammatory. The production of fibrin degradation products as the end result of coagulation and fibrinolysis, causes the systemic elaboration of IL-6 (and the up regulation of liver proteins, such as coagulation factors), which is most likely the mechanism by which the body counters consumption of factors. We have, therefore, a circular mechanism: inflammation begets coagulation, which begets more inflammation. Inflammation appears to be associated with atherothrombotic disease throughout the natural history of this process, from the early stages of fatty streak development, to the rapture of complex plaque with resultant coronary (or peripheral) artery occlusion (depicted on the left side of Figure 6).

Atherosclerosis seems to be a chronic inflammatory condition. Atherosclerosis is then converted to an acute clinical event by the induction of plaque rupture (a stable atherosclerotic plaque is converted to an unstable and potentially occlusive lesion), which in turn leads to thrombosis (Berliner *et al.*, 1995; Haverkate, 2002; Held *et al.*, 2000; Ridker, 1997a).

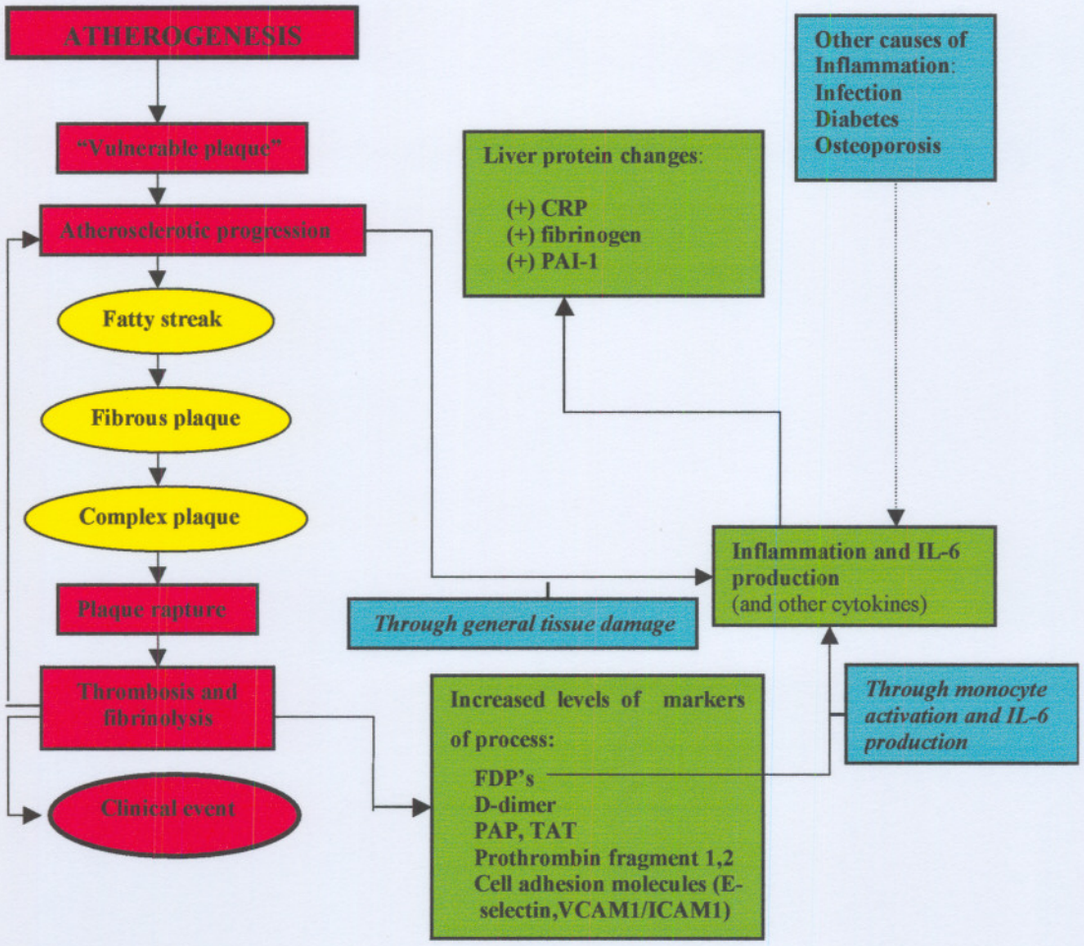

Figure 6 **Schematic representation of the interaction between atherosclerosis, coagulation, fibrinolysis and inflammation.**

FDP: Fibrinogen degradation product; **IL-6:** Interleukin 6; **CRP:** C-reactive protein; **PAI-1:** Plasminogen activator inhibitor 1; **PAP:** Plasmin-antiplasmin complex; **TAT:** Thrombin-antithrombin complex; **VCAM-1:** Vascular cell adhesion molecule 1; **ICAM-1:** Intercellular adhesion molecule 1 (composed from Berliner *et al.*, 1995; Ridker, 1997a; Ridker, 1997b; Seljeflot *et al.*, 1998; Tracy, 1999).

Atherosclerotic lesions are heavily infiltrated with cellular components associated with inflammation (Ridker, 1997b). These lesions occur principally in large and medium-sized elastic and muscular arteries and can lead to ischaemia of the heart, brain, or extremities, resulting in

infarction. Furthermore, both macrophage growth factor and MCP-1 are preferentially expressed in atherosclerotic arteries and several cellular adhesion molecules are over expressed among patients with unstable coronary disease and acute ischaemic stroke (Ridker, 1997a; Ridker, 1997b). According to Ross (1999), atherosclerotic lesions may be present throughout a person's lifetime. In fact, the earliest type of lesions, the so-called fatty streak, which is common in infants and young children, is a pure inflammatory lesion consisting only of monocyte-derived macrophages and T lymphocytes. In persons with hypercholesterolaemia, the influx of these cells is preceded by the extra-cellular deposition of amorphous and membranous lipids.

Inflammation, as a major factor in CVD, has been suggested in the response-to-injury model by Ross and colleagues (Ross, 1999). The mechanism postulated is that activation of the endothelial lining of an affected artery results in cellular infiltration and proliferation and ultimately in the development of advanced atheroslerotic lesions. Activated EC and infiltrating phagocytic leukocytes are a major source of reactive oxygen metabolites, which are able to initiate and/or amplify inflammation *via* the up regulation of different genes involved in inflammatory response, such as those that code for pro-inflammatory cytokines and adhesion molecules.

Numerous pathophysiologic observations in humans and animals led to the formulation of the above mentioned response-to-injury hypothesis of atherosclerosis, which initially proposed that endothelial denudation was the first step in atherosclerosis. The most recent variation of this hypothesis emphasises endothelial dysfunction rather than denudation. Whichever process is at work, each characteristic lesion of atheroslcerosis represents a different stage in a chronic inflammatory process in the artery; if unabated and excessive, this process will result in an advanced, complicated lesion. Possible causes of endothelial dysfunction leading to atherosclerosis include: elevated and modified low density lipoprotein (LDL), free radicals caused by cigarette smoking, hypertension and diabetes mellitus, genetic alterations, elevated plasma homocysteine concentrations, infectious micro-organisms (herpes viruses or chlamydia pneumoniae) and combinations of these and other factors (Ross, 1999; Oosthuizen, 1999). Endothelial injury may also be caused by oxidised LDL-particles released from macrophages within the sub-endothelial space (Berliner *et al.*, 1995). Therefore, systemic inflammation is intrinsically associated with oxidative stress, endothelial dysfunction/activation and haemostatic activation which are all important mediators of atherosclerosis and thrombotic events.

In light of the above mentioned association of systemic inflammation with oxidative stress, one should take note of several components that may display anti-inflammatory properties (e.g. ubichinons, flavonoids, enzymes and some phytotherapeutics). Moreover, it would be of further importance to note that dietary antioxidants may protect against oxidant-mediated inflammation and tissue damage by scavenging free radicals and by inhibiting the activation of oxidant-sensitive transcription factor such as the nuclear factor-$_k$B, resulting in an attenuated inflammatory response (Berliner *et al.*, 1995). Indeed in studies in patients with inflammatory diseases, antioxidant nutrients reduced the inflammatory symptoms. The amount and the relation of pro- and antioxidative nutrients had an impact on inflammation in rheumatic disease (Adam, 1995; Berliner *et al.*, 1995).

D. FIBRIN NETWORK STRUCTURE

Knowledge of the structure of fibrinogen and of the properties of the fibrin polymers that are formed on activation of fibrinogen by thrombin or other enzymes has expanded considerably during the past decade. Evaluation of fibrin network properties has become easy and accessible in almost any standard laboratory. Methods based on turbidity and permeability have become available for measurement of FNS properties (Nair *et al.*, 1991a). Little is known about the relationship between fibrin gel structure properties and coronary atherosclerosis and thrombosis in man. In examining cardiovascular risk, fibrinogen concentration is important but there are also other aspects which must be considered, such as the complex interplay between fibrinogen and other risk factors producing altered network structures. It has been established that the quality of fibrin networks may be an important risk factor for the development of CHD. Fibrin clots associated with CAD have a proneness to form tight, rigid and space-filling FNS with small pores (long thin fibres) (Blombäck *et al.*, 1992; Fatah *et al.*, 1992; Veldman *et al.*, 1997). The physical properties of fibrin networks depend upon the polymerisation conditions (Blombäck *et al.*, 1992).

The FNS forms the 'hinge' of the haemostatic balance. The initiation of coagulation result in fibrinogen forming fibrin monomeres. These monomeres polymerise to form fibrin threads leading to the formation of FNS, which consist of a lattice-work of fibrin threads. This lattice-work forms the basis in which red blood cells and platelets are trapped, resulting in the formation of blood clots (known as thrombi when they become dislogded) (Blombäck, 1996). FNS with either long and thin threads, or short and wide threads are formed (Blombäck & Okada, 1983). Networks formed with long, thin threads are more resistant to lysis than networks consisting of short and thick threads (Gabriel *et al.*, 1992). Once the FNS starts forming, the fibrinolysis system is activated to dissolve the blood clots that have been formed.

According to Blombäck *et al.* (1994) linear or branched polymers, are formed by association of bi-functional fibrin monomers. These initial structures were thought to grow to various thicknesses by lateral aggregation of protofibres. Random interaction between these fiber strands growing in different directions in space would then together with water establish a gel. The gel (or clot) is an infinite network structure having the macroscopic dimension of the sample. The clotting time signals the formation of this structure.

Depending on the conditions prevailing during activation, the gels formed may vary between the extremes 'fine, transparent' and 'coarse, turbid' structures. The fine networks are composed of thin fiber strands with small liquid spaces and are prone to become rigid. In the coarse gels, the fiber strands are thick and the liquid spaces large and they are, therefore, deformable and plastic (Blombäck *et al.*, 1994).

Plasma is an aqueous solution containing large amounts of proteins and other dissolved substances. Changes in any of these constituents can directly influence the characteristics of the FNS, which can be divided into two categories (Blomäck *et al.*, 1992):

1) kinetic factors
2) modulating factors.

The kinetic factors are thrombin and fibrinogen concentrations. When thrombin and fibrinogen interact, fibrin monomer is generated according to the relative amounts of enzyme and substrate (Nair et al., 1986). Changes in the fibrinogen concentration will change the affinity between the enzyme and substrate and, therefore, affect the clotting potential of thrombin. The rate of activation of fibrinogen by thrombin will increase significantly with increasing fibrinogen concentration and this leads to a drastic change in the fibrin gel structure. Raising the concentration of fibrinogen might be expected to lead to thicker fibres because of the decreased thrombin-to-fibrinogen ratio reflected in longer clotting times. However, the decreased fiber diameters may be attributed to accelerated rates of monomer formation because of higher substrate concentrations (Ryan *et al.*, 1999). Furthermore, the permeability of fibrin networks is inversely related to the concentration of fibrin in the gel. Long clotting times and low fibrinogen concentrations will favour elastic networks with a high porosity which easily yield to stress. These networks may be non-thrombogenic but they may be haemostatically less efficient. On the other hand, short clotting times and high fibrinogen concentrations will favour tight and rigid networks. These networks may have high haemostatic efficacy but may be thrombogenic (Blombäck *et al.*, 1983). The permeability of fibrin networks increase logaritmically with decreasing fibrin concentrations. Therefore, the porosity of the fibrin networks, that is the spaces between the fibres, increases with decreasing fibrin concentrations. In gels formed at high concentrations of fibrinogen, or with a high amount of thrombin, the spaces between the fibres decrease, indicating a decrease of gel porosity (Blombäck *et al.*, 1990). The common feature of a normal fibrin gel structure is an ordered network composed of straight rod-like fiber elements that cross each other in space and often originate in nodes (Figure 7). Increasing the kinetic factors

will, therefore, result in tighter, less porous networks with thinner fibres and a higher density of nodes. These structures are supposedly more rigid since the flow of liquid through them is impaired. Conversely, low concentrations of the kinetic factors result in porous networks with thick fibres and fewer nodes. These structures are deformable and plastic, since fluid easily escapes from the structure (Blombäck, 1994; Carr & Hardin, 1987).

However, it is known that other factors in blood, modulating factors, affect the network structures as they are formed with otherwise constant fibrinogen and thrombin concentrations. These factors change the architecture of the network by either interacting with the water in the medium during fibrin formation or by binding to either fibrinogen or to the fibrin strands in the established network (Blombäck, 1994). The modulating factors affect the fibrin structures as they are formed with otherwise constant kinetic factors. Such factors include proteins and ions in the direct surrounding of the fibrinogen molecule (Blombäck *et al.*, 1994; Blomäck *et al.*, 1992). Most of the modulating factors favour formation of more porous, plastic networks e.g. calcium ions, albumin, dextran (Blombäck, 1994) (other examples are zinc, magnesium, fibronectin, gamma-globulins, protamine, leukocyte cationic proteins and the carboxyl terminus of platelet factor 4). Increased ionic strength on the other hand favours the formation of tight, rigid structures. At extreme ionic strengths of higher than 2.4, a swelling of fibres is, however, observed due to decreased protein-protein interaction (Blombäck, 1994). The effects of some modulating factors such as AT are not yet clear. The presence of erythrocytes does not seem to influence the network structure, other than that it increases gel pore-size (Blomäck *et al.*, 1992; Blombäck, 1994; Blombäck *et al.*, 1994; Veldman, 1996).

The network structures formed in recalcified plasma by thrombin, as compared to those formed in the purified system in the presence of calcium, are much more porous with wider fiber strands. This is partly due to the presence of albumin and fibronectin in the plasma, but other factors may also play a role (Blombäck *et al.*, 1994).

D.1 Fibrin network formation

As mentioned previously, formation of fibrin networks is initiated by the enzymatic conversion of fibrinogen to fibrin by thrombin and these fibrin monomers are generated according to the relative amounts of enzyme and substrate. Fibrin assembly occurs in an orderly fashion from fibrin monomer to protofibril to fiber. The pore size can be estimated as a function of the fiber

size. Therefore, as the fiber size increases, pore size increases (Carr & Hardin, 1987). Turbidity studies show a lag phase, phase of increasing turbidity and the equilibrium phase (Nair *et al.*, 1986). These phases collectively represent the breakdown of fibrinogen to fibrin monomer, the initial aggregation of monomer to protofibrils and the growth of protofibrils to an opaque network. The lag phase corresponds to the time required for the overall action of thrombin on fibrinogen until the appearance of turbidimetrically detectable fibrin and includes the enzymatic breakdown of fibrinogen and the initial aggregation to protofibrils. The fibrinogen solution forms a gel during the early part of the second phase during which turbidity rises rapidly. This phase represents growth of the protofibril network already laid, although some further protofibrils may continue to appear. The main increase in turbidity is on account of growth in the thickness of the initial network of protofibrils (Nair *et al.*, 1986). Thrombin concentration, pH, temperature and ionic strength influence the initial protofibril network formation and its subsequent growth in thickness by two separate mechanisms. Ions other than divalent cations do not affect the network structure. Both Ca^{++} and Mg^{++} increase turbidity, but the effect of Ca^{++} on permeability is dependent on the presence of FXIII as a contaminant. It seems that pH and ionic strength affect the network structure by influencing fibrin assembly while lowered temperature (like concentration of thrombin) influences both the rate of fibrin monomer generation and fibrin assembly. Both permeability and turbidity are decreased as pH and ionic strength is increased (Nair *et al.*, 1986). The effect of higher thrombin concentrations on diminishing fiber size are observed in the results of permeability experiments (Blombäck *et al.*, 1990), turbidity experiments (Blombäck *et al.*, 1990; Shah *et al.*, 1985) and electron microscopy (Weisel *et al.*, 1992). Lowering the concentration of the enzyme reduces the rate of fibrinopeptide cleavage, resulting in a slower production of fibrin monomers. Existing protofibrils grow longer and aggregate laterally as monomers are slowly produced, resulting in a network of long, thick fibres (Weisel *et al.*, 1992). At higher concentrations, the rate of lateral and lengthwise fiber growth is slow compared to the rate of fibrinopeptide cleavage. Thus, several short oligomers are formed before they can join lengthwise or associate laterally, resulting in a network of thin, short fibres.

The variations of pore size with fiber diameter are shown in Figure 7 (Carr & Hardin, 1987).

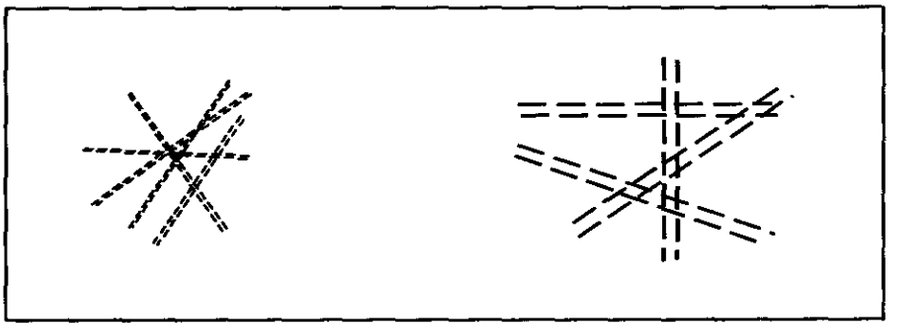

Figure 7 **Variation in fiber diameter (Carr & Hardin, 1987)**

Left represents a gel composed of thin fibres. Right represents a gel composed of thick fibres. As the mass is concentrated into thick fibres, the spaces or pores between them increase in size (Carr & Hardin, 1987).

D.1.1 ***Fibrinogen structure and it's association sites***

Fibrinogen is defined as the soluble protein in blood and tissue extract which in presence of thrombin, is transformed into an insoluble FNS. Fibrinogen is estimated to have a molecular weight of about 340 kDa (Blombäck, 1996).

Fibrinogen is a tridomainal (two outer D domains and a central E domain) disulfide-bridged molecule more or less 45 nm in length, composed of two symmetrical half molecules, each half consisting of a set of three different polypeptide chains termed Aα, Bβ, and γ (Figure 8). The two halves are covalently joined in the central amino-terminal E domain by five disulfide bridges (Blombäck *et al.*, 1976; Mosesson, 1998). A schematic model of fibrinogen and fibrin showing the major domains can be seen in Figure 8.

Enzymatic conversion of fibrinogen to fibrin by thrombin results in the release of FPA and FPB. FPA and FPB are released from the amino-termini of the Aα and Bβ chains respectively, thereby revealing recognition site for aggregation with the γ chain and exposure of E_A en E_B polymerisation sites respectively (in the E domain). FPA is always released at the fastest rate. It seems that FPA needs to be released for the activated molecule to be incorporated in the growing polymer. The product of the proteolysis is the formation of a fibrin monomer (Blombäck *et al.*, 1978).

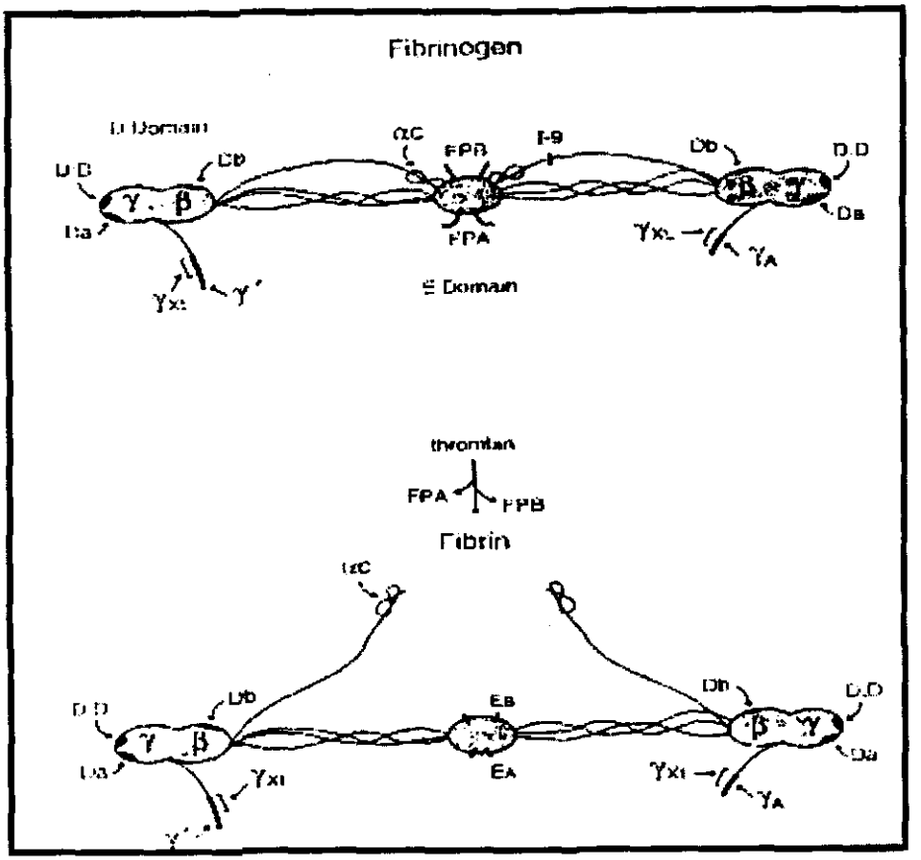

Figure 8 **Schematic model of fibrinogen and fibrin showing the major domains (D and E) (Mosesson, 1998)**

At least four different complementary cleavage site with aceptor-donor pairs are responsible for fibrin formation. Two of these primary sites are known as the 'A' and 'a' sites. They control linear polymerisation and protofibril formation. They are situated in the D and E domain of fibrinogen. The other two sites, 'B' and 'b', control lateral association of the fibrin monomers. Arrangement of the monomers is mainly anti-parallel (Blombäck *et al.*, 1976; Mosesson, 1998). Although thrombin and FXIIIa catalyse the formation of cross-linked fibrin, APC inhibits fibrin formation by regulating thrombin generation (Bouma *et al.*, 2000). Research by Gruber *et al.* (1994) led to the hypothesis that APC, *in vivo*, may enhance the efficacy of thrombolysis by altering the internal matrix structure of maturing thrombi and by inhibiting deposition of new fibrin on thrombi.

D.1.2 The assembly of fibrin networks

The following schematic mechanism represents the different phases of polymerisation (Veldman, 1996):

1. **Fibrinogen**→→activation: thrombin→→**fibrin monomer**→→linear polymerisation→→**protofibrils**
   FPA released
2. **Protofibrils**→→lateral association→→**fibrin network**
   FPB released

Fibrin polymerisation begins with thrombin cleavage of precursor fibrinogen molecules to release FPA. This is followed by an orderly process of intermolecular association to form linear and branched fibrin fibrils, lateral fibril associations to from a branched fiber network, subsequent release of FPB and concomitant covalent cross-linking by FXIIIa to form the mature stabilised clot (Mosesson, 1998). As mentioned, FPA is always released at the fastest rate and at the time of gel formation (*id est* clotting time), only a small amount of FPB has been released. The release of FPB is greatly accelerated subsequent to gel formation and this acceleration may be associated with conformational changes occurring as a result of polymer organisation (Blombäck, 1996).

FPB is released by thrombin at a slower rate than FPA. A second set of polymerisation sites is now becoming operative. The release of FPB from polymeric nuclei appears to allow for higher functionality of the latter. This means that polymerisation in different spatial directions commences and gelation occurs at an earlier stage of activation than when only FPA is released. Release of FPB, therefore, serves as a switch to speed up gelation and minimising the concentration of soluble polymeric species that otherwise would be required to induce gelation (Blombäck, 1994).

After the cleavage of FPA, exposing the polymerisation site E_A, each fibrin E_A site subsequently combines with a constitutive complementary site (D_a) in the D domain of neighboring molecules. These initial E_A:D_a associations result in the formation of double-stranded twisting fibrils in which the fibrin molecules become aligned in an end-to-middle, staggered, overlapping domain arrangement. These fibrils now form lateral associations and form branches that result in a complex fibrin network (Mosesson *et al.*, 1989). Two mechanisms account for the branched structures that constitute a three-dimensional fibrin matrix. The first type of branch consists of a pair of double stranded fibrils that converge to form a 'tetramolecular' branch point. This type of

lateral fibril association also accounts for the formation of thick fiber bundles. The second type of branch junction, termed the 'trimolecular' branch point, constitutes the junction of three double-stranded fibrils. Trimolecular branching probably plays an important, possibly dominant, role in the initial and ultimate structuring of a three-dimensional fibrin matrix (Mosesson, 1998).

The product that is formed after cleavage of FPA is called desA fibrin monomers. The formation of these polymers enhances the cleavage of the second peptide, FPB. FPB is, therefore, removed primarily from molecules that have already been assembled into protofibrils and fibres. This final product is called desAB fibrin (Blombäck *et al.*, 1978).

As mentioned previously, FPB release occurs more slowly than the release of FPA and exposes an independent polymerisation site, E_B (in the E-domain). The E_B site is utilised through interactions with a complementary D_b site located in a carboxy-terminal β-chain segment of the D domain. The E_B:D_b interaction is not absolutely required for lateral fibril and fiber association, but it contributes to this process through co-operative interactions resulting from alignment of D domains in the firbrin polymer. Therefore, many fibrin monomer molecules polymerise within seconds into long fibrin threads that form the reticulum of the clot. In the early stages of polymerisation, the fibrin monomer molecules are held together by weak non-covalent bonds, mostly hydrogen and/or hydrophobic and the threads are not cross-linked with each other, therefore, the resultant clot is weak and can be broken apart with ease. However, still another process occurs during the following few minutes that greatly strengthens the fibrin reticulum. This involves a globulin, fibrin-stabilising factor that is normally present in small amounts in plasma but is also released from platelets entrapped in the clot. The fibrin-stabilising factor must be activated before it can have an effect on the fibrin threads. Thrombin that causes fibrin formation also activates the fibrin-stabilising factor. This activated substance operates as an enzyme to cause covalent bonds between the adjacent fibrin threads, therefore, adding tremendously to the three-dimensional strength of the fibrin network structure. The formed fibrin latticework entraps blood and platelets to form the red blood clot (Mosesson, 1998; Mosesson, 1999). After cleavage of FPB, αC domains become available for self-association with other αC domains, thereby promoting lateral fibril association and fiber assembly. Concomitant activation of FXIII to FXIIIa by thrombin result in the introduction of ε-(-γ-glutamyl) lysine cross-links between C-terminal $_{\gamma XL}$ sites (thickness between D domains, Figure 8) mainly within fibrils to from γ- dimers, γ-trimers and γ-tetramers form by interfibril γ-chain cross-linking (Mosesson, 1998).

It is generally believed that fibres and fiber bundles eventually interact to form a three-dimensional network structure of the matured fibrin gel/FNS. Fibrin always occupies a small part of the clot volume and it is mainly the water kept in the gel network that has the volume filling capacity. The elasticity of this structure would determine the occlusive power of it. Tight network structures with small pores and thin fibrin strands are rigid. They are also brittle and during strain, rather break than give off water trapped in the clot. These clots are considered thrombogenic. Clots with thicker strands and large fiber meshes give off water when strained and deform in the process. These clots are non-thrombogenic (Blombäck, 1996).

D.2 Methods for the determination of fibrin network structure

As mentioned previously, methods based on turbidity and permeability have become available for measurement of FNS properties. In the past, investigations into the role of FNS in haemostatic disorders in thrombosis and other clinical areas have been hampered on account of lack of suitable methods for study (Nair *et al.*, 1991a).

Fiber size can be measured by gel perfusion or by turbidity techniques. The larger and more extended a molecule, the more light it will scatter. Thus gel turbidity is a function of fiber thickness. Advantages of turbidity measurements include simplicity and relative ease of interpretation. A major disadvantage has been the requirement of a scanning spectrophotometer. By turbidity measurement one can obtain a measure of the average fiber mass-length ratio (MLR) and fiber diameters of the hydrated fibres in fibrin gels (Blombäck & Okada, 1982; Nair *et al.*, 1991a).

The measurements of permeability of FNS give measurement of porosity. The permeability of clots is a function of pore size, which can be directly measured by perfusing liquid through the FNS (Blombäck & Okada, 1982). Fibrin clots with decreased permeability are usually also described as having long, thin fibres causing the network to be tight, rigid and with small pores (Blombäck *et al.*, 1992; Fatah *et al.*, 1992). Permeability of the clot is, therefore, dependent on the shape of the fibrin fibres. The amount of cross-linking in the network (as measured by compaction) determining the tensile strength of the fibres also influences the rate of permeation of fibrinolytic enzymes through the clot (Nair *et al.*, 1991b). As been mentioned before, fibrin networks can be influenced by kinetic and modulating factors, of which kinetic factors are the

most important in determining final gel structure (Blombäck, 1996). Upon addition of thrombin to fibrinogen, there is a lag-phase, which corresponds to the time required for the overall action of thrombin on fibrinogen until the appearance of turbidimetrically detectable fibrin. The second phase is a phase of sudden increased permeability due to the gelation of the activated fibrinogen. The permeability eventually reaches a plateau when all the fibrinogen has been concerted to fibrin (Nair *et al.*, 1986). The rate of fibrinogen activation (which is indicated by the duration of the lag phase or the clotting time) is, therefore, the most important factor in determining the final structure. The longer the time, the more porous the networks. Fibrin incorporated after the clotting time does not alter the FNS, but is only incorporated into the already existing scaffolding (Blombäck *et al.*, 1992). Pore size can also be estimated as a function of fiber size. If the amount of fibrin in a given volume remains constant, pore size will increase as fiber size or MLR increase. When fibrin is concentrated in a few large fibres, the distances or voids separating them will be increased (Blombäck *et al.*, 1990). Additionally to calculating the MLR and permeability, the fiber density, fibrin content and tensile strength of the fibrin fibres can be determined.

Of the above mentioned methods, only MLR, compaction and fibrin content were used in this study. Each of these will be discussed shortly hereafter.

D.2.1 Mass length ratio from turbidity

Networks containing thin fibres are transparent with closely interlocking fibres, making them tight, rigid and brittle. They, therefore, have small pores. The networks containing thick fibres are, however, opaque, elastic and are more porous (Shah *et al.*, 1982). There is a correlation between MLR and network permeability (τ) (Nair *et* al., 1991a). According to related literature, there are two ways of mathematically calculating MLR. The one way is *via* a transformation of the K_S-value (permeability of a clot), where:

$K_S = (Q \times L \times \mu)/(t \times A \times P)$. Q is the volume of buffer having a viscosity μ flowing through the chamber with the length L, A is area of cuvette, t the time for permeation of fluid under pressure gradient P (Fatah *et al.*, 1992; Nair *et al.*, 1991a), while the other is calculated using turbidimetric measurements. This method is based on the fact that fibrin fibres are thin, rod-like shaped structures and that their pattern of light scattering would be the same as that predicted theoretically for rod-like shapes. The MLR from the optical density can be calculated using the intercept, A of the plot where $C/T\lambda^3$ was plotted as a function of $1/\lambda^2$, where T is turbidity (2.303 x optical density), λ is the wavelength and C (mg/ml) is the concentration of fibrinogen. The

intercept *A* on the y-axis of this linear plot was used to calculate the average MLR, μ_T, according to the following equation as described by Carr and Hermans (1978) and Nair *et al.*, (1991a):

$$\text{MLR } (\mu_T) = \frac{10}{1.48A} \times 10^{12} \text{ daltons/cm}$$

In this study, turbidity (optical density x 2.303) was recorded continuously making use of unclotted citrated plasma in the reference cell at wavelengths between 600nm and 800nm with 2nm intervals with a Shimadzu UV 2100 spectrophotometer.

D.2.2 Compaction of fibrin networks

Compaction is one of the three methods used for the characterisation of the FNS and this method describes the tensile strength of the fibrin fibres. This simple method of compaction depends on the number and strength of the primary cross-links and branch points in the network. Compaction is not dependent on the size of the major fibres of the network nor on the organisation of the minor network (Nair *et al.*, 1991a). Compaction can be inversely correlated to Young's modulus of the elasticity of the networks and the final strength at break point (Dhall *et al.*, 1976). Initially fibrin fiber in networks are held together by non-covalent bonds, making them easily disruptable. It is only after FXIIIa covalent cross-links form that the networks become stable. The ability of a network to withstand the shear forces *in vivo* largely depends upon the rigidity of the network that arises from the cross-linking (Nair & Shats, 1997). Therefore, compaction/collapsibility of networks seems to be a measure of primary cross-links and branching in the network. Nair *et al.* (1991a) found statistically significant correlations between permeability (τ) and compaction, but not between turbidity (MLR, μ_T) and compaction.

Compaction is determined by making plasma clots in microcentrifuge tubes and centrifuging them for 45 seconds, at 800g, in order to compact the clot. The expelled supernatant is then measured and expressed as a percentage of the total initial volume (Dhall *et al.*, 1976).

D.2.3 Network Fibrin content (FC)

The FC of the network is directly dependent on the amount of clottable fibrinogen in the plasma (Nair *et al.*, 1991a). The method of Ratnoff & Menzie (1951) is often used for FC determination. Plasma clots are made in glass tubes and left overnight for maximum polymerisation. In order to get rid of all excess unbound and other dissolved proteins, the clots are then washed with saline

and centrifuged (this process is repeated several times). Sodium hydroxide is then added and samples are incubated overnight (30 °C) to ensure maximum hydrolisation of fibrin. The following day, water and sodium carbonate are added just before adding Folin & Ciocalteu's reagent and absorbency is measured. The concentration is then determined from a standard curve. A pure fibrinogen solution is the best standard to use for the preparation of a standard curve. Other proteins such a DL-Tyrosine (Veldman, 1996) or albumin (used in this study) can also be used, although they might decrease the accuracy of the procedure since the amount and sequence of amino acid of these proteins differ from that of fibrin.

Nair *et al.* (1991) found significant correlations between FC with both network fibrin thickness (μ_T) and permeability (τ). This reinforces the concept that the structural integrity and network characteristics are strongly related to the FC of the networks.

D.3 Fibrin network structure and fibrinolysis

The fibrin network has a much more complicated role than just providing the scaffolding of the thrombus or being the target of fibrinolysis. Impaired fibrinolytic function secondary to raised plasma PAI-1_{act} levels is associated with abnormal fibrin gel structure (Fatah *et al.*, 1996). Abnormal FNS has been associated with hypofibrinolysis, which is detrimental for patients with CVD (Gabriel *et al.*, 1992). By constructing three-dimensional structures using confocal microscopy, Collet *et al.* (2000) were able to demonstrated, that this so-called thrombogenic fibrin, consisting of thin fibres organised in a tight three-dimensional network is thrombogenic because of its resistance to lysis that arises directly from its network architecture. Furthermore, it has been demonstrated that in peripheral vascular disease, diabetes, hypercholesterolaemia (Nair *et al.*, 1993; Nair *et al.*, 1991) and MI (Fatah *et al.*, 1992; Fatah *et al.*, 1996), fibrin networks are composed of thin fibres (tight, rigid and space-filling FNS with small pores) with increased tensile strength (thus thrombogenic). This network has significantly reduced permeability and is highly resistant to lysis, raising possibilities of re-infarction. Coagulation mechanisms are normally active at low levels even without tissue injury. Small elevations in coagulation factor activities and concentrations result in a hypercoagulable state associated with increased atherosclerosis and thrombosis. The study done on the plasmin-resistant tight fibrin structure in Dusart syndrome further emphasizes the potential significance of fibrin network morphology in thrombolysis (Collet *et al.*, 1993).

As mentioned before, APC inhibits fibrin formation by regulating thrombin generation. Gruber *et al.* (1994) found that FNS were altered by the anti-thrombotic plasma enzyme APC and that it may enhance the efficacy of thrombolysis by reducing the relative mass of fibrin within maturing thrombi. The addition of APC before clotting, but not after clotting, accelerated clot lysis. Rate of increase in the turbidity of clotting plasma was reduced by APC (reduction in turbidity indicated clot lysis, whereas increase in turbidity indicated fibrin assembly). Furthermore, APC reduced the diameter and relative number of fibrin fiber in plasma clots during gel assembly (less fibrinogen was converted and incorporated into the gel when plasma was clotted in the presence of APC and lower turbidity of the clots formed in the presence of APC corresponded to less fibrin, suggesting further that APC reduce the relative quantity of fibrin (fibrin mass to clot volume ratio) in clots) (Gruber *et al.*, 1994).

Fibrinolysis is likely to be related to the fiber size in the gel matrix. This is because coarse fibres are, in contrast with fine fibres, composed of a larger number of protofibrils. Enhanced local concentration of pro-fibrinolytic components in coarse fibres combined with decreased diffusion distances between the protofibrils (within a fiber) result in relatively faster lysis of these fibres. Increased fibrinogen concentration decreases porosity of gels in an exponential manner. Fiber numbers are increased but not fiber diameter. The combined effect of these events will decrease fibrinolytic potential. In addition, in the tightest clot structures diffusive flow may limit access of fibrinolytic components to the binding sites on the fiber (Blombäck, 1996).

Fibrin is not only the substrate of fibrinolytic enzymes, but it also plays an active part in the regulation of its own lysis. Fibrin is able to bind plaminogen activators and plasminogen. It has a stimulating effect on plasminogen activation and protects plasminogen activators and plasmin against inhibition by proteinase inhibitors (Collet *et al.*, 1993; Collet *et al.*, 2000; Nieuwenhuizen, 2001). Fibrin, but not fibrinogen, enhances the rate of activation of plasminogen by tPA. Fibrin polymerisation also exerts regulatory effects. These effects include: exposure of the rate enhancing sites, mutual positioning of the tPA and plaminogen binding sites and effects on the kinetic properties of tPA and plasminogen (Nieuwenhuizen, 2001). It, therefore, appears as though fibrin concentrates and correctly orientates tPA and plasminogen on its surface, inducing conformational changes that lead to higher catalytic efficiencies.

Although much is known about the molecular basis of fibrinolysis, the role of FNS in fibrinolysis still needs clarification. Several studies have shown that clots with long, thin fibres and a tight conformation have slower lysis rates than clots with coarse fibres and loose conformations (Collet *et al.*, 1993; Carr & Alving, 1995). This was usually attributed to the different fiber widths. Fibrin has been believed to be digested from the outside inwards, with degradation products released layer by layer (Gabriel *et al.*, 1992). Increased fiber thickness was associated with increased plasmin-mediated fibrinolysis (Carr & Alving, 1995; Gabriel *et al.*, 1992). This was explained by suggesting that thicker fibres have a larger surface area, therefore providing more binding sites for tPA, plasminogen and plasmin. It was also suggested that long thin fibres not only have less binding sites, but steric factors due to increased curvature of the fibres also prohibit binding of fibrinolytic components (Gabriel *et al.*, 1992).

This model was based on the concept that plasma fibrin degradation takes place in two sequential phases (Sakharov *et al.*, 1996). During the first phase (pre-lysis), plasminogen gradually accumulates onto the relative immobile fibrin of the clot. This is a consequence of the gradual increase in plaminogen binding sites as a result of fibrin nicking by plasmin. During the second phase (final lysis), fragmentation of the plasminogen-loaded fibrin takes place causing the network to become mobile, collapse and shortly after be dissolved completely.

According to Collet *et al.* (2000), fibrin is in fact not digested from the outside inwards. They found that although tight fibrin networks are dissolved at a slower rate than loose ones, the thin fibres themselves are cleaved at a faster rate than the thick fibres. They were able to construct three-dimensional structures using confocal microscopy and specially designed computer software. They were also able to collect images during ongoing fibrinolysis. These techniques allowed them to measure lysis not just with respect to fiber diameter (as was the case with precious studies), but also with respect to the network configuration. According to Collet *et al.* (2000), fibrin fibres are transected laterally across the fiber and not digested uniformly from the outside inward.

Collet *et al.* (2000) ascribes this apparent paradox of tight networks being cleaved more slowly while thin fibres themselves are being cleaved faster and *vice versa* to two reasons. Firstly, it is likely that fibrin configuration, rather than fiber diameter determines fibrinolysis speed. Therefore, although thin fibres are digested more rapidly, plasma clots with a tight network configuration display a significantly higher fibrin fiber density than loose clots, but with the same

amount of total protein. Secondly, although the mechanism is unknown, fibrin fiber retraction phenomena that occur in the prelysis zone of plasma clots are another potential explanation. Impaired retraction in plasma clots with a tight network conformation may contribute to hindered lysis.

It should also be considered that the thicker fibres may have a greater potential than the thin fibres for the local enhancement and acceleration of lysis (enzymes can move faster through the larger pores), while the time needed for plasmin to reach new fibres in tight networks is longer, even though the thin fibres are cleaved faster (Collet *et al.*, 1993; Collet *et al.*, 2000; Carr & Alving, 1995). Fibrin network architecture rather than fibrin fiber diameter regulates the distribution of fibrinolytic components during the course of fibrinolysis and may account for this apparent paradox (Collet *et al.*, 2000).

Although it is agreed in the literature that tight fibrin networks are digested more slowly than loose, coarse networks, controversy regarding the method behind the lysis of networks still seems to exist.

E. THE PROTECTIVE EFFECTS OF ANTIOXIDANTS

Free radicals are any species capable of an independent existence that contain one or more unpaired electrons. An antioxidant is any substance that when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substance. In healthy individuals, the generation of reactive oxygen species (ROS) should be in balance with antioxidant defenses. When imbalances occur this is referred to as oxidative stress. Oxidative stress can be caused by: depletion of antioxidant levels, e.g. malnutrition lowers antioxidant vitamin and glutathione levels, or increased ROS formation, e.g. caused by toxic chemicals and drugs and at sites of inflammation (as a result of phagocyte oxidative burst). In many cases free radicals are not the major cause of disease but rather a consequence and complicating component of the underlying disease pathology leading to lipid peroxidation as a consequence rather than a cause of cell injury (Wiseman, 1996).

Scientific evidence strongly suggests that oxidative modification of LDL-particles may play an important causative role in the development of atherosclerosis (Ross, 1999; Ridker, 1997a). This theory, known as the oxidative modification hypothesis (Odeh *et al.*, 1995), led to the development of many clinical and epidemiological trials. These trials served the purpose of examining the effects of various natural antioxidants (such as vitamin E, β-carotene, vitamin C, selenium, flavonoids) on this disease, and ultimately, on the clinical events resulting from atheroslerosis, such as CVD.

Vitamin C is a reductant (antioxidant) and minimises damage by oxidative processes. Furthermore, vitamin C, or L-ascorbic acid, is water soluble and is present in its deprontonated state under most physiologic conditions. It is considered to be the most important antioxidant in extra-cellular fluids and also has many roles as an intracellular antioxidant. As an antioxidant, vitamin C donates electrons in both intracellular and extra-cellular reactions. It is well suited for this role because its free radical intermediate, semidehydroascorbic acid, is non-reactive compared with other free radicals and thus chain reactions are prevented. Vitamin C has both enzymatic and chemical functions. It is a cofactor for several enzyme reactions involved in collagen, catecholamine, carnitine and amino acid metabolism, in which it acts as an electron donor. In many cases, vitamin C transfers single electrons to transitional metal such as copper (Harats *et al.*, 1998). Vitamin C (as chemical reductant) increases iron absorption in the gastrointestinal tract. As an antioxidant, vitamin C may reduce harmful oxidants and

nitrosamines in gastric juices, decreases LDL-oxidation, oxidative deoxyribonucleic acid (DNA) and/or protein damage, lipid peroxidation, as well as extra-cellular oxidants from neutrophils. Vitamin C can also improve endothelium-dependent vasodilation (Levine *et al.*, 1999). Chambers *et al.* (1999) showed an increase in flow-mediated dilation after methionine load with pre-treatment of vitamin C. This effect of vitamin C was attributed to its antioxidant property that scavenges superoxide anion-radical. Nappo *et al.* (1999), found similar results with antioxidant pretreatment (vitamin C and vitamin E), and also concluded that the adverse effects of homocysteine on vascular EC are mediated through oxidative stress mechanisms (Chambers *et al.*, 1999; Nappo *et al.*, 1999). The protective function of vitamin C, according to Padh (1991) is two fold: already oxidised groups in prosthetic centers of enzymes are reduced and oxidants and free radicals are removed.

It is important to note that there is no definitive data showing that vitamin C concentrations directly enhance biochemical or molecular function in human tissues, or that higher vitamin C concentrations confer benefit. Only indirect information is available regarding dose-function relationships (reviewed by Levine *et al.*, 1999). To aid in the following discussion, reference can be made to Table 1 that summarises studies that investigated the effects of vitamin C on haemostatic variables.

E.1 Vitamin C and cardiovascular disease

Many epidemiological studies have shown a significant association between vitamin C intake and protection against CVD (Gaziano, 1999; Greco *et al.*, 1982; Khaw & Woodhouse, 1995; Ness *et al.*, 1996; Odeh *et al*, 1995). In general, an increased intake of vitamin C through a diet rich in fruits and vegetables, was associated with a decreased risk and mortality from CVD. Moreover, Khaw & Woodhouse (1995) found that 60mg vitamin C/day was associated with a decrease in fibrinogen levels, which is equivalent to a 10 % reduction in IHD risk.

E.2 Vitamin C and haemostatic variables

The supplement used in this study (discussed in Chapter 2) was a mixture of vitamin C, flavonoids, magnesium and vitamin B. Most studies were conducted on vitamin C, a few on bio-flavonoids and very little on magnesium and vitamin B. No studies depicting the effects of vitamin C supplementation on FNS could be found. The focus of this discussion will, therefore, fall on the effects of vitamin C supplementation on haemostatic variables.

Vitamin C and flavonoids share a common function: both are antioxidants. Moreover, flavonoids inhibit lipidperoxidation, platelet aggregation, capillary permeability and fragility and activity of enzyme systems including cyclo-oxygenase and lipoxygenase. Flavonoids exert these effects as antioxidants, free-radical scavengers, and chelators of divalent cations (Aviram & Hayek, 1997; Cook & Samman, 1996; Da Silva *et al.*, 2000; Edwin, 1997; Fuhrman *et al.*, 2002; Prior & Cao, 2000; Yoshida *et al.*, 1999). Therefore, it could be further noted that the effects found with flavonoid supplementation, largely mirrors that of vitamin C supplementation with regard to its protective function in CVD (may also protect against atherosclerosis and thrombotic tendencies).

Table 1 gives a summary of studies that investigated the physiological effects of vitamin C on haemostatic variables.

It is of interest to note that most of the studies investigated the effect of antioxidants on platelet- and endothelium function, few looked at the effect on the other haemostatic variables and none could be found that were conducted on FNS. Hereafter follows a discussion on some of the studies done on the above mentioned fields of interest.

E.3 Vitamin C and platelet function

Supplementation with vitamin C of 3 g/day, compared to placebo, showed a reduction in platelet aggregation and platelet adhesiveness (Bordia & Verma, 1985). Bordia (1980) also found a reduction in platelet adhesion and aggregation with supplementation of 2g vitamin C/day, compared to placebo. However, Tofler *et al.* (2000) found no effect of vitamin C (2g/day) supplementation on aggregation or adhesion of platelets, compared to placebo. Crawford *et al.* (1975) found no effect of 1g vitamin C/day on platelet adhesiveness compared to placebo. Calzada *et al.* (1997) could also not find an effect on platelet function, but did, however, note that there was a trend towards reduction in platelet aggregation and increased sensitivity to inhibitor PGE-1 after supplementation with 250mg vitamin C/day compared to placebo. No decrease in ADP-induced platelet activity or blood viscosity was found with vitamin C and vitamin E intake after a methionine load (Nappo, *et* al., 1999). Antioxidant supplementation of men with low antioxidant status and high fat intake reduced lipidperoxidation, the capacity of platelets to aggregate and to produce TXA_2, and *in vivo* platelet activation compared to placebo (Salonen *et al.*, 1991).

E.4 Vitamin C and endothelium function

Hyperlipidaemic states (hypercholesterolaemia) exhibit systemic signs of an inflammatory response, including leukocytosis, lymphocytosis, activation of the complement and kinin system and elevation of acute phase reactants, such as fibrinogen. One distinct characteristic of altered vasomotor regulation in the presence of hypercholesterolaemia is an impairment of endothelium-dependent vasodilation, which limits vasodilation evoked by increases in arterial flow (Henry *et al.*, 1995).

Studies on the effects of antioxidant therapy on endothelial function have been inconsistent. Hornig *et al.* (1997) studied the effects of vitamin C on conduit artery endothelial function in patients with chronic heart failure. He observed a significant improvement in radial artery flow-mediated dilatation in a subset of 5 patients examined after 4 weeks of high dose oral vitamin C supplementation (2g/day), compared to placebo. Recently, Gocke *et al.* (1999) studied the effects of short (2 hours, 2g) and long-term (30 days, 500mg/day) oral vitamin C therapy on endothelial function in patients with established CAD and observed an improvement in endothelial function (compared to placebo). Contrary to a study done by Raitakari *et al.* (2000), Hornig *et al.* (1997) and Gocke *et al.* (1999) found a sustained beneficial effect on endothelial function after 1 month of therapy with 500mg ascorbic acid/day compared to placebo. Raitakari *et al.* (2000) could only provide an improvement in endothelium function in the short-term in healthy young smokers (single 2g dose of vitamin C), but failed to find a beneficial effect of oral vitamin C therapy in the long-term (1g vitamin C/day for 8 weeks). Hyperhomocysteinemia is a major independent risk factor for CVD and venous thrombosis, associated with an acute impairment of vascular endothelial function. According to Chamber *et al.* (1998), this effect, after a methionine load, can be prevented with pretreatment with vitamin C (1g/day for 1 week) in healthy subjects, showing an increase in flow-mediated dilatation as compared to placebo. This improvement in endothelial function, after methionine load, was confirmed by Nappo *et al.* (1999), with pretreatment of vitamin C and vitamin E in healthy subjects.

E.5 Other haemostatic variables

Coagulation factors (fibrinogen, FVII, FVIII, FXI), coagulation inhibitor activity (ATIII, PC, PS), and coagulation activation markers (prothrombin fragment 1 + 2 and TAT) were measured in the Third MONICA Survey (Woodward *et al.*, 1997). Serum vitamin C was inversely associated with coagulation factors. Coagulation activation markers were also inversely related with serum vitamin C, possibly because of a more consistent relation of vitamin C to clotting factors than

clotting inhibitors. This finding further suggests that coagulation activation may be one mechanism through which low vitamin C levels are associated with CVD (Woodward *et al.*, 1997). Because of the powerful antioxidant properties exerted by vitamin C it may be beneficial in limiting excessive oxidative tissue damage caused by phagocytes during infection with subsequent attenuation of the acute phase response. This is consistent with findings that show a strong negative association of PAI-1_{act} with serum vitamin C concentrations (Woodhouse *et al.*, 1997), which mirrors similar associations of ascorbate with plasma fibrinogen and FVII (Khaw & Woodhouse, 1995). Low vitamin C levels could, therefore, contribute to a pro-thrombotic state because it allows greater activation during respiratory infections (high plasma PAI-1 and fibrinogen levels) (Woodhouse *et al.*, 1997; Khaw & Woodhouse, 1995). Nilsson *et al.* (1991) also showed that a frequent intake of food rich in vitamin C (fruits, vegetables, and root vegetables), is associated with lower PAI-1_{act} levels. This is consistent with increased activity of the fibrinolytic system, and, therefore, a reduced risk for thromboembolic and CVD in subjects who exhibit this food intake pattern. Photo-dynamic treatment generates singlet oxygen which causes inactivation of fibrinogen and FVIII. Parkkinen *et al.* (1996) studied this photo-dynamic treatment of FVIII and fibrinogen in fresh frozen plasma and found that ascorbate is a primary antioxidant against photo-oxidation in plasma and that it effectively prevents oxidative damage to coagulation factors and other proteins.

However, Harats *et al.* (1998) (50mg vitamin C/day, compared to control groups (controlled feeding)) and Tofler *et al.* (2000) (2g vitamin C/day) could not demonstrate an effect of vitamin C on the haemostatic factors, fibrinogen and $FVII_{ag}$, compared to placebo. Tofler *et al.* (2000) did, however, find a decrease in tPA_{ag} levels with increased vitamin C intake, compared to placebo. Nappo *et al.* (1999) also found a decrease in PAI-1_{ag}, tPA_{ag} as well as prothrombin fragment 1 + 2. Neither Crawford *et al.* (1975) nor Rifici *et al.* (1997) could confirm these findings.

Table 1 Summary of studies investigating the effect of vitamin C on haemostatic variables

REFERNCE	STUDY DESIGN	SUBJECTS	SUPPLEMENT	FISIOLOGICAL EFFECTS
BORDIA & VERMA, 1985	Study was done in two parts: **Part I** Controlled post-prandial study, no randomisation. Fasting blood samples collected in morning and again 4 hours after a fatty breakfast. It was repeated after 2 days but with 1g of vitamin C added. **Part II** Randomised, placebo controlled intervention study for10 days. Blood samples collected in the beginning, on the 5th and 10th day.	**Part I** 10 healthy males 30 – 55 years non-smokers. **Part II** 20 CAD patients with hyperlipidaemia.	**Part I** Control group: Fatty breakfast. Experimental group: breakfast + 1g vitamin C. **Part II** Experimental group: 1g vitamin C every 8 hours for 10 days (3g/day; n=10).	**Part I** Vitamin C prevented an increase in platelet adhesiveness and platelet aggregation that occurred after the fatty meal (↓ platelet adhesiveness). **Part II** ↓ in platelet adhesiveness and platelet aggregation. ↑ in serum vitamin C levels.
BORDIA, 1980	Controlled double blind, randomised, 2 part intervention study.	**Part I** 40 males with CAD. **Part II** 40 patients with	**Part I** *Group I* (no Vitamin C) (n=10). *Group II* = 1g vitamin C/day, divided in 2 doses	**Part I** Group I: no effect. Group II: • ↑ serum ascorbic acid by 22 % • ↔ In fibrinolytic activity • ↔ in blood lipids

	Part I Samples were collected, initially and every 2 months (for 6 months) and finally 2 months after stopping vitamin C. **Part II** Supplementation for 20 days. Blood samples collected every 10th day during 40 day follow up.	acute MI. All patients had low normal ascorbic acid levels at the beginning. Effect of vitamin C observed must be considered a pharmacological action rather than nutritional supplement.	(n=10). *Group III* = 2g vitamin C/day, divided in 2 doses (n=20). **Part II** 2g vitamin C/day (2 doses) (n=20). Placebo (n=20).	• ↔ platelet adhesive index. Group III (2g vitamin C/day) • ↑ ascorbic acid by 96 % • ↑ fibrinolytic activity by 45 % • ↓ in platelet adhesive index by 27 % • ↓ serum cholesterol by 12 % • ↓ in serum β-lipoproteins • ↑ in α-fraction. **Part II** • 2g Vitamin C/day ↑ serum ascorbic acid by 96 % • ↑ fibrinolytic activity by 62.5 %.
CALZADA *et al.* 1997	Double blind, randomised, placebo controlled study for 8 weeks.	40 healthy volunteers: 24 men, 16 women 20 –50 years.	Supplements: Vitamin E (300mg/day) (n=10), vitamin C (250mg/day) (n=8), or β-carotene (15mg/day) (n=9) or placebo with breakfast.	Supplementation with vitamin C ↔ platelet function although trends towards ↓ platelet aggregation and an ↑ sensitivity to the inhibitor PGE-1.
CHAMBERS *et al.*, 1999	Randomised placebo controlled intervention study, performed on 3 separate days, at least 2 weeks apart.	17 healthy hospital staff (10 male, 7 female) 21-59 years.	2 hours and 4 hours after fasting (1) oral methionine (L-methionine 100mg/kg), (2) oral methionine preceded by vitamin C (1g/day,	Vitamin C after methionine load ↑ flow-mediated dilatation, thus endothelial function ↔ on homocysteine concentrations after methionine. Adverse effects of homocysteine on vascular EC are mediated through oxidative stress mechanism.

			for 1 week), and (3) placebo on separate days and in random order.	
CRAWFORD *et al.* 1975	Randomised, placebo controlled human intervention trial for 3 months.	36 adults (18 men, 18 women) • < 25 years • All in good health • Received no drug therapy (medical and technical staff and medical students). 12 of each gender received vitamin C and 6 acted as controls. All 18 men completed the study, 2 women (each of control and vitamin C groups withdrew (inter-current illness).	1g/day vitamin C (n=24) placebo (n=12).	↔ serum cholesterol, tPA_{act}, plasminogen, fibrinogen, partial thromboplastin time, platelet adhesiveness, α_1-antitrypsin or α_2- macroglobin.
GOKCE *et al.* 1999	Double blind, randomised, placebo controlled study. Flow mediated dilation of the branchial artery	55 subjects with CAD. 8 excluded before data were unblinded (poor image control). 1 excluded due to	Single dose ascorbic acid :2g. Then continued treatment with long-term ascorbic acid 500mg/day	Both single dose (2g) and long-term treatment (500mg/d) with ascorbic acid improved flow mediated dilation, thus improved endothelial function. Improvement was accompanied by: ↑ in plasma ascorbic acid concentration, ↔ blood pressure, lipoprotein profile or systemic markers of oxidative damage.

	was examined by high resolution vascular ultra-sound at baseline, 2 hours after a single dose of vitamin C, and 30 days after long-term treatment.	medical reasons. 46 remained • 4 menopausal women in ascorbic acid group, none in placebo; • Trends to-wards lower fasting glucose concentration in placebo group; • no use of vitamin C or E supplements.		
HARATS *et al.*, 1998	Randomised parallel 2 month intervention study, run-in period of 1 month.	36 healthy male students.	50mg vitamin C/day in low vitamin C diet during run-in period and controls (n=17). 500mg vitamin C/day in high vitamin C diet group (n=19) Diets were provided – controlled feeding.	↑ LDL-C during ↑ vitamin C diet (high vitamin C diet) ↔ TC, HDL-C ↔ Fibrinogen, FVII.
HORNIG *et al.*, 1997	Randomised, placebo controlled 4 week study. High-resolution	15 patients with coronary heart failure and 8 healthy volunteers.	Patients were randomised (ratio 2:1) to receive: intra-arterial infusion of	Vitamin C improves radial artery flow-dependant dilation in a subset of 5 patients with coronary heart failure, after 4 weeks. Thus improves endothelial function.

	ultrasound and Doppler was used to measure radial artery diameter and blood flow.		25mg/minute over 10 minute or placebo, and oral therapy with 2mg vitamin C/day for 4 weeks (n=5) or placebo (n=5).	
KHAW & WOODHOUSE, 1995	Longitudinal study of individuals seen at intervals of 2 months over one year.	47 men and 49 women 65-74 years.	Subjects were visited at intervals of 2 months for 1 year (winter to summer).	Serum ascorbate concentration : **inversely related** to fibrinogen, FVII, CRP, α_1-anti-chymotrypsin. ↑ in dietary vitamin C of 60mg/day was associated with : ↓ fibrinogen concentrations, equivalent to a 10 % ↓ in risk of IHD.
LEVINE *et al.* 1996	Placebo controlled, blind post-prandial study. Brachial artery endothelium-dependent dilation in response to hyperemia was assessed by high-resolution vascular ultra-sound before and 2 hours after oral administration of either 2g ascorbic acid or placebo. Endothelium-dependent, flow-mediated dilation of the brachial artery was	46 patients with documented CAD. Vasoactive medications were withheld for at least 12 hours before study. All long acting vasoactive indications were withheld for at least 24 hours.	2g Vitamin C.	Plasma ascorbic acid concentration ↑ 2.5 fold 2 hours after treatment. In the prospectively defined group of patients with an abnormal baseline response (< 5 % dilation), ascorbic acid ↑ dilation, placebo had no effect. Ascorbic acid ↔ on hyperemic flow, and ↔ on arterial dilation to sublingual nitroglycerin.

	determined from 2-dimensional ultrasound images.			
NAPPO *et al.*, 1999	Observer blinded, randomised crossover intervention study.	20 healthy hospital staff (10 women, 10 men) 25-45 years.	Subjects were given each of 3 loads in random order at 1 week interval: Oral methionine, 100mg/kg in fruit juice; The same methionine load immediately following ingestion of antioxidant vitamin E, 800 IU and ascorbate acid, 1000mg; and methionine-free fruit juice (placebo) 10 of the 20 subjects also ingested a placebo load with vitamins.	Vitamin C, vitamin E after methionine load: ↓ d-dimer ↓ $PAI-_{ag}$ ↓ tPA_{ag} ↓ prothrombin fragment 1 + 2 ↓ sVCAM-1 ↓ sICAM-1 ↓ FPA ↔ ADP induced platelet aggregation ↔ Blood viscosity ↑ Endothelium function. Thus: Impairment of endothelial function by acute homocysteinemia, reversed by antioxidant vitamins (vitamin C, vitamin E). Adverse effects of homocysteine on vascular EC are mediated through oxidative stress mechanism.
NILSSON *et al.*, 1990	Population-based cross-sectional study (Swedish domestic fare). Assessment of the relation between food intake habits and factors of the	260 subjects 30-60 years.	Food frequency questionnaire. Focused on questions concerning intake of fiber and vitamin C-rich items in relation to	**High consumers** showed **lowest** PAI-1levels, higher activity of the fibrinolytic system. **Low consumers** showed **highest** PAI-1 levels. **Medium consumers** showed **intermediate** PAI-1 values. tPA levels did not differ between the groups.

	fibrinolytic system were conducted. Subjects were grouped as high, low or medium consumers of fruits and rootvegetables.		fibrinolytic system.	
PARKKINEN *et al.* 1996	Studied the effect of photo-oxidation on the activity of coagulation factors in fresh frozen plasma. In vitro study. Blood was collected from normal blood donors.	Blood was collected by normal blood donors.		There was an inverse correlation between the extent of coagulation factor inactivation during photo-dynamic treatment and the plasma ascorbate concentration. The coagulation factor activities were protected from inactivation in a dose-dependend manner when exogenous ascorbate was added to the plasma before photo-oxidation. ↓ Fibrinogen and FVIII.
RIFICI *et al.* 1997	Randomised, double blind, placebo controlled crossover study, with a 4 week washout period. Subjects refrained from exercise 24 hours and fasted for 12 hours before blood samples were obtained.	15 males with central obesity (hip to waist ratio 0.8) • mean age: 48.6±3.5years • BMI: 31.8±0.7 kg/m^2 • No altered plasma lipoprotein metabolism or haemostasis. • Did not take any vitamin supplements	Combination of 250mg vitamin C, 200 IU vitamin E and 6mg β-carotene, 4 time/day or placebo.	↓ in production of thiobarbituric acid reactive substances VLDL + LDL protein. ↑ in lag phase of conjugated diene formation. ↑ in reactivity of lysine residues demonstrating a ↓ in the susceptibility of lipoproteins to oxidation. ↔ on plasma PAI-1_{act}, PAI_{ag}, tPA_{act}, tPA_{ag}, fibrinogen and fibrin degradation products.

		for 8 weeks prior to the start of study.		
RAITAKARI *et al.*, 2000	Randomised, placebo controlled, double-blind crossover study.	20 healthy smokers (6 – 40 pack-years) 18-50 years.	Each subject was studied at baseline, 2 hours after a single dose of 2g vitamin C, and 8 weeks after taking 1g vitamin C/day and after placebo.	• Oral vitamin C therapy improves endothelial function in the short term in healthy young smokers: after single 2g dose of vitamin C flow-mediated dilation improved. • Oral vitamin C therapy had no long-term beneficial effects: after 8 weeks of 1g vitamin C/day, flow-mediated dilation did not significantly improve. • ↔ serum TC, HDL-C, TG.
SALONEN *et al.*, 1991	Randomised, pair-matched, placebo controlled, double blind study for 5 months.	78 men with low antioxidant status: 54 non-smokers 26 smokers paired into these strata.	3 capsules/day: Antioxidant capsule (n=39) – 200mg ascorbic acid, 100mg α-tocopherol, 9mg β-carotene, and 25µg selenium in selenium enriched yeast. Placebo (n=39).	20 % ↓ in serum lipid peroxidase 24 % ↓ in ADP-induced platelet aggregation 42 % ↓ in the rate op ATP release during aggregation 51 % ↓ in serum (platelet-produced) TXA_2 29 % ↓ in plasma β-thromboglobulin concentrations.
TOFLER *et al.* 2000	Randomised, placebo controlled, crossover study for 16 weeks (6 weeks intervention, 4 weeks washout, and 6 weeks intervention)	18 healthy non-smoking male volunteers (aged 30 to 65 years). No aspirin or other medication during the prior 3 weeks.	2g vitamin C supplementation/ day or placebo.	Vitamin C compared to placebo: ↑ plasma vitamin C ↓ TC, HDL-C, LDL-C ↔ TC/HDL ratio, TG ↔ platelet adhesion and platelet aggregation ↔ fibrinogen, FVII, PAI-1, and viscosity ↑ vWF tPA_{ag} levels were inversely related to plasma vitamin C levels, thus ↓ risk.

WOODHOUSE *et al.* 1997	Prospective study. Each subject was visited at home 7 times over a 14-month period.	47 men and 49 women aged 65 – 74 years.	-	Multiple regression analysis found an inverse relationship between PAI-1_{act} and serum ascorbate. Persisted even when vitamin C supplement takers and or smokers were excluded from the analysis. Serum ascorbate was strongly related to estimated dietary intake of vitamin C.

ADP: adenosine 5'-diphosfate sodium salt; **ATP**: adenosine 5'-triphosphate disodium salt; **CAD**: Coronary artery disease; **MI**: Myocardial infarction; **IHD**: Ishaemic heart disease; **TXB_2**: Thromboxane B_2; **CRP**: C reactive protein; **tPA_{act}**: Tissue plasminogen activator activity; **tPA_{ag}**: Tissue plasminogen activator antigen; **FVII**: Factor VII; **FVIII**: Factor VIII; **VLDL**: Very low density lipoprotein; **LDL-C**: Low density lipoprotein cholesterol; **PAI-1**: Plasminogen activator inhibitor 1; **PAI-1_{act}**: Plasminogen activator inhibitor 1 activity; **PAI-1_{ag}**: Plasminogen activator inhibitor 1 antigen; **FPA**: fibrinopeptide A; **TC**: Total cholesterol; **HDL-C**: High density lipoprotein cholesterol; **TG**: Triglyceride; **vWF**: von Willebrand factor; **sVCAM-1**: soluble vascular cell adhesion molecule 1; **sICAM-1**: soluble intercellular adhesion molecule 1; ↑: increase; ↓: decrease; ↔: no effect

F. RECOMMENDATIONS FOR VITAMIN C INTAKE

Vitamin C has become popular because of its role as an antioxidant, which potentially offers protection from some diseases as mentioned previously. Furthermore, because vitamin C can't be stored in the body, it is important to replenish it by taking the recommended amount of vitamin C each day (Levine *et al.*, 1999).

Revised recommendations for vitamin C intake are based on new data, new criteria for devising recommendations, new recommendations and new guidelines termed Dietary References Intakes (DRI) Levine *et al.* (1999). The DRI for vitamin C released in April 2000 by the Food and Nutrition Board, United States, National Academy of Sciences for men was determined as 90mg per day and 75mg per day for females (in both sexes these values increased from 60mg/day) (Monsen, 2001). But the DRI for women was based on data for men. An estimated average requirement (EAR) of 75mg daily was determined from depletion-repletion data. Because similar data were not available for women, an EAR had to be extrapolated on the basis of body weight differences between sexes and hence a DRI of 75mg daily was derived. However, recently Levine *et al.* (2001) presented data that can be used to calculate a new DRI specifically for young women. By using an inpatient depletion-repletion design, vitamin C in healthy young women subjects was measured at steady state for each of seven doses (30, 60, 100, 200, 400, 1 000, and 2 500mg) in plasma, circulating cells and urine for the entire hospitalization. By using Food and Nutrition Board guidelines, Levine *et al.* (2001) indicate that the DRI for young women should be increased to 90mg daily. Furthermore, the data from their study describes for the first time the relationship between vitamin C doses and steady-state concentrations in healthy young women. Their findings also indicate that daily vitamin C intake of 100 to 200mg produces near saturation of plasma and tissues.

The tolerable upper intake levels (UL) is the highest daily level of nutrient intake that does not pose risk or adverse health effects to almost all individuals in the population (Levine *et al.*, 1999). The UL-level of vitamin C intake for men and women is proposed to be less than 1g (1 000mg) daily (Levine, *et al.*, 1999; Monsen, 2001). Taking too much vitamin C can cause side effects such as nausea, stomach cramps, diarrhea, possibly kidney stones and possibly retention of iron stores (Levine *et al.*, 1999).

CHAPTER 2

FOODSTATE VITAMIN C COMPLEX® MAY BENEFICIALLY AFFECT HAEMOSTASIS AND FIBRIN NETWORK STRUCTURES IN HYPERLIPIDAEMIC PATIENTS

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Number of tables: 5 Tables included in this manuscript

Table 1: Baseline characteristics of subjects (n=25)

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Table 3: Median [25, 75 percentiles] plasma haemostatic factors during the study

Table 4: Median [25, 75 percentiles] fibrin network structures during the study

Table 5: Mean [95 % CI] serum lipids and lipoprotein (a) levels during the study

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ABSTRACT

This randomised, placebo controlled, double blind, crossover study on 25 free-living hyperlipidaemic volunteers aspired to prove the hypothesis that supplementation for 8 weeks with a FoodState Vitamin C complex® (500mg vitamin C, 160mg bioflavonoids, 600mg magnesium and 900mg vitamin B complex) may improve haemostatic factors and fibrin network structures (FNS). Of the haemostatic factors measured, only median plasmin-antiplasmin complex (PAP) (marker of plasmin generation – initiate fibrinolysis) and thrombin-antithrombin complex (TAT) (marker of thrombin generation – initiate coagulation) levels were both significantly decreased with FoodState Vitamin C complex® compared to placebo (PAP: -4.05[-23.39, -0.23]% *vs* 1.81 [-8.95, 8.09]%; TAT: -5.81[-18.47, 0.39]% *vs* 0.12[-8.03, 13.5]%). As for FNS, only compaction was significantly increased (49.95[47.55, 53.70]% to 51.85[48.55, 56.65]%) by FoodState Vitamin C complex® supplementation. No significant changes were found in plasma fibrinogen, plasminogen activator inhibitor 1 activity, tissue plaminogen activator antigen, d-dimer, serum lipids or lipoprotein (a) levels. In conclusion, FoodState Vitamin C complex® may in part be protective of cardiovascular disease by causing a new reduced steady state of haemostatic balance (TAT and PAP were significantly decreased), and less rigid clots (increased compaction).

Keywords: Haemostasis; TAT; PAP; Fibrin network structure; Compaction; Vitamin C.

INTRODUCTION

Evidence has accumulated over the past few years that factors involved in haemostasis are directly implicated both in the evolution of coronary atherosclerosis and in promoting thrombotic occlusion of arteries with atherosclerotic lesions, as well as stroke and cardiovascular disease (CVD). It is hypothesised that part of this association may be mediated by the quality of the fibrin network structures (FNS) formed when fibrin monomers polymerise, since the fibrin network is the end-product of coagulation and also the target of fibrinolytic enzymes **[1]**. A change in any of the constituents of plasma could influence the characteristics of the fibrin network *via* effects on metabolism, possible direct steric effects or altered fibrinogen conversion. This may cause the clot to be tight, rigid, and more atherogenic, or loose elastic and more easily lysable. Atherogenic, tight, and rigid fibrin clots have been associated with CVD risk factors such as increased fibrinogen and serum lipoproteins (low density lipoprotein (LDL) and very low

density lipoprotein) **[2;3].** These fibrin clots are not lysed easily and may, therefore, increase the clotting potential, raising the possibility of re-infarction **[4].** Furthermore, the type of fibrin deposited may influence the outcome **[5]**. Thick fibrin fibers may influence initial disorganisation of endothelium cells, promotion of a more pro-fibrinolytic/anti-thrombotic environment, fibrinolysis and finally the endothelium cells are re-organised. In contrast, thin fibrin fibers promote a more anti-fibrinolytic/pro-thrombotic environment and may induce a further exposure of sub-endothelium, resulting in increased platelet adhesion, migration of monocytes and growth of atherosclerotic plaques. Therefore, the formation of more porous, less rigid, and more lysable clots (with thick fibrin fibers) may have a protective effect on the risk for CVD as these FNS creates a pro-fibrinolytic/anti-thrombotic environment **[4;5;6].**

It has become clear that the relationship between coagulation and inflammation is not unidirectional, but instead, an interaction between the systems occurs by which activation of coagulation will also affect inflammatory activity. In particular, vascular endothelium cells seem to play a pivotal mediatory role in the coagulation response to systemic inflammation and the interaction between them **[7;8].** Hyperlipidaemic states exhibit systemic signs of an inflammatory response, including leukocytosis, lymphocytosis, activation of the complement and kinin system, and elevation in acute phase reactants, such as fibrinogen **[9].** Furthermore, hypercholesterolaemia is associated with atherosclerosis, a process that is looked upon as a chronic inflammation in the vessel wall, accompanied by endothelial dysfunction **[10].**

Ascorbate is a primary antioxidant against photo-oxidation in plasma and it effectively prevents oxidative damage to coagulation factors, endothelium cells and other proteins **[11]** and has been implicated in the protection against CVD.

Several observational studies have shown an inverse relationship between dietary vitamin C or serum vitamin C levels and CVD **[12]**. In general, an increased intake of vitamin C through a diet rich in fruits and vegetables were associated with a decreased risk and mortality for CVD. This relationship between vitamin C intake and CVD may, in part, be mediated through effects on serum lipid levels **[13;14]**, oxidation of LDL **[15]** and coagulation factors **[16;17]**. Scientific evidence strongly suggests that oxidative modification of the LDL particle may play an important causative role in the development of atherosclerosis **[18;19]**. This theory, known as the oxidative modification hypothesis **[20]** has led to the development of many clinical and epidemiologic trials to examine the effects of various natural antioxidants (vitamin E, β-carotene, vitamin C,

selenium, flavonoids) on this disease and ultimately, on the clinical event resulting from atheroslerosis, such as CVD. Because of the powerful antioxidant properties of vitamin C it may be beneficial in limiting excessive oxidative tissue damage caused by phagocytes during infection, with subsequent attenuation of the acute phase response **[20]**. **Parkkinen et al. [11]** found that vitamin C is a primary antioxidant that effectively prevents oxidative damage to coagulation factors and other proteins. In the Third Glasgow MONICA Survey **[13]**, serum vitamin C showed a significant negative correlation with thrombin-antithrombin complex (TAT – a marker of thrombin generation). Furthermore, **Khaw & Woodhouse [16]** found that 60mg vitamin C/day were associated with decreased fibrinogen levels, which are equivalent to a 10 % reduction in ischaemic heart disease risk. Vitamin C also showed a strong negative association with factor VII antigen ($FVII_{ag}$) **[16]** and plasminogen activator inhibitor 1 activity ($PAI\text{-}1_{act}$) **[17]**. **Nilsson et al. [21]** also showed that a frequent intake of food sources rich in vitamin C (fruits, vegetables, and root vegetables) is associated with lower $PAI\text{-}1_{act}$ levels. This may indicate increased activity of the fibrinolytic system, and possibly a reduced risk for thromboembolic events and CVD in subjects who exhibit this food intake pattern. **Harats et al. [22]** (50mg vitamin C/day) and **Tofler et al. [23]** (2g vitamin C/day) could, however, not show an effect on plasma fibrinogen and $FVII_{ag}$ levels with intake of vitamin C, compared to a control diet or placebo respectively. Although **Tofler et al. [23]** found a decrease in tissue type plasminogen activator antigen (tPA_{ag}) levels with increased vitamin C intake, neither **Crawford et al. [24] nor Rafici et al. [25]** could confirm this finding.

The effects of vitamin C on FNS has, however, not been investigated before. Due to the anti-thrombotic effects exerted by vitamin C, it could be hypothesised that the FNS might also be positively affected.

The main aim of this study was to investigate the effects of FoodState Vitamin C complex® supplementation on haemostatic factors and FNS in hyperlipidaemic adults in a randomised, placebo controlled, double blind, crossover study. In addition to these, the effects on serum lipids and lipoprotein (a) (Lp(a)) were also investigated.

METHODS

1. Subjects

The study was approved by the Ethics Committee of the Potchefstroom University for Christian Higher Education (CHE) (**Ethics no**. HHK3M3-92). Informed consent forms were signed by 30 hiperlipidaemic patients who regularly attended the Lipid Clinic, Potchefstroom University for (CHE), and who voluntarily participated in this study. The results of 25 subjects were statistically analysed (dropouts discussed later). The baseline characteristics of the subjects are summarised in Table 1.

Table 1

Baseline characteristics of subjects (n=25)

Characteristics	Mean±SD
Men/women	15/10
Age	49.2±9.96 (23-46 years)
BMI (kg/m²)	23.9±3.27
Diastolic BP (mmHg)	79±8.6
Systolic BP (mmHg)	125±14
Serum Lp(a) (mg/dL)	60.59±35.03
Serum TC (mmol/L)	6.97±1.25
Serum TG (mmol/L)	1.10±0.46
Lipid lowering medication	2 patients (statins)
Haematocrit (%)	45.2±2.93
Haemoglobin (mmol/L)	8.9±1.63

SD: Standard deviation; BMI: Body mass index; BP: Blood pressure; Lp(a): Lipoprotein (a); TC: Total cholesterol; TG: Triglyceride.

The inclusion criteria for selection of subjects were: adult men or women, baseline serum Lp(a) > 30mg/dL, baseline serum total cholesterol (TC) > 5.0mmol/L, baseline serum triglyceride (TG) < 4mmol/L, no diabetic patients, no familial hypercholesterolaemic patients. Two of the subjects were using statins chronically. They continued with the medication for the duration of the study without changing the prescription.

2. Study design

This study was done under free living conditions using a randomised, placebo controlled, double blind, crossover study design. The subjects were randomly assigned to one of 2 groups, (group A or group B) receiving either the FoodState Vitamin C complex® supplement or the placebo. For the duration of the study the subjects were asked to continue with their normal daily routine (alcohol consumption, physical activity and diet) and to exclude vitamin supplements. The identity of the treatments was made known after the statistical analysis of the data.

After a run-in period of 4 weeks, during which subjects refrained from using any vitamin supplements, group A received treatment A, while group B received treatment B for 8 weeks. After a wash-out period of 8 weeks, during which the subjects received no treatment, the experimental intervention was crossed-over, and continued for another 8 weeks.

Fasting blood samples were drawn and anthropometric measurements (weight and height), as well as blood pressure and temperature were taken at the beginning and end of each experimental period. Body mass index (BMI) (kg/m^2) was calculated. Because of the very large intra- and interindividual variations reported with Lp(a), double blood samples (drawn one week apart) were collected at the beginning and end of each experimental period (that is 8 blood samples). The means of the two samples were calculated.

Adherence was determined by pill counting.

3. Supplement

Sportron International supplied the supplement and placebo. The subjects received 2 tablets per day, one in the morning and one in the evening of either the FoodState Vitamin C complex® or the placebo. Two tablets of FoodState Vitamin C complex® provided 500mg vitamin C, 160mg bioflavonoids, 600mg magnesium food complex and 900mg vitamin B complex. Two

tablets of placebo provided 883mg sorbitol, 9mg magnesium stearate, 4.5mg aerosil and 66.7mg cacao.

4. Dietary intakes

Dietary intakes were estimated with a validated food frequency questionnaire at the beginning and end of each experimental period. The computer program Dietary Manager (Scharf, 1999 Program Management, Johannesburg, South Africa), which is based on the American food composition tables with added South African foods, was used to analyse nutrient content.

5. Blood samples

Fasting (12 hours) venous blood samples were drawn by a qualified nursing sister using a 21-gauge scalp infusion set. To avoid the effects of diurnal variation, samples were drawn with minimal stasis between 07:00 and 10:00h. For the lipid analysis, ±10ml blood was drawn and left to clot. For the determination of coagulation factors, 10ml citrated blood was drawn. The clotted blood and citrated blood were centrifuged for 15 minutes at 2000g to yield serum and plasma respectively. EDTA blood was used for the analysis of haematocrit and haemoglobin. For the determination of FNS, another 50ml of citrated blood (3.8 % citrate) with added Trasylol® (35µl/10ml blood) were drawn. This citrated blood was centrifuged twice for 15 minutes at 3660g to yield platelet free plasma. Plasma was divided for analysis of compaction, fibrin content and mass-length-ratio. Aliquots of serum and plasma were stored at –82 °C until the analysis was done at the end of the study.

6. Experimental methods

Plasma fibrinogen was measured with the Clauss method using the ACL-200 automated coagulation analyser and reagents from Instrumentation Laboratories (IL) (Milan, Italy) (Coefficient of variance of laboratory analysis (CV) = 5.11 %). Plasma PAI-1_{act} was measured with an indirect enzymatic method (Biopool, Umeå, Sweden, Cat. no. 101201) (CV = 2.54 %). Plasma TAT, plasmin-antiplasmin complex (PAP) and d-dimer were determined with ELISA methods (Behring, Cat. no's. OWMG15, OQBM11 and OQBC11, respectively) (TAT: intra CV = 2.8 %, inter CV = 4.4 %; PAP: intra CV = 5.6 %, inter CV = 7.4 % and d-dimer: intra CV = 14.2 %, inter CV = 16.6 %). Plasma tPA_{ag} was measured with an ELISA method (Biopool, Umeå, Sweden, Cat. no. 101005) (intra CV = 5.3 %, inter CV = 5.6 %).

Fibrin network compaction was measured in pentad using the method described by **Nair et al. [6], Nair & Shats [26]** as well as **Dhal et al. [27]** (CV = 2.06 %).
The method of **Ratnoff & Menzie [28]** was used for duplicate determination of all network fibrin content (CV = 8.74 %).
The mass length ratio (μ_T) from turbidity was determined in pentad using the turbidimetric method described by **Nair et al. [6]** (CV = 2.48 %).

Serum total cholesterol (TC) and TG concentrations were determined with enzymatic methods (Randox, Antrim, UK, Cat. no's. CH200 and TR210, respectively) (CV = 7.3 % and 2.5 %, respectively). Serum high density lipoprotein cholesterol (HDL-C) was determined with a precipitation method (Randox, Antrim, UK, Cat. no. CH204) followed by the cholesterol method described above (CV = 2.92 %). Serum low density lipoprotein cholesterol (LDL-C) was calculated by using the Friedewald formula: LDL-C (mmol/L) = TC – TG/2.2 – HDL-C. Serum Lp(a) was determined with the IMMAGE® Immunochemistry systems (Beckman Coulter) (CV = 6.37 %).
Haematocrit was determined with the capillary tube method and haemoglobin with a colorimetric method (Boehringer Mannheim, Cat. no. 124729).

7. Statistical analysis

The computer software package Statistica® was used for the analysis of the data. Significant differences from baseline to end and between treatments in variables that were normally distributed were determined with the t-test for dependent variables. These variables are presented as means [95 % confidence intervals (CI)]. Differences in variables that were not normally distributed were determined with the Wilcoxon matched pairs test and are presented as medians (25, 75 percentiles). Correlations between baseline FNS values of (1) compaction and network fibrin content (2) compaction and mass length ratio and (3), network fibrin content and mass length ratio were determined in order to ascertain internal consistency between the variables by using Spearman correlations. A p-level of ≤ 0.05 was regarded as statistically significant.

EXPERIMENTAL RESULTS

The responses in biochemical variables with intake of FoodState Vitamin C complex® or placebo during the 2 periods for men and women were always in the same directions. The results

obtained during the 2 periods and for men and women are, therefore, reported combined. Baseline is taken as the levels at the beginning of each experimental period.

There were 2 dropouts from the study. One subject experienced nausea from the supplement and another subject underwent an emergency diaphragm operation. The results from 3 subjects were excluded because 2 of the subjects started weight reducing diets during the study, even though they were instructed not to change their normal diet routines and another subject suffered from diarrhoea for a month during the study.

Adherence, as determined by pill counting with FoodState Vitamin C complex® was 78 ± 16 % and 81 ± 14 % for the placebo.

Anthropometric measurements did not change during the study (data not shown). Haematocrit levels were also not affected, but mean haemoglobin levels decreased significantly with FoodState Vitamin C complex® intake from 10.65[9.8,11.50]mmol/L to 9.80[9.21,10.39]mmol/L with a mean change of -0.85[-1.7, -0.01]mmol/L (p 0.05). This decrease was probably not an independent effect of FoodState Vitamin C complex® because the haemoglobin levels decreased to the same extent with intake of placebo from 10.31[9.25,11.37]mmol/L to 9.43[8.70, 10.15]mmol/L, with a mean change of -0.89[-2.15, 0.37]mmol/L although not statistically significant (p=0.07).

As shown in Table 2, total energy intake and energy distribution (% E) of macronutrients did not change during the intake of FoodState Vitamin C complex®. During the placebo period, however, % E from fat increased significantly and % E from carbohydrates as well as added sugar decreased significantly. Although statistically significant, these changes are small and probably did not influence the results. There were no significant changes in dietary vitamin C intake (excluding supplement). The Dietary Reference Intake (DRI) for vitamin C as reported by the Panel on Dietary Antioxidants and Related Compounds released mid-April 2000, were increased from 60mg/day in adult women and men to 75 mg/day and 90 mg/day, respectively **[29]**.

Table 2

Mean [95 % CI] energy, energy distribution of macronutrients and vitamin C intake during the study

Variable	FoodState Vitamin C complex® (n=25)			Placebo (n=25)		
	Mean	95 % CI		Mean	95 % CI	
Energy (kJ)						
Baseline	10993	9508	12478	10987	10144	11829
End	11152	9929	12375	10551	9524	11577
Protein (%)						
Baseline	16.89	15.86	17.93	16.17	15.22	17.12
End	16.50	15.50	17.52	16.49	14.87	18.12
Fat (%)						
Baseline	32.80	30.38	35.27	31.47[a]	29.50	33.44
End	33.30	31.16	35.51	33.90[a]	31.75	36.05
Cholesterol (mg)						
Baseline	324.4	274.3	374.5	300.5	262.2	338.7
End	337.8	288.2	387.3	313.2	269.9	356.5
Carbohydrate (%)						
Baseline	51.20	48.09	54.49	53.90[b]	50.88	56.93
End	51.20	48.66	53.93	50.87[b]	47.95	53.78
Dietary fibre (g)						
Baseline	34.54	26.74	42.33	34.00	28.10	39.89
End	31.27	25.47	37.08	30.76	25.92	35.59
Added sugar (%)						
Baseline	2.50	1.65	3.34	2.35[c]	1.78	2.93
End	2.60	1.70	3.50	1.77[c]	1.32	2.22
Dietary vitamin C (mg) (excluding supplement)						
Baseline	134.4	91.0	177.9	128.3	88.1	168.5
End	113.1	83.4	142.7	111.2	86.1	136.3

a, b, c: Means with the same symbol differed significantly (t-test for dependant variables, $p \leq 0.05$)
CI: Confidence interval.

In general the subjects in this study consumed ample vitamin C with mean baseline intakes of 160[98, 222]mg/day for men and 166[72,260]mg/day for women. Data on intake of other micronutrients are not included, but can be obtained from the authors.

In this study, initial vitamin C status probably did not affect the results. Mean biochemical levels of subjects with a low dietary vitamin C status (lower than 100 % of the DRI) (n=10) did not differ from subjects with a normal vitamin C status (greater than 100 % of the DRI) (n=15) (data not shown).

Changes in plasma haemostatic factors during the study are illustrated in Table 3.

Table 3

Median [25, 75 percentiles] plasma haemostatic factors during the study

Variable	FoodState Vitamin C complex® (n=25)			Placebo (n=25)		
	Median	25, 75 Percentiles		Median	25, 75 Percentiles	
PAI-1_{act} (U/mL) (Normal 12.8±12.1U/mL)*						
Baseline	17.97	14.03	27.16	17.49	12.95	25.81
End	16.93	13.87	25.96	18.31	11.76	23.64
% Change (baseline to end)	-0.51	-14.59	16.25	-5.46	-14.14	24.30
D-Dimer (µg/L) (Normal 7-78 µg/L)*						
Baseline	11.25	10.07	13.48	10.87[a]	9.70	12.38
End	11.49	10.44	13.27	11.92[a]	9.94	13.31
% Change (baseline to end)	-0.76[b]	-10.16	6.99	5.04[b]	-3.49	17.56
Fibrinogen (g/L) (Normal 2-4 g/L)*						
Baseline	2.67[c]	2.56	2.90	2.72[d]	2.40	2.96
End	2.59[c]	2.43	2.85	2.58[d]	2.30	2.86
% Change (baseline to end)	-3.40	-8.80	1.17	-6.34	-13.78	4.38
TAT (µg/L) (Normal 1.0-4.1 µg/L)*						
Baseline	6.14[e]	5.73	7.51	5.91	5.70	6.18
End	5.83[e]	5.65	5.99	5.81	5.64	6.56
% Change (baseline to end)	-5.81[f]	-18.47	0.39	0.12[f]	-8.03	13.15
PAP (µg/L) (Normal 99-368 µg/L)*						
Baseline	116.04[g]	104.89	162.21	114.97	104.66	134.77
End	113.13[g]	103.32	126.24	112.85	105.92	138.08
% Change (baseline to end)	-4.05[h]	-23.39	-0.23	1.81[h]	-8.95	8.09
TPA_{ag} (µg/L) (Normal 3-10 µg/L)*						
Baseline	5.62	2.83	10.73	6.32	4.28	11.66
End	5.65	4.01	8.82	6.73	4.49	12.13
% Change (baseline to end)	1.06	-6.18	16.77	5.25	-14.60	25.90

a, b, c...Medians with the same symbol differed significantly (Wilcoxon matched pairs test, $p \leq 0.05$) * Normal ranges given by the producers of the diagnostic test kits.
$PAI\text{-}1_{act}$: Plasminogen activator inhibitor-1 activity; TAT: thrombin-antithrombin complex; PAP: plasmin-antiplasmin complex; tPA_{ag}: tissue plasminogen activator antigen.

Compared to the normal ranges given by the producers of the diagnostic kits used for the analysis of haemostatic factors in this study, d-dimer, fibrinogen, PAP and tPA_{ag} levels fell within these proposed normal ranges. However, $PAI\text{-}1_{act}$ as well as TAT levels were higher than the normal range of 12.8±12.1U/mL and 1.0-4.1µg/L respectively. FoodState Vitamin C complex® had no effect on median $PAI\text{-}1_{act}$, d-dimer or tPA_{ag} levels. D-dimer levels increased significantly with placebo and this change was significantly different compared to the change during FoodState Vitamin C complex® intake. Fibrinogen levels decreased significantly with intake of both FoodState Vitamin C complex® and placebo. This decrease was probably caused by factors other than the interventions. TAT and PAP were both significantly decreased from baseline to end with intake of FoodState Vitamin C complex®. When compared to the placebo group these decreases were also significant.

As shown in Table 4, there were no significant changes in fibrin content or mass-length-ratio with either FoodState Vitamin C complex® or placebo. There was, however, a significant increase in compaction from baseline to end with FoodState Vitamin C complex®. This change did not differ significantly from the change with intake of placebo. Baseline compaction and baseline fibrin content correlated significantly ($R = -0.49$, $p = 0.01$).

Table 4

Median [25, 75 percentiles] fibrin network structures during the study

Variable	FoodState VitaminC complex® (n=25)			Placebo (n=25)		
	Median	25, 75 Percentiles		Median	25, 75 Percentiles	
Compaction (%)						
Baseline	49.95[a]	47.55	53.70	50.05	46.90	53.15
End	51.85[a]	48.55	56.65	49.85	47.55	54.25
Change (baseline to end)	1.75	-0.30	3.65	0.50	-2.20	4.30
Fibrin content (g/L)						
Baseline	2.58	2.26	2.99	2.56	2.35	2.88
End	2.34	2.16	2.91	2.45	2.09	2.97
Change (baseline to end)	- 0.08	- 0.18	0.04	- 0.23	- 0.60	0.03
Mass length ratio (dalton/cm x 10^{-12})						
Baseline	29.61	27.00	34.98	28.99	26.01	34.80
End	30.39	25.83	43.30	29.47	27.09	36.29
Change (baseline to end)	0.02	- 1.82	9.19	2.01	- 4.34	4.11

a: Medians with the same symbol differed significantly (Wilcoxon matched pairs test, $p \leq 0.05$)

Changes in serum lipids and Lp(a) levels during the study are illustrated in Table 5. FoodState Vitamin C complex® intake had no effects on mean TC, TG, HDL-C, LDL-C or Lp(a) levels. LDL-C levels, however, decreased and HDL-C levels increased significantly in the placebo group. These changes did not differ significantly from the changes with intake of FoodState Vitamin C complex®.

Because of the large inter-individual variations reported for Lp(a), 25 subjects do not provide enough statistical power to study the effects on Lp(a). This limitation of the study was partly compensated for by taking double blood samples, one week apart, at the beginning and end of each phase so that individual responses of Lp(a) could be examined. The amount of subjects that showed increases, decreases or no effect in Lp(a) during the study was the same during the intake of both FoodState Vitamin C complex® and placebo (increase (> 10 %): 9 *vs* 10; decrease (> 10 %): 6 *vs* 7; no effect: 10 *vs* 8).

Table 5

Mean [95% CI] serum lipid and lipoprotein (a) levels during the study

Variable	FoodState Vitamin C complex® (n=25)			Placebo (n=25)		
	Mean	95 % CI		Mean	95 % CI	
TC (mmol/L)						
Baseline	6.58	6.12	7.05	7.07	6.50	7.64
End	6.57	6.14	7.00	6.88	6.40	7.35
Change (baseline to end)	-0.01	-0.36	0.28	-0.19	-0.49	0.10
LDL-C (mmol/L)						
Baseline	5.17	4.72	5.61	5.77[a]	5.19	6.35
End	5.06	4.63	5.50	5.40[a]	4.94	5.85
Change (baseline to end)	-0.10	-0.39	0.18	-0.37	-0.70	-0.03
HDL-C (mmol/L)						
Baseline	0.81	0.72	0.90	0.78[b]	0.71	0.86
End	0.82	0.73	0.90.	0.85[b]	0.75	0.94
Change (baseline to end)	0.01	-0.81	0.10	0.06	0.01	0.94
TG (mmol/L)						
Baseline	1.20	0.96	1.45	0.97	0.82	1.11
End	1.24	0.97	1.51	1.27	1.04	1.49
Change (baseline to end)	0.03	-0.15	0.22	0.30	0.09	0.51
Lp(a) (mg/dL)						
Baseline	51.57	42.22	60.92	54.08	43.19	64.96
End	55.00	45.18	64.81	54.68	45.04	64.32
Change (baseline to end)	3.42	-1.25	8.11	0.60	-3.34	4.55

a, b: Means with the same symbol differed significantly (t-test for dependant variables, $p \leq 0.05$)
TC: Total cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; Lp(a): Lipoprotein (a).

DISCUSSION

The salient observations in this randomised, placebo controlled, crossover study in 25 hyperlipidaemic subjects were that PAP (marker of plasmin generation – initiate fibrinolysis) and TAT (marker of thrombin generation – initiate coagulation) levels were both significantly decreased with intake of FoodState Vitamin C complex® compared to placebo. FoodState Vitamin C complex® intake, furthermore, had a possible beneficial effect on FNS by increasing

compaction. No statistical significant effects on plasma fibrinogen, $PAI\text{-}1_{act}$, tPA_{ag}, d-dimer, serum lipids or Lp(a) were seen.

An adherence of 78 % would have resulted in an intake of 195mg vitamin C twice daily. We argued that this level of intake was probably sufficient, because plasma vitamin C levels are tightly controlled and high doses of vitamin C results in decreased bioavailability and increased renal excretion **[30]**. Pharmacokinetic data on vitamin C has shown that at doses of > 200mg/day little changes in ascorbic acid concentrations in plasma and circulating cells occurred, with saturation between 200 and 400mg daily **[30;31;32;33]**.

A shortcoming of this study was that the FoodState Vitamin C complex® supplement used contained other constituents apart from vitamin C, namely flavonoids, magnesium and vitamin B. In the literature we found that most studies were conducted on vitamin C and few on bioflavonoids. Vitamin C and flavonoids share a common function: both are antioxidants. Moreover, flavonoids inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, it may reduce damage from ischaemia and reperfusion by inhibition of LDL-oxidation and it also reduces activity of enzyme systems including cyclo-oxygenase and lipoxygenase. Flavonoids exert these effects as antioxidants, free-radical scavengers and chelators of divalent cations. Flavonoids may, therefore, act as effective antioxidants in prevention of coronary heart disease through the anti-thrombotic effects exerted by them **[34;35;36;37;38;39]**. It can further be noted that the effects found with flavonoid supplementation largely mirrors that of vitamin C supplementation with regard to its protective function in CVD and reduced platelet aggregation. Very few studies could be found on the effects of magnesium and vitamin B. Regarding haemostasis, from the limited available literature on magnesium and vitamin B **[40;41;42;43]**, it seems that they principally influence platelet function via effects on thromboxane metabolism, giving rise to an anti-thrombotic effect by reducing platelet aggregation. No studies depicting the effects of vitamin C supplementation on FNS could be found.

Diet plays an important role in primary and secondary prevention of CVD. The notion that diet influences haemostasis is not new. However, the relationships of the total diet, as well as specific food and nutrients, with different haemostatic variables are far from clear **[1]**. Normal haemostasis is a balance between formation and breakdown of blood clots in the circulation. Measurements of TAT and PAP reflect this balance between coagulation and fibrinolysis respectively. The reduced TAT and PAP levels in the FoodState Vitamin C complex® group

might indicate a reduced steady state of haemostatic balance, which in turn may lead to a lower initiation of coagulation and compensatory fibrinolytic activity. This new lowered state of haemostatic balance may play an important role in the prevention and treatment of arteriosclerosis, thrombosis and resultant CVD **[1;7;19;20]**. These effects might be due to the antioxidant properties of vitamin C. It can be assumed that a reduction in the inflammatory response to injury of the vessel wall accrued which is associated with a reduction in atherosclerosis/lesion formation and coagulation **[8;44]**. The reduction in TAT levels are supported by the findings of the third MONICA survey **[13]**, which indicated that serum vitamin C were inversely associated with TAT levels (in men and women). The other haemostatic markers measured in this study remained constant. This is consistent with the findings of other studies done on the probable effect of vitamin C intake on haemostasis. In these studies, as well as in the present one, levels of plasma fibrinogen **[23;25]**, PAI-1_{act} **[23;25]**, tPA_{ag} and fibrin degradation products **[25]** were not affected by vitamin C intake.

Very little is known about the effects of diet on FNS. **Veldman et al. [45]** investigated the effect of dietary pectin on FNS and **Moss et al. [46]** the effects of exercise and different pre-exercise meals on FNS. To our knowledge, no other work investigating dietary intervention and FNS has been published. In this study, characteristics of fibrin networks were described by investigating clot rigidity (compaction), mass-length-ratio (turbidimetrically) and fibrin content of the fibrin fibers. Intake of FoodState Vitamin C complex® resulted in a statistically significant increase in compaction, probably increasing the lysability of these clots. Increased compaction can therefore possibly reduce atherosclerosis and have a positive effect on the risk for CVD, due to the creation of a pro-fibrinolytic environment **[2;3;5;6]**. During fibrin clot formation, covalent bonds form between fibrin fibers through the action of activated factor XIII. This cross-linking alters the visco-elastic characteristic of networks and they become more rigid **[26]**. Rigidity is also dependent on the amount of branch points present in the clot **[47]**. The ability of a network to withstand the shear forces in vivo largely depends upon the rigidity of the network (compaction, therefore, is a measure of amount of the cross-links and branch points) **[26]**. The increased compaction could therefore be due to either the formation of thicker fibers with less branch points and/or decreased cross-linking. To determine the specific reason for increased compaction, one would have to use extensive quantitative analysis of network features (fiber lengths, diameters, branching point densities and fiber densities). This can be achieved by using a recently developed method of constructing computerised three-dimensional wire frame models of fibrin networks from stereo electron micrographs, as described by **Ryan et al. [47] [adapted from**

Baradet et al. 1995]. Stereo viewing of the networks is particularly useful for measuring fiber lengths in three dimensions and distinguishing branch points from fibers that merely crossed each other at different points. Therefore, scanning electron microscopy (SEM) of fibrin clots can give an indication of the clot fiber diameter, as well as fiber and branch point densities. Electrophoretic experiments involving gel electrophoresis (SDS-PAGE) can furthermore be used to analyse the cross-linking profiles of clots in order to determine the amount and type of cross-linking **[47]**.

In this study, the significant inverse correlation between compaction and fibrin content indicates internal consistency in the results obtained for the measurement of the FNS. Increased compaction is indicative of fewer branch points and/or less cross-linking. Lower fibrin content is also consistent with fewer branch points (possibly due to formation of thicker fibers), both of which imply lower clottability, resulting in higher compaction **[47]**. The clinical relevance of vitamin C on FNS is, however, unclear. Furthermore, although compaction increased in the FoodState vitamin C complex®, this gives no indication whether it will in vivo, lead to protection against formation of thrombin or increased lysis. It is, therefore, necessary to correlate the in vitro laboratory analysis of FNS with in vivo processes associated with CVD.

Results from prior studies of vitamin C on lipid levels have been inconsistent. As found in the present study other randomised placebo controlled trials could also not demonstrate significant effects of vitamin C on lipid levels **[48;49;50;51]**. In a placebo controlled study with hyperlipidaemic subjects who took 500mg vitamin C supplements per day a significant increase in HDL-C and decrease in LDL-C levels were seen after 12 months **[52]**. In another double blind placebo controlled study with 1g of vitamin C/day for 8 months HDL-C was significantly increased in a sub-group of subjects with low initial vitamin C status **[48]**. Decreased TC and HDL-C levels with no change in TC/HDL-C ratio were reported with the intake of 2g/day vitamin C for 6 weeks **[23]**. Two randomised double blind placebo controlled studies could not demonstrate any effect of vitamin C intake on Lp(a) levels **[51;53]**. In the study of **Bostom et al. [51]**, 19 subjects with a history of CHD took 4.5g vitamin C/day for 12 weeks. The study of **Jenner et al. [53]** included healthy men and women of which 49 subjects took 1g vitamin C/day for 8 months.

In conclusion, FoodState Vitamin C complex® supplementation, reduced TAT and PAP concentrations and thereby may have resulted in a new reduced steady state of haemostatic

balance that may be protective of CVD. FoodState Vitamin C complex® supplementation may have resulted in less rigid clots as indicated by the increased compaction levels. FoodState Vitamin C complex® supplementation had no effect on plasma fibrinogen, $PAI\text{-}1_{act}$, tPA_{ag}, d-dimer, serum lipids or Lp(a).

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