REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2006 Name of Author KONSTANTOULAS C.J.

COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS

Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).

B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.

C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.

D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.

☑ This copy has been deposited in the Library of UCL

☐ This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

GENETIC VARIANTS IN AN ENDOTHELIAL CELL MEMBRANE PROTEIN (THROMBOMODULIN) PARTICIPATING IN THE PROTEIN C PATHWAY: CLINICAL STUDIES OF HEART DISEASE & *IN VITRO* ANALYSIS

Constantine J Konstantoulas

A thesis submitted in accordance with the regulations of the University of London for the degree of Doctor of Philosophy

March 2006

Centre for Cardiovascular Genetics

Department of Medicine

Royal Free and University College Medical School

University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

TO MY PARENTS

JANET AND MICHAEL

ABSTRACT

Thrombin is the key enzyme in the formation of a fibrin clot. Control of its activity and generation forms an important haemostatic function preventing occlusive blood clot formation. Deregulation of this control process results in increased thrombin generation and thus increased clot formation in thrombotic conditions. Thrombomodulin (Tm) is an important thrombin-regulatory gene expressed at the endothelial cell surface, and as such may have a role in modifying susceptibility to occlusive thrombotic disease.

This thesis focuses on the role of gene variants, within Tm, in determining risk of coronary heart disease (CHD). Contribution to risk of CHD by variants in the Tm gene was assessed in a case-control study (HIFMECH), a large prospective study of heart disease (NPHSII) and a cross sectional study of type 2 diabetes (EDSC). Tm gene variant interaction with clinical and plasma markers of CHD was also studied. The consequences of Tm gene variants upon thrombin generation and inflammation were also addressed. Tm antigen levels and cofactor activity for protein C (PC) activation were assessed to determine whether the overall contribution to heart disease by dysfunctional variants could be estimated. *In vitro* functional studies were performed, to determine the molecular mechanisms of the effect of the variants showing strong effects on risk and to further our understanding of the role of Tm in the pathogenesis of CHD.

The work included in this thesis demonstrates that genetic variation in the Tm gene may influence risk for CHD in an environment of metabolic syndrome. It adds to a growing body of evidence suggesting a contribution to CHD risk caused by variants or mutations in the Tm gene.

ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr Helen Ireland, for her assistance, support and guidance throughout my project. Her experience and whole hearted support made possible the completion of this thesis. I would also like to thank Professor Steve Humphries for giving me the opportunity to pursue a higher degree in science and for his ideas and continuous support during my research fellowship. I would like to thank all members of the CVG laboratory, past and present, for initial guidance in laboratory techniques and for making my PhD a pleasant and enriching experience. Special thanks go to Professor Shinichiro Kurosawa for supervising and allowing me to measure plasma soluble thrombomodulin (sTm) at the Oklahoma Medical Research Foundation, Oklahoma City, USA. Many thanks go to Dr Ann Kristin Ohlin for developing in house assays for Tm antigen and the cofactor activity for protein C activation and measuring plasma sTm levels in participants of the EDSC study. I would also like to acknowledge everyone at the Advanced Biotechology Centre (ABC), Imperial College London for their help and advice with sequencing. I must also thank my fellow PhD students and post doctoral fellows in the CVG laboratory for enthusiastic discussions and 'thought provoking' conversations throughout my project. I am also grateful to Dr Birgit Dorfmeister who generously dedicated time to read and improve the manuscript.

The financial support of the British Heart Foundation (BHF Grant Code: FS/02/085/14756) is gratefully acknowledged.

Thanks to all my friends for their continued encouragement, understanding and support. Finally, all my love and gratitude goes to my parents Janet and Michael Konstantoulas and my sister Natalie for their unwavering support and belief in me, without them I would never be where I am today. Thank you.

DECLARATION

All the work presented in this thesis is my own, or has been carried out as part of a collaboration in which I played a major part. All collaborative work has been acknowledged in the text; see page 327. No part of this thesis has been submitted for a degree, diploma or other qualification at any other University.

Constantine J Konstantoulas

PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS

Full papers

Konstantoulas CJ, Hawe E, Saut N, Yudkin JS, di Minno G, Margaglione M, Hamsten A, Humphries SE, Juhan-Vague I, Ireland H. Common variants in the thrombomodulin gene as a risk for myocardial infarction in the north of Europe (HIFMECH Study). Thrombosis and Haemostasis 2004; 91(3): 628-630.

Konstantoulas CJ, Cooper J, Warnock G, Miller GJ, Humphries SE, Ireland H. A combination of two common thrombomodulin gene variants (-1208/09TT>delTT and A455V) influence risk of coronary heart disease: a prospective study in men. Atherosclerosis 2004; 177(1): 97-104.

Submitted paper

Konstantoulas CJ, Ohlin AK, Humphries SE, Goodall AH, Toh C-H, Mather H, Ireland H. Measurements of Soluble Thrombomodulin Activity and Antigen: Factors Contributing to Variance, and association with Risk for Coronary Heart Disease in Type II Diabetes. Manuscript submitted to Thrombosis and Haemostasis 2006.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AC	Afro-Caribbean
Act	Activity
Ag	Antigen
α_2M	Alpha 2-macroglobulin
α_1AT	Alpha 1-antitrypsin
APC	Activated Protein C
AT	Antithrombin
BAEC	Bovine Aortic Endothelial Cells
BASMC	Bovine Aortic Smooth Muscle Cells
CABG	Coronary Artery Bypass Graft
Ca-CaM	Ca-calmodium Complex
CaMKII	Calmodium Kinase II
cAMP	Cyclic Adenosine Monophosphate
CHD	Coronary Heart Disease
CRP	C-reactive Protein
DBP	Diastolic Blood Pressure
DS sTm ag	Diagnostica Stago soluble Thrombomodulin antigen
EC	Endothelial Cells
ECG	Electrocardiogram
EDSC	Ealing Diabetic Study of Coagulation
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor kinase
EMSA	Electrophoretic Mobility Shift Assays
EPCR	Endothelial cell Protein C Receptor
EPI	Extrinsic Pathway Inhibitor
EW	European White
FV	Factor V
FVa	Activated Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FIX	Factor IX

FIXa	Activated Factor IX
FX	Factor X
FXa	Actvated Factor X
FXI	Factor XI
FXIa	Activated Factor XI
FXIII	Factor FXIII
FXIIIa	Activated Factor XIII
F1+2	Prothrombin Fragments F1+2
FPA	Fibrinopeptide A
GAG	Glycosaminoglycans
GAPDH	Glyceraldehydes-3-phosphate Dehydrogenase
Gla	γ-carboxy-glutamic acid
GP	Glycoprotein
GPCR	G-protein Coupled Receptor
HAEC	Human Aortic Endothelial Cells
HDL	High Density Lipoprotein
HIFMECH	Hypercoagulability and Impaired Fibrinolytic Functions Mechanisms
HRT	Hormone Replacement Therapy
HSVEC	Human Saphenous Vein Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
HCII	Heparin Cofactor II
IH sTm act	In-house soluble Thrombomodulin Activity
IH sTm ag	In-house soluble Thrombomodulin antigen
IA	Indian Asian
IL-1	Interleukin-1
IL-6	Interleukin-6
IP3	Inositol 1,3,5-triphosphate
LACI	Lipoprotein Associated Coagulation Inhibitor
LDL	Low Density Lipoprotein
MADGE	Microtitre Array Diagonal Gel Electrophoresis
MI	Myocardial Infarction
NF-κβ	Nuclear Factor kappa beta
NO	Nitric Oxide
NOS3	Nitric Oxide Synthase 3
NPHSII	Second Northwick Park Heart Study

Ox-LDL	Oxidised Low Density Lipoprotein
PAI-1	Plasminogen Activator Inhibitor type 1
PAR	Protease Activated Receptor
PC	Protein C
PCI	Protein C Inhibitor
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-3 Kinase
PL	Phospholipid
PS	Protein S
PTCA	Percutaneous Transluminal Coronary Angioplasty
PZ	Protein Z
p300/CBP	Cyclic Adenosine Monophosphate Response Element Binding Protein
RA	Retinoic Acid
RARE	Retinoic Acid Response Element
RVG	Rabbit jugular Vein Grafts
SDS-PAGE	Sodium Dodecyl-Sulfate-Polyacrylamide gel
SBP	Systolic Blood Pressure
SSRE	Shear Stress Response Element
SMC	Smooth Muscle Cells
sTm	soluble Thrombomodulin
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TAFIa	Activated Thrombin Activatable Fibrinolysis Inhibitor
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF	Transforming Growth Factor beta
THP-1	Monocytic Cell Line
Tm	Thromobmodulin
TNF-α	Tumour Necrosis Factor alpha
t-PA	Tissue Type Plasminogen Activator
u-PA	Urokinase Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
ZPI	Protein Z dependent Protease Inhibitor

One and three letter codes for amino acids

A (Ala) alanine	K (Lys) lysine	T (Thr) threonine
C (Cys) cysteine	L (Leu) leucine	V (Val) valine
D (Asp) aspartic acid	M (Met) methionine	W (Trp) tryptophan
E (Glu) glutamic acid	N (Asn) asparagine	Y (Tyr) tyrosine
F (Phe) phenylalanine	P (Pro) proline	
G (Gly) glycine	Q (Gln) glutamine	
H (His) histidine	R (Arg) arginine	
I (Ile) isoleucine	S (Ser) serine	

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1 Haemostasis

A complex physiological system has evolved to guarantee blood fluidity and to prevent blood loss, thereby restoring the integrity of the circulatory system following vessel damage (Furie and Furie, 1992) (van Hinsbergh, 2001). This system, termed haemostasis, represents a group of balanced activities consisting of acute vasoconstriction, platelet adhesion and aggregation, blood coagulation (resulting in the formation of a fibrin clot), natural anti-coagulant mechanisms, which prevent generalised activation of coagulation, thereby localising thrombus formation to the site of injury, and fibrinolysis, which dissolves the fibrin clot. Under physiological conditions, the haemostatic system favours the anti-coagulant state, which maintains blood fluidity. This balance is shifted towards a pro-coagulant state in response to vascular injury, due to the presence of triggering molecules that become available after damage. If such molecules become available in intact vessels at places distant from the wound, intravascular coagulation can occur. This process may result in the development of mural or occlusive thrombi, which contribute to the pathology of venous and arterial thrombosis.

This thesis will focus on one endothelial cell membrane associated protein (Thrombomodulin; Tm) participating in a natural anti-coagulant mechanism (the protein C anti-coagulant pathway). The aim of this thesis is to determine whether variants or mutations in this gene contribute to coronary heart disease (CHD), and if so, how these are dysfunctional. The introduction will be divided into three main parts. The first will address the biochemical aspects of the coagulation cascade, its activation and its

regulation by natural anti-coagulant mechanisms, thus providing the framework for the interpretation of the roles of endothelial cell membrane associated proteins in haemostasis. The second part will focus on the pathogenesis of arterial thrombotic disease, predominantly looked at from the point of view of the haemostatic involvement. The third part will centre upon known structural, functional and molecular genetic aspects of Tm.

1.2 Blood coagulation

1.2.1 Activation of blood coagulation

The blood coagulation cascade is initiated as a consequence of endothelium rupture (Dahlback, 2000) (Butenas and Mann, 2002). The anti-coagulant nature of vascular endothelial cells is shifted towards a pro-coagulant state in response to either mechanical injury or inflammatory stimuli (van Hinsbergh, 2001) (Butenas and Mann, 2002). Endothelial cell denudation exposes the subendothelial collagen matrix and tissue factor (TF) on the cells of the intima (Rapaport and Rao, 1992) (Davie, 1995) (Mann et al., 1998). Inflammatory cytokines are also able to induce the expression of TF by vascular endothelial cells (Nemerson, 1995). Following vascular injury, the exposed connective tissue matrix allows the adhesion of platelets. Platelet adhesion is primarily mediated by the interaction of glycoprotein (GP) Ib-IX-V, on the platelet surface, with von Willebrand factor (vWF), which is tethered to damaged endothelium and exposed subendothelial collagen (Lopez et al., 1988) (Hickey et al., 1989) (Kroll et al., 1996) (Ruggeri, 1997) (Nakamura et al., 1999). Platelet-collagen interaction is also facilitated by other GPs including GP Ia/IIa, GP IV and GP VI. Once bound, platelets are activated by a number of agonists such as thrombin, adenosine 5'-disphosphate,

collagen, vWF and shear stress (Cines et al., 1998) (Selwyn, 2003). Platelet activation exposes integrin GP IIb-IIIa (Marguerie et al., 1979), which binds fibrinogen with high affinity (Parise and Phillips, 1985) (Furie and Furie, 1992). Fibrinogen is thought to act as a bridge between GP IIb-IIIa molecules on different platelets, promoting platelet aggregation. This process leads to primary platelet plug formation, which is stabilised by the formation of an insoluble fibrin network, generated by thrombin (Bennett and Vilaire, 1979) (Marguerie et al., 1979) (Mann et al., 1990) (Dahlback, 2000) (Selwyn, 2003). Platelet plug formation is co-ordinated with the coagulation system responses (Dahlback, 2000). A detailed account of platelet adhesion and the role platelets play in thrombin generation and fibrin clot formation lies outside the scope of this thesis. Excellent reviews exist on this topic; see (Sixma et al., 1995) (Heemskerk et al., 2002) (Monroe et al., 2002).

Thrombin generation is the result of a series of stepwise enzymatic reactions involving plasma proteins (Davie, 1995); see Figure 1.1. Coagulation proteins circulate as inactive zymogens, which are converted to their active serine protease forms by proteolytic cleavage (Mann et al., 1990) (Furie and Furie, 1992). The protease of one reaction catalyses the activation of the next zymogen in a process termed the "coagulation cascade". It is thought that the exposure or expression of TF provides the trigger for initiation of physiologic coagulation (Bevilacqua et al., 1984) (Broze, Jr. et al., 1990) (Mann, 1999). TF binds to activated factor VII (VIIa), which constitutes 1-2% of circulating FVII, thereby altering FVIIa's catalytic site, enhancing the enzymatic activity of this coagulation factor >100-fold (Morrissey et al., 1993) (Nemerson, 1995). The coagulation cascade is propagated by the formation of 3 main complexes, two 'tenase' complexes required for factor X (FX) activation and the 'prothrombinase' complex, which activates prothrombin (Davie and Ratnoff, 1964) (Macfarlane, 1964)

(Mann, 1999). These pro-coagulant complexes consist of the serine protease activated in the previous step, a cofactor and the substrate. Formation of these enzyme complexes significantly enhances the activity of the serine protease towards the substrate. Complex assembly and activity requires membranes containing acidic phospholipids, as well as calcium ions. Damaged vascular endothelial cells, activated platelets and inflammatory cells provide phospholipid binding sites for coagulation proteins (Bevilacqua et al., 1984). TF binds to VIIa and forms the 'extrinsic tenase' complex, which activates zymogens factor IX (FIXa) and factor X (FXa). FXa is able to generate low amounts of thrombin, which enhance the coagulation process by activating platelets and cleaving pro-cofactors factor V (FV) and factor VIII (FVIII), yielding the active cofactors FVa and FVIIIa. FVIIIa forms the 'intrinsic tenase' complex with FIXa, which is 50-fold more efficient at activating FX than the 'extrinsic tenase' (Mann et al., 1990) (Mann, 1999). FXa together with FVa form the 'prothrombinase' complex, which is the primary activator of prothrombin (Tracy et al., 1985). Thrombin produced is able to amplify its own generation by activating factors V, VIII, X, XI and platelets (Gailani and Broze, Jr., 1991) (Dahlback, 2000). Furthermore, catalytic cleavage of fibrinogen by thrombin leads to the production of fibrin monomers, which polymerise to form the fibrin network (Brummel et al., 1999). Activated factor XIII (FXIIIa), which is also generated by thrombin, stabilises the newly formed fibrin network by forming covalent cross-links between fibrin units (Gerth et al., 1974) (Roberts et al., 1974) (Lorand et al., 1980) (Naski et al., 1991).

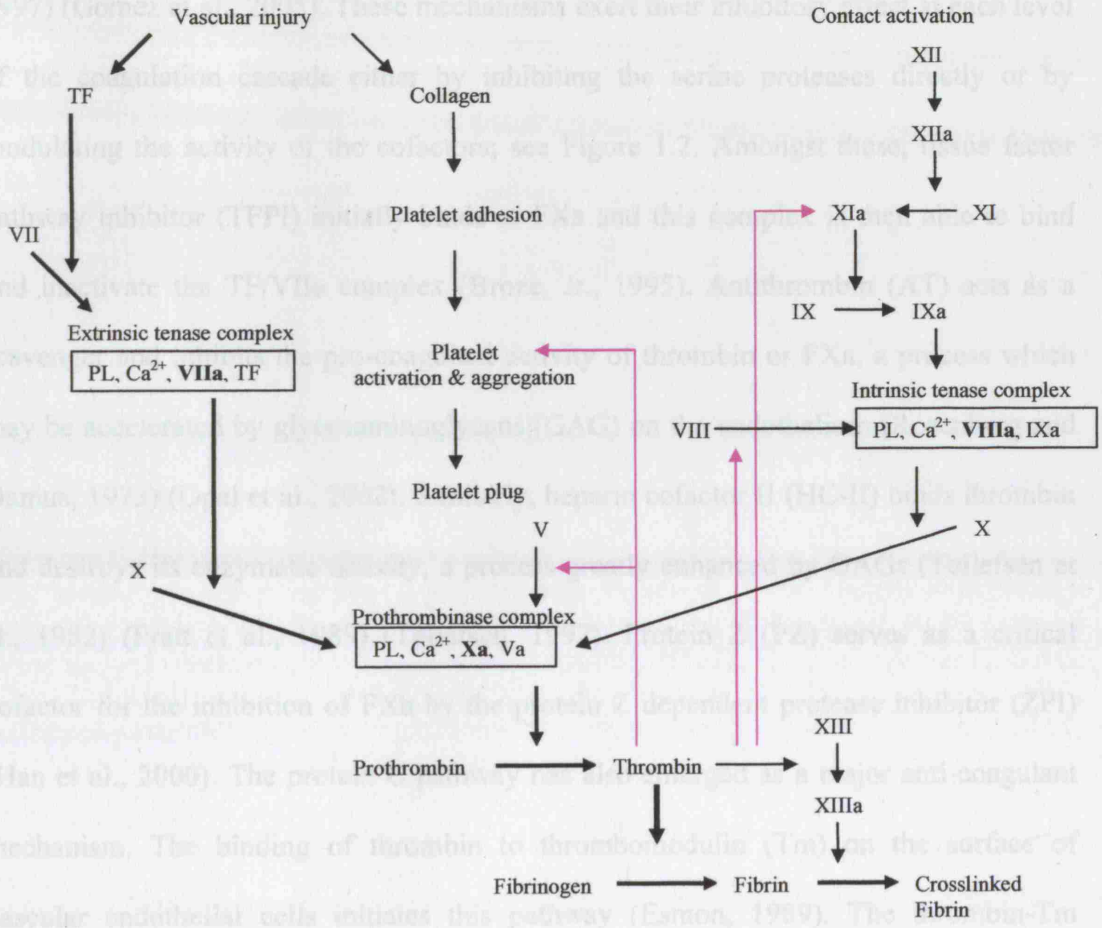


Figure 1.1 The coagulation pathway.
Consists of the extrinsic branch (initiated by TF) and the intrinsic branch (initiated by contact activation). Bold arrows represent the physiologically important extrinsic cascade. Pink arrows represent the thrombin positive feedback loop. Boxes represent the 'tenase' and 'prothrombinase' complexes. TF/VIIa complex is also able to directly activate factor IX. **PL**: Phospholipid surface.

1.2.2 Regulation of blood coagulation

A number of anti-coagulant mechanisms have evolved to stop coagulation from becoming generalized, thereby preventing excessive fibrin formation (Bombeli et al., 1997) (Gomez et al., 2005). These mechanisms exert their inhibitory effect at each level of the coagulation cascade either by inhibiting the serine proteases directly or by modulating the activity of the cofactors; see Figure 1.2. Amongst these, tissue factor pathway inhibitor (TFPI) initially binds to FXa and this complex is then able to bind and inactivate the TF/VIIa complex (Broze, Jr., 1995). Antithrombin (AT) acts as a scavenger and inhibits the pro-coagulant activity of thrombin or FXa, a process which may be accelerated by glycosaminoglycans (GAG) on the endothelium (Rosenberg and Damus, 1973) (Opal et al., 2002). Similarly, heparin cofactor II (HC-II) binds thrombin and destroys its enzymatic activity, a process greatly enhanced by GAGs (Tollefsen et al., 1982) (Pratt et al., 1989) (Tollefsen, 1997). Protein Z (PZ) serves as a critical cofactor for the inhibition of FXa by the protein Z dependent protease inhibitor (ZPI) (Han et al., 2000). The protein C pathway has also emerged as a major anti-coagulant mechanism. The binding of thrombin to thrombomodulin (Tm) on the surface of vascular endothelial cells initiates this pathway (Esmon, 1989). The thrombin-Tm complex then activates the vitamin K dependent zymogen, protein C (PC), to generate the active form of the protein (activated protein C; APC) (Kisiel, 1979), a process enhanced by the endothelial cell protein C receptor (EPCR) (Stearns-Kurosawa et al., 1996) (Fukudome et al., 1998). APC then interacts with protein S (PS) to give rise to a complex capable of inactivating coagulation factors Va and VIIIa, thereby attenuating thrombin generation (Kisiel et al., 1977).

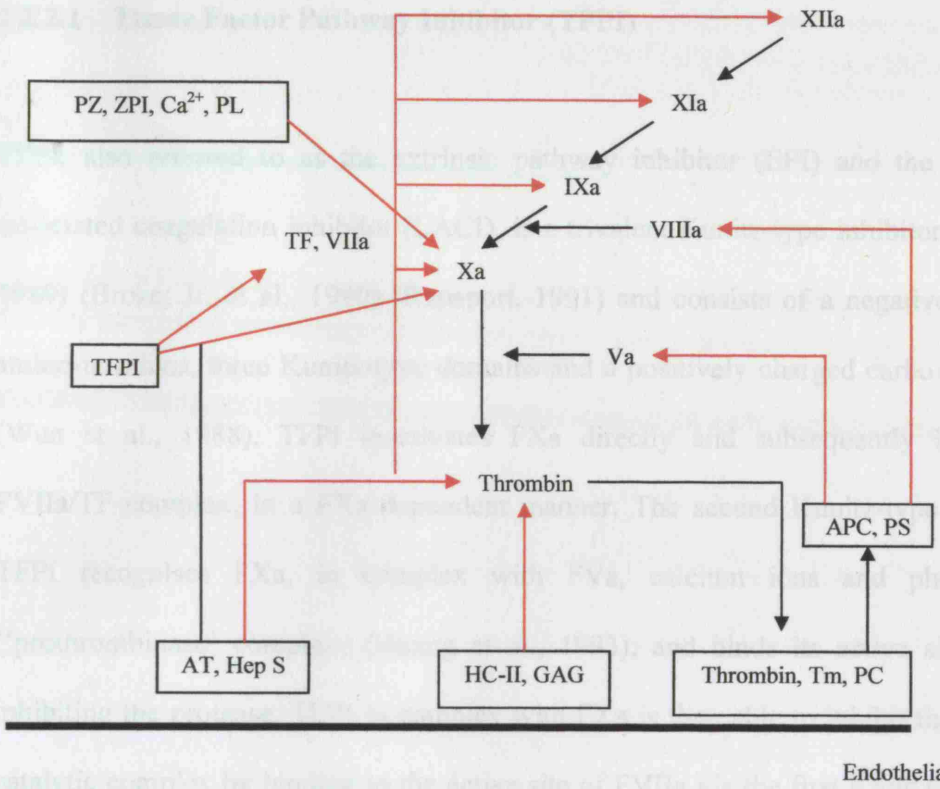


Figure 1.2 Natural anti-coagulant pathways.
Red arrows represent inhibitory activity. **PZ**: Protein Z; **ZPI**: Protein Z dependent protease inhibitor; **PL**: Phospholipid; **Ca^{2+}**: Calcium ions; **TFPI**: tissue factor pathway inhibitor; **AT**: antithrombin; **Hep S**: heparan sulfate; **HC-II**: Heparin cofactor II; **GAG**: glycosaminoglycan; **Tm**: thrombomodulin; **PC**: protein C; **PS**: protein S; **APC**: activated protein C.

1.2.2.1 Tissue Factor Pathway Inhibitor (TFPI)

TFPI, also referred to as the extrinsic pathway inhibitor (EPI) and the lipoprotein associated coagulation inhibitor (LACI), is a trivalent Kunitz-type inhibitor (Rapaport, 1989) (Broze, Jr. et al., 1990) (Rapaport, 1991) and consists of a negatively charged amino-terminus, three Kunitz-type domains and a positively charged carboxy-terminus (Wun et al., 1988). TFPI inactivates FXa directly and subsequently inhibits the FVIIa/TF complex, in a FXa dependent manner. The second Kunitz-type domain of TFPI recognises FXa, in complex with FVa, calcium ions and phospholipids ('prothrombinase' complex) (Huang et al., 1993), and binds its active site, thereby inhibiting the protease. TFPI in complex with FXa is then able to inhibit the FVIIa/TF catalytic complex by binding to the active site of FVIIa via the first Kunitz-type repeat (Broze, Jr. et al., 1988) (Girard et al., 1989). TFPI is primarily synthesised on the surface of vascular endothelial cells, where it is thought to exert its anti-coagulant function (Bajaj et al., 1990). Small amounts of TFPI have also been found in other cells, including platelets (Bajaj et al., 2001). TFPI also circulates in plasma, at a concentration of 54-124 ng/ml (Novotny et al., 1991), either in a free form or in association with lipoproteins (low-density lipoprotein (LDL), high-density lipoprotein (HDL) and lipoprotein (a)) (Broze, Jr. et al., 1994). Heparin treatment may contribute to a portion of TFPI found in plasma, since it has been found to induce the release of endothelial cell surface associated TFPI (Sandset et al., 1988) (Lupu et al., 1999). Plasma TFPI circulating in association with lipoproteins has a truncated carboxy-terminus, lacking a portion of the carboxy-terminus, including part of the third Kunitz-type domain, and is characterised by reduced anti-coagulant potential (Broze, Jr. et al., 1994). The physiological relevance of TFPI in inhibiting thrombus formation is as yet uncertain. Animal models have, however, demonstrated that depletion of plasma TFPI levels

sensitized rabbits to TF and endotoxin induced disseminated intravascular coagulation (Sandset et al., 1991a) (Sandset et al., 1991b). More recently, expression of exogenous TFPI, through retrovirus mediated gene transfer, was able to inhibit intravascular thrombus formation in stenotic and injured rabbit carotid arteries (Golino et al., 2001). These studies would suggest a potential role for TFPI in regulating thrombotic complications, arising from the exposure of blood to TF.

1.2.2.2 Antithrombin (AT)

AT is a hepatically synthesised serine protease inhibitor (Mammen, 1998), predominantly found in plasma, circulating at a concentration of 110-140 mg/l (Murano et al., 1980). AT forms a 1:1 stoichiometric complex with serine protease clotting factors, blocking their active sites and destroying their enzymatic activity (Eisele and Lamy, 1998) (Mammen, 1998). AT is able to directly inhibit factors XIIa, XIa, Xa, IXa and thrombin (Rosenberg et al., 1975) (Stead et al., 1976) (Eisele and Lamy, 1998) (Mammen, 1998). Recently, it has been suggested that AT may also function together with heparin and TFPI to inactivate the FVIIa/TF catalytic complex (Jesty et al., 1996). In addition to a serine protease binding domain, AT also contains a GAG binding domain in close proximity to its active site. Heparin, as well as heparan sulfate, expressed on the endothelial cell surface, are able to accelerate the inhibition of serine protease clotting factors by AT (Damus et al., 1973) (Rosenberg et al., 1975) (Stead et al., 1976) (Metcalfe et al., 1979) (Marcum and Rosenberg, 1984). Highly acidic sulfated pentasaccharides, found in GAGs, bind to the positively charged binding domain of AT, altering its three dimensional structure, which in turn enhances AT enzymatic activity 1000-fold. The binding of GAGs to AT results in an arginine residue (R393), normally located near the carboxy-terminus of AT, being brought into close proximity to AT's

active site. This arginine residue forms a covalent bond with a serine moiety within the active sites of serine proteases, resulting in their inactivation (Pomerantz and Owen, 1978) (Griffith, 1982) (Olson et al., 1993) (Frebelius et al., 1994) (Mammen, 1995) (Levi and ten Cate, 1999).

1.2.2.3 Heparin cofactor II (HC-II)

HC-II is a 66.5 kDa protein, synthesised exclusively in the liver and found in normal blood plasma at a concentration of 1.2 μM (Tollefsen and Pestka, 1985). HC-II is a very specific inhibitor of thrombin, but no other serine protease in blood coagulation (Parker and Tollefsen, 1985). The HC-II reactive site peptide bond is Leu444-Ser445 (Griffith et al., 1985) (Blinder et al., 1988). Like AT, HC-II forms a 1:1 molar complex with thrombin, blocking its active site and destroying its enzymatic activity (Tollefsen et al., 1982). HC-II mediated inhibition of thrombin is greatly enhanced following binding to GAGs, as is the case for AT (Tollefsen, 1997) (Pratt et al., 1989). In addition to heparin, heparin sulfate and dermatan sulfate, other polyanions, including polyphosphates, polysulfates and polycarboxylates, are also able to accelerate HC-II inhibition of thrombin (Tollefsen, 1997) (Pratt et al., 1989). The GAG binding domain is located in close proximity to its active site (Baglin et al., 2002). GAG binding is thought to activate HC-II by displacing the acidic amino-terminus from an intramolecular interaction with the basic GAG binding site, thus freeing it for binding to thrombin via anion-binding exosite I (Van Deerlin and Tollefsen, 1991) (Liaw et al., 1999). The recent determination of the crystal structures of both native HC-II and HC-II in complex with inactive thrombin, has led to an alternative mechanism for GAG activation of HC-II being proposed. In this model, GAG binding is thought to result in the expulsion of the buried reactive centre loop normally located near the amino-terminus, thus altering

the amino-terminal tail interaction to promote binding to thrombin anion-binding exosite I (Baglin et al., 2002). The physiological activator of HC-II is assumed to be extravascular dermatan sulfate, suggesting that HC-II has a major role in thrombin regulation at extravascular tissue sites following vessel injury (Tollefsen et al., 1983) (McGuire and Tollefsen, 1987) (Maimone and Tollefsen, 1990) (He et al., 2002).

1.2.2.4 Protein Z (PZ) & Protein Z dependent protease inhibitor (ZPI)

PZ is a 62 kDa vitamin K dependent protein (Miletich and Broze, Jr., 1987), synthesised in the liver and found predominantly in plasma (Kemkes-Matthes and Matthes, 1995). PZ plasma levels span a broad range, with a mean of 2.9 $\pm$ 1.0 μg/ml (Miletich and Broze, Jr., 1987). Its structure shows considerable homology to many other serine proteases, such as factors VII, IX, X and protein C. However, PZ is not a zymogen for a serine protease, because the histidine and serine residues of the canonical catalytic triad are absent (Ichinose et al., 1990). As such, the physiological function of PZ remained uncertain for several years. Subsequent experiments showed that incubation of PZ with FXa, phospholipids and Ca^{2+}, reduced FXa pro-coagulant activity in a one stage coagulation assay, an effect due in part to a previously unidentified plasma proteinase inhibitor termed protein Z dependent protease inhibitor (ZPI) (Han et al., 1998). ZPI is a 72 kDa hepatically synthesised serine protease inhibitor, with tyrosine 387 thought to be the P_1 residue in its reactive centre. It is predominantly found in plasma and circulates at a concentration of 3.8 μg/ml (Han et al., 1999) (Han et al., 2000). In the presence of PZ, phospholipids and Ca^{2+}, ZPI produces rapid inactivation of FXa (half-time < 10 seconds) (Kemkes-Matthes and Matthes, 1995). It is thought that PZ forms a calcium ion dependent complex with FXa at the phospholipids surface, which is subsequently recognised by ZPI, thus forming a tertiary complex containing FXa, PZ

and ZPI at the phospholipids surface (Kemkes-Matthes and Matthes, 1995). ZPI is able to inhibit FXa pro-coagulant activity in the absence of PZ, though the rate of inhibition is reduced more than 100-fold (half-time = 210). Whereas the combination of PZ and ZPI reduces the initiation and rate of thrombin generation in mixtures containing prothrombin, FV, phospholipids and Ca2+, no inhibition was observed in mixtures containing FVa. This observation would suggest that PZ and ZPI act to dampen the early stages of the coagulation response, prior to the formation of the 'prothrombinase' complex (Han et al., 2000). ZPI does not appear to produce significant inhibition of thrombin, meizothrombin, FVIIa, FIXa, APC, tissue-type plasmigogen activator (t-PA), urokinase plasminogen activator (u-PA), plasmin, trypsin, leukocyte elastase, chymotrypsin or cathepsin G (Han et al., 1999). However, studies have shown that in addition to inhibiting the pro-coagulant activity of FXa, ZPI is also able to inhibit FXIa in the absence of PZ, phospholipids and Ca^{2+}. Both factor X and XIa are responsible for the inactivation of ZPI by proteolytic cleavage at the carboxy-terminus of the molecule, thus regulating its anti-coagulant potential (Han et al., 2000).

1.2.2.5 The Protein C Pathway

The protein C (PC) pathway has emerged as a major regulatory mechanism of the coagulation cascade.

PC is a vitamin K dependent plasma zymogen, and circulates at a concentration of 65 nM. It is synthesised in the liver as a single chain molecule. Removal of the Arg157-Thr158 dipeptide in the Golgi apparatus converts this single chain molecule into a two chain molecule, linked by a disulfide bond between Cys 141 and Cys 277. The light chain of PC consists of a γ-carboxy-glutamic acid (Gla) domain and two epidermal

growth factor (EGF)-like domains, whereas the heavy chain consists of a short activation peptide and the serine protease domain (Kisiel et al., 1977).

Following activation of the coagulation system, the resulting thrombin activates PC by cleaving the Arg169-Leu170 bond, releasing a dodecapeptide (activation peptide) from the amino terminus of the heavy chain (Kisiel, 1979). Rapid PC activation requires the formation of a high affinity complex between thrombin and Tm (Esmon and Owen, 1981) (Owen and Esmon, 1981) (Esmon et al., 1982b) (Esmon, 1989), a vascular endothelial cell surface glycoprotein, which is discussed in detail later on and is the focus of this thesis; see section 1.4. Furthermore, the formation of the thrombin-Tm complex alters thrombin substrate specificity (Musci et al., 1988) (Ye et al., 1991) (Ye et al., 1992), thus converting thrombin enzyme activity from pro- to anti-coagulant (Esmon et al., 1982a) (Esmon et al., 1982b) (Esmon et al., 1983). PC binds Ca^{2+} in the EGF domains and protease domain, altering the conformation of PC, including the region containing the scissile bond. This conformational change is important for PC activation by the thrombin-Tm complex (Ohlin and Stenflo, 1987) (Ohlin et al., 1988) (Stearns et al., 1988) (Rezaie et al., 1992) (Rezaie and Esmon, 1994a) (Rezaie and Esmon, 1994b) (Rezaie et al., 1994). Although PC can be activated by the thrombin-Tm complex in solution, it has been demonstrated that activation is greatly accelerate by membrane surfaces. The PC Gla domain is thought to bind to negatively charge phospholipids in a Ca^{2+} dependent manner (Xu et al., 1999). However, negatively charged phospholipids are not exposed on the surface of healthy vascular endothelial cells (Fukudome and Esmon, 1994). It is thought that *in vivo* the PC Gla domain binds to the transmembrane endothelial cell protein C receptor (EPCR) directly, thus increasing the local concentration of PC and augmenting the activation process (Stearns-Kurosawa et al., 1996) (Fukudome et al., 1998) (Taylor, Jr. et al., 2001).

EPCR is thought to be of particular relevance in large vessels, where it is likely to compensate for the low effective Tm concentration found in such vessels (Maruyama et al., 1985b) (Laszik et al., 1997) (Fukudome et al., 1998); see section 1.4.2.

Once generated, APC is capable of inactivating coagulation factors Va and VIIIa, by proteolytic cleavage, thereby attenuating thrombin generation and downregulating the coagulation cascade (Kisiel et al., 1977). Three APC cleavage sites have been identified in FVa, at positions Arg 306, Arg 506 and Arg 679. Cleavage at Arg 506 reduces FVa activity, whereas subsequent cleavage at Arg 306 completely inactivates FVa (Mather et al., 1996). Cleavage at Arg 679 does not appear to play an important role in the inactivation process (Egan et al., 1997). Similarly, FVIIIa inactivation requires cleavage at three separate positions Arg 336, Arg 562 and Arg 740 (Fay et al., 1991) (Fay, 1999). APC mediated inactivation of factors Va and VIIIa, in plasma, requires the presence of protein S (PS) as a cofactor. PS is a vitamin K dependent protein consisting of a Gla-domain, a thrombin sensitive region, four EGF-like repeats and two laminin G-type domains (Lane et al., 1996a). PS circulates both in complex with C4bBP and free, and needs to be in its free form to be able to function as a cofactor for APC (Dahlback et al., 1986) (Dahlback, 1991). PS serves as a cofactor primarily by increasing the affinity of APC for phospholipid surfaces by forming a complex on the surface of endothelial cells and platelets (Walker, 1980) (Harris and Esmon, 1985). When PS binds to APC, the distance of the APC active site to the membrane surface is decreased by 10 Å, stimulating FVa cleavage at Arg 306 (Rosing et al., 1995) (Yegneswaran et al., 1997). In addition, PS and intact FV appear to work together, as cofactors for APC in the inactivation of VIIIa (Shen and Dahlback, 1994).

APC is itself inactivated and cleared from the circulation quite slowly, with a half-life of 15 minutes (Esmon, 2000), which will allow APC time to inactivate factors Va and VIIIa before inhibition occurs (Hermans and Stone, 1993). Protein C inhibitor (PCI), α1-antitrypsin (α_1AT) and α2-macroglobulin (α_2M) are responsible for the inhibition of APC activity (Suzuki et al., 1983) (Heeb and Griffin, 1988) (Heeb et al., 1991) (Espana et al., 1991) (Scully et al., 1993). All three APC inhibitors are found in normal blood plasma and circulate at a concentration of 88 nM, 40 μM and 3 μM, respectively. PCI and α_1AT are the major inhibitors of APC in plasma, with α_2M having a minor role (Heeb and Griffin, 1988) (Heeb et al., 1991). Both PCI and α_1AT are members of the serpin superfamily and as such display a protease inhibition profile for arginine specific serine proteases (Hermans and Stone, 1993). PCI inhibition of APC is accelerated by a range of GAGs and polyanions. The mechanism appears to involve the formation of a ternary complex, with the GAG bridging the serpin and protease (Pratt and Church, 1992) (Pratt et al., 1992). In contrast, α_1AT mediated inhibition of APC does not require GAGs for reversible inhibitor serpin-protease complex formation (Heeb and Griffin, 1988). α_2M inhibits APC in a calcium ion stimulated reaction. Unlike PCI and α_1AT, α_2M does not inhibit APC by altering the protease active site upon complex formation. Instead, α_2M is thought to entrap the protease in a cage-like structure (Barrett and Starkey, 1973) (Feldman and Pizzo, 1986) (Heeb et al., 1991). The protease, which retains some of its enzymatic activity, causes a conformational change by cleaving a region of α_2M, thus stimulating covalent attachment between reactive thioester groups of α_2M and NH2 groups of the protease to close the cage (Heeb et al., 1991).

1.2.2.6 Fibrinolysis

Fibrin, the major proteinaceous component of the clot, is removed upon activation of the fibrinolytic system. The process of fibrin deposition and removal help maintain blood fluidity and prevent excess blood loss at the site of injury (Collen and Lijnen, 1991) (Davie et al., 1991) (Irigoyen et al., 1999). During fibrinolysis, glu-plasminogen and t-PA bind to the surface of partially degraded fibrin. This binding is mediated by carboxy-terminal lysine residues on the surface of partially degraded fibrin, which are recognized by lysine binding sites in plasminogen and t-PA, resulting in the formation of a ternary complex (Christensen, 1985) (Fleury and Angles-Cano, 1991). Fibrin acts as a cofactor in the whole fibrinolytic cascade, since fibrin-bound plasmin is protected from inactivation by α2-antiplasmin (Sakharov and Rijken, 1995). Furthermore, fibrin enhances the conversion of glu-plasminogen to lys-plasminogen, which is a better substrate for t-PA, thus increasing the efficiency of plasmin generation (Hoylaerts et al., 1982). Plasmin subsequently cleaves fibrin through proteolytic cleavage at specific arginine and lysine residues (Collen and Lijnen, 1991). Plasmin is also able to activate metalloproteinases, which in turn degrade the vessel wall extracellular matrix (Birkedal-Hansen, 1995) (Lijnen and Collen, 1999).

The fibrinolytic cascade is in turn regulated by specific serine protease inhibitors, plasminogen activator inhibitor type 1 (PAI-1) and α2-antiplasmin (Robbie et al., 1993). α2-antiplasmin is responsible for the inactivation of free circulating plasmin. PAI-1 binds t-PA to form an activator-inhibitor complex, which is subsequently removed from the circulation (Nordenhem and Wiman, 1998). A new inhibitor of fibrinolysis has recently been identified. This metallocarboxypeptidase, thrombin activatable fibrinolysis inhibitor (TAFI), circulates in plasma as a zymogen (Eaton et al., 1991)

(Bajzar et al., 1995). TAFI is activated to TAFIa, by trypsin, plasmin and thrombin, by a single proteolytic cleavage at Arg 92 (Tan and Eaton, 1995) (Marx et al., 2002). Thrombin-mediated activation of TAFI is greatly accelerated by Tm (Bajzar et al., 1996a) (Bajzar et al., 1998). TAFIa, acts by cleaving the carboxy-terminal lysine residues off the surface of partially degraded fibrin, preventing glu-plasminogen and t-PA binding, thus hindering clot lysis (Sakharov et al., 1997) (Mutch et al., 2003).

Although the processes of coagulation, fibrinolysis and anti-coagulant mechanisms have been presented as somewhat independent events, *in vivo*, the picture is completely different, with the aforementioned processes forming an integrated response to vessel injury. When properly regulated each mechanism performs its physiological function extraordinarily well. An imbalance in regulation, however, has profound pathophysiological consequences, reflected in bleeding and thrombotic disorders. The next section will discuss the pathogenic aspects of thrombotic complications and particularly CHD, focussing on the role of the coagulation cascade.

1.3 Coronary Heart Disease (CHD)

1.3.1 Pathogenesis of CHD

The current consensus is that atherosclerosis is an inflammatory disease (Ross, 1999) and that inflammatory mediators play a key role in the initiation, progression and thrombotic complications of atherosclerotic plaques. The clinical manifestation of CHD results from the development of atherosclerotic plaques in the coronary arteries, which when ruptured lead to occlusive thrombus formation.

In the widely accepted response-to-injury hypothesis, endothelial injury, leading to compromised endothelial integrity and function, is a crucial early event in the development of atherosclerosis (Ross, 1999). Numerous factors may contribute to impaired endothelial function, including oxidised lipoproteins (Dart and Chin-Dusting, 1999), bacterial toxins (Ngeh et al., 2002), cigarette smoking products (Raij et al., 2001), diabetes mellitus (Yu et al., 2001), genetic alterations (Kinlay et al., 2001), hemodynamic forces (Gimbrone, Jr. et al., 2000), homocysteinaemia (Lentz, 1997), hypertension (Taddei et al., 2000) and a combination of these and other factors (Ross, 1999). Endothelial permeability is increased as a result of injury, leading to LDL accumulation in the subendothelial matrix, which is subsequently modified by oxidation, lipolysis, proteolysis and aggregation (Navab et al., 1996) (Steinberg, 1997). The accumulation of modified LDL, and oxidised LDL (ox-LDL) in particular, in the intima layer of the artery results in increased adhesion and migration of inflammatory leukocytes (monocytes and T-lymphocytes) across the endothelium. Ox-LDL stimulates the expression of adhesion molecules by the overlying endothelial cells (selectins, intercellular adhesion molecules and vascular adhesion molecules) that act as receptors for integrins and glycoconjugates present on inflammatory leukocytes (Lusis, 2000). Leukocytes subsequently migrate across the endothelium in response to chemoattractant molecules (monocyte chemotactic protein 1, osteopondin) (Rajavashisth et al., 1990). Uptake of modified LDL particles by monocyte-derived macrophages, leads to the formation of foam-cells (macrophages containing large amounts of cholesterol esters), which together with T lymphocytes form a fatty streak (Navab et al., 1996) (Steinberg, 1997) (Ross, 1999). These monocyte-derived macrophages are highly activated and secrete a range of cytokines and growth factors important for smooth muscle cell (SMC) migration and proliferation. A high proportion of foam cells die with time, to make up the lipid rich necrotic core of the lesion. This, together with the accumulation of

extracellular lipid, SMCs and SMC derived extracellular matrix elements (namely collagen) gives rise to a fibrous plaque, which is separated from the lumen by a collagen-rich fibrous cap. Calcification and neovascularisation further stabilise the atherosclerotic lesion, leading to the development of advanced plaques (Lusis, 2000).

Advanced lesions, lead to progressive narrowing of the vessel wall and blocking blood flow, potentially giving rise to ischaemic symptoms. It is, however, generally accepted that plaque rupture, and subsequent platelet adhesion and aggregation, together with thrombosis, results in clinical manifestations of CHD (Lusis, 2000) (Khrenov et al., 2002). The likelihood of rupture and thrombosis is dependent on the structure and cell content of the atherosclerotic plaque. Plaques likely to rupture are characterised by a large lipid core, increased number of inflammatory cells, decreased number of SMCs and a thin fibrous cap. Rupture of the atherosclerotic plaque exposes the thrombogenic lipid core of the lesion, abundant in cholesterol ester and TF, to blood components, with subsequent platelet adhesion and thrombus formation, ultimately resulting in myocardial infarction (MI) and stroke (Ross, 1999) (Davies, 2000) (Lusis, 2000) (Glass and Witztum, 2001).

1.3.2 Risk factors for CHD

CHD has emerged as the major cause of death in the Western society to date. It is the single most common cause of death in the USA and the principal cause of death in Europe and much of Asia (Braunwald, 1997) (Breslow, 1997). British Heart Foundation statistics published in 2001 showed that CHD is responsible for 120,000 deaths per year in the UK, with the majority of deaths caused by MI.

Epidemiological studies of CHD conducted in the 1940s and 1950s, such as the Framingham study, gave rise to the concept of cardiovascular risk factors (Castelli et al., 1986b) (Kannel, 2000). A prospective epidemiological approach demonstrated a consistent association of characteristics observed in apparently healthy individuals who went on to develop CHD. Although a statistically significant association between a trait and disease does not provide proof of causality or establish a pathophysiological relationship, such observations contribute to our understanding of pathophysiology. CHD is a multifactorial disorder and a number of well-accepted factors associated with increased risk of developing CHD have been identified to date. These risk factors can be divided into non-modifiable risk factors, such as age, gender, family history, and into modifiable risk factors including cholesterol, increased LDL-cholesterol, decreased HDL-cholesterol, hypertension, obesity, diabetes mellitus, lack of physical activity and cigarette smoking.

The risk of CHD increases with age in both men and women, most probably due to increased acquisition and exposure to risk factors (Stamler et al., 1999). Men below the age of 60 develop CHD at more than twice the rate of women (Hochner-Celnikier et al., 2002). Pre-menopausal women are at a significantly lower risk of CHD than men, developing CHD about 10 years later (Jousilahti et al., 1999). The difference in CHD risk for men and women diminishes and disappears after menopause, suggesting a role for hormonal factors in CHD risk determination. Oestrogen-replacement therapy has been shown to cause a reduction in cardiovascular risk (Kafonek, 1994), although more recent reports have shown that hormone replacement therapy (HRT) increases risk of venous thromboembolism, stroke and early CHD (Beral et al., 2002) (Lowe, 2004).

A family history of CHD is a very good predictor of the disease in individuals (Goldbourt and Neufeld, 1986). Most early cardiovascular disease events (< 40 years of age) have been shown to occur in families with a positive history of CHD, and in particular in individuals with a first-degree relative who has suffered a CHD event below the age of 65 (Pohjola-Sintonen et al., 1998). In addition, a large family based study, the Health Family Tree study, demonstrated that a high percentage of individuals with early CHD and early stroke have a positive family history (Koski et al., 2000) (Williams et al., 2001). In addition, genetic factors were found to influence death from CHD at younger age in both women and men, in a study of monozygotic and dizygotic twins (Marenberg et al., 1994). The observation that cardiovascular risk tends to cluster in affected families demonstrates the importance of genetic factors in the aetiology of CHD (Grant, 2003).

Environmental factors are also implicated in the manifestation of vascular disease. Numerous migration studies have indicated that migrants initially have the mortality of the country of origin but subsequently go on to acquire the risk of the country of destination. Such an observation was made in Finnish migrants to Sweden, where mortality rates decreased after 20 years (Hammar et al., 2002), in Asians migrating from China to Australia where an increase in mortality was seen after 5 years (Hsu-Hage and Wahlqvist, 1993) and in Japanese migrants to California, who when compared with native Japanese subjects, acquired a doubling of risk (Marmot et al., 1975). It is thought that environmental factors alter risk by interacting with numerous genetic factors thereby modifying synthesis and secretion of proteins associated with CHD risk.

Blood pressure shows a linear and graded correlation with CHD. Risk of CHD increases with elevated blood pressure and an additive effect on risk has been demonstrated for

elevated systolic (SBP) and diastolic (DBP) blood pressure (Kannel, 1986a) (Kannel, 1986b). Clinical trials have demonstrated a beneficial effect of successful blood pressure reduction on CHD risk (Hansson et al., 1998) (Himmelmann et al., 1998).

Changes in the plasma lipid profile of individuals are associated with CHD (Haim et al., 1999). LDL is the major carrier of cholesterol in blood, whereas HDL transports excess cholesterol back to the liver where it can be removed. The association of elevated levels of LDL-cholesterol and reduced levels of HDL-cholesterol with CHD has been demonstrated in many epidemiological studies (Armstrong et al., 1986) (Castelli et al., 1986a) (Tanaga et al., 2002). High levels of total plasma cholesterol are a strong predictor of CHD but may be misleading, since it may be due to increased HDL-cholesterol levels. The use of HDL/LDL ratio or total cholesterol/HDL-cholesterol ratio are therefore preferred as predictive values (Real et al., 2001). Elevated triglyceride levels have also been associated with increased incidence of CHD (Assmann et al., 1996) (Cullen, 2000). Clinical trials have demonstrated a reduced incidence of CHD upon cholesterol and triglyceride reduction (Maron et al., 2000).

Patients with diabetes have a two- to three-fold increased incidence of CHD (Garcia et al., 1974) and it is estimated that by the year 2010, the number of people in the world with diabetes will be around 221 million (Orchard, 1998), rising to 300 million by the year 2025 (King et al., 1998). Type 2 diabetes and CHD are thought to be closely linked disease entities (Pradhan and Ridker, 2002). The observation that atherosclerosis can precede the development of type 2 diabetes (Haffner, 2003) has led to the speculation that both conditions share common aetiological mechanisms (Stern, 1995) (Pradhan and Ridker, 2002), genetic or environmental, rather than being related as underlying disease (diabetes) and complication (atherosclerosis) (Stern, 1996). Recently, data have been

presented supporting a possible role for inflammation in insulin signaling and hence the development of type 2 diabetes (Yudkin, 2000). Increased sub-clinical inflammation, as determined by C-reactive protein (CRP) levels, a marker of acute inflammation (Hirschfield and Pepys, 2003), has been associated with insulin resistance and has been demonstrated to act as a good predictor of type 2 diabetes and CHD (Pradhan and Ridker, 2002) (Haffner, 2003). Furthermore, the clustering of insulin resistance with established cardiovascular risk factors, such as increased triglyceride levels, increased SBP, increased LDL-cholesterol levels, decreased HDL-cholesterol levels, obesity and hyperglycemia, referred to as the metabolic syndrome (Kaplan, 1989) (Haffner et al., 1992), is recognized as a common precursor of overt type 2 diabetes (Pradhan and Ridker, 2002). The above evidence supports the thesis that both type 2 diabetes and atherosclerosis share a common inflammatory basis (Haffner, 2002) (Pradhan and Ridker, 2002).

Obese individuals have a higher risk of developing CHD compared to lean individuals (Lamarche, 1998) (Stevens et al., 1998) and there is increasing evidence that adipose tissue in general and abdominal adiposity in particular, is a key player in the inflammatory process which contribute to the progression of atherosclerosis (Yudkin, 1999). Adipose tissue has been demonstrated to secrete pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999). Furthermore, a positive correlation between serum TNF-α levels and body mass index (BMI ; a measure of obesity) has been demonstrated, and in a separate study elevated circulating TNF-α levels have been observed in obese individuals (Mendall et al., 1997) (Ziccardi et al., 2002). It is, however, plausible that the observed increased risk in CHD

associated with obesity may actually be mediated via other risk factors, namely those that make up the metabolic syndrome with which obesity often clusters.

Of the behavioural risk factors, diet, cigarette smoking and physical inactivity have been extensively investigated. Atherosclerosis is characterised by the accumulation of cholesterol, inflammatory cells, fibrous tissue and calcium in the arteries. A diet high in fats, particularly saturated fats, results in an excess of cholesterol and triglycerides in the blood and has been associated with CHD risk (Schonfeld et al., 1982). Saturated fatty acids are the most important determinants of LDL-cholesterol, whereas monounsaturated and polyunsaturated fats have been found to lower LDL-cholesterol (Kris-Etherton et al., 1999). Excess sodium in the diet can also have detrimental effect on blood pressure (Weinberger, 1988). In contrast, a diet high in fresh fruit and vegetables has been shown to be beneficial in CHD prevention (Liu et al., 2001). The exact mechanism for this is unclear, but may be partly due to the beneficial effects of increased fibre, and antioxidants such as vitamin E, C and β-carbonate (Herbaczynska-Cedro et al., 1994) (Bullon et al., 1996) (Rinne et al., 2000).

The association between CHD and smoking is very strong and consistent, with a three-fold increased risk for CHD, and independent of other risk factors (Doll and Hill, 1966). Smoking can damage the endothelium, directly, leading to increased secretion of adhesion molecules, which enhance platelet and monocyte adhesion to the vessel wall, promoting thrombosis and atherosclerosis (Blann, 1992) (Blann et al., 1997). Smoking has been associated with lipid abnormalities, with smokers having reduced HDL-cholesterol levels but increased LDL-cholesterol and triglyceride levels (Craig et al., 1989) (Dullaart et al., 1994). Smoking may also favour the oxidation of LDL, since reduced levels of antioxidants have been determined in people who smoke (Fickl et al.,

1996). Smokers have raised plasma fibrinogen levels, prothrombin fragment 1+2 (F1+2) levels, increased FVII activity and reduced plasminogen levels (Meade et al., 1986) (Mendez et al., 1998), which may contribute to the adverse effects of smoking.

As for physical activity, it is hard to assess whether lack of exercise is an independent risk factor for CHD (Kannel et al., 1986). Regular exercise has, however, been shown to lower CHD risk and favourably effects blood pressure, weight, glucose tolerance, HDL-cholesterol, triglyceride levels and fibrinogen levels (Hardman, 1996).

1.3.3 The role of coagulation in CHD

Although thrombosis has been established as the major factor responsible for atherosclerosis-related mortality, factors contributing to increased thrombogenicity of atherosclerotic lesions have received little attention compared to factors associated with atherogenesis.

1.3.3.1 Relationship between haemostatic factors and disease

Endothelial injury is an early event in the development of the atherosclerotic plaque (Ross, 1999), which results in impaired endothelial integrity and function, shifting the anti-thrombotic nature of endothelial cells towards a pro-thrombotic state. The release of vasodilators, including nitric oxide (NO), prostacyclin, prostaglandin E2 and adenosine, is altered following endothelial cell injury and the anti-thrombotic nature of the endothelium is also altered following endothelial injury (Becker et al., 2000) (van Hinsbergh, 2001). Inflammatory cytokines, as well as bacterial products such as endotoxin, induce the expression of TF, responsible for the initiation of the coagulation

cascade (Moore et al., 1987) (Nawroth et al., 1986) (Nemerson, 1995) (Speiser et al., 2001). Inflammatory cytokines are also able to elicit a direct downregulatory effect on the expression of natural anti-coagulants, including Tm (Nawroth et al., 1986) (Moore et al., 1989) (Archipoff et al., 1991) (Lentz et al., 1991) (Speiser et al., 2001) (Nan et al., 2005), EPCR (Gu et al., 2000b) and TFPI (Shimokawa et al., 2000). In addition, mediators of inflammation have been shown to cause deregulation of natural fibrinolytic components. For example, TNF-α and interleukin-1 (IL-1), have been found to increase PAI-1 activity and expression *in vitro,* which would lead to decreased plasmin generation, reducing clot lysis efficiency (Orbe et al., 1999).

The initial response to vascular injury is the adhesion of platelets, which constitute the main component of coronary thrombi; see section 1.2.1. This is in part due to reduced NO, prostacyclin, prostaglandin E2 and adenosine release from endothelial cells (Becker et al., 2000) (van Hinsbergh, 2001), which are able to counteract platelet adhesion and activation (Cines et al., 1998). In addition, endothelial damage exposes the subendothelial collagen matrix, which allows platelet adhesion (Nakamura et al., 1999).

Soluble fibrin polymerises into fibrin strands, which stabilises the thrombi and blocks blood loss. Fibrin is deposited following the activation of the coagulation cascade and removed upon activation of the fibrinolytic system (Nesheim, 2003). Numerous genes involved in these pathways have been implicated in cardiovascular disease. Elevated plasma fibrinogen levels have consistently been associated with increased incidence of CHD in a number of prospective studies (Wilhelmsen et al., 1984) (Meade et al., 1986) (Heinrich et al., 1994). Meta-analysis of six prospective studies has suggested that healthy individuals with a fibrinogen level in the highest tertile were 2.3 times more likely to suffer a cardiovascular event than those in the lowest tertile of fibrinogen

distribution (Ernst and Resch, 1993). Whether fibrinogen is a causal factor in the development of atherosclerosis and its complications is still a matter of debate. Fibrinogen is incorporated into the atherosclerotic lesion where it is degraded, giving rise to fibrin and fibrinogen degradation products. Fibrinogen, as well as its degradation products, have been found to induce SMC proliferation and migration (Thompson and Smith, 1989) (Smith et al., 1990). Fibrinogen is also the major determinant of platelet aggregation (Bennett and Vilaire, 1979) (Marguerie et al., 1979) (Selwyn, 2003) and plasma viscosity (Ernst and Resch, 1993). Fibrinogen may thus play a direct role in the development of atherosclerosis, as well as its thrombotic complications. It should, however, also be kept in mind that fibrinogen is an acute phase protein (Ernst and Resch, 1993) (Sehnal and Slany, 2002). As aforementioned, atherosclerosis is an inflammatory disease (Ross, 1999) and many of the inflammatory mediators, found in plaques, may induce acute-phase proteins. As such, inflammatory cytokines, namely IL-6, have been found to increase hepatic synthesis and plasma levels of fibrinogen (Libby and Simon, 2001), and are thought to explain the observed association between cigarette-smoking and plasma fibrinogen levels (Woodward et al., 1998). This may suggest that fibrinogen does not represent a causal risk factor, but instead acts as a marker for atherosclerosis.

Of the proteins involved in the coagulation cascade, there has been much interest in the relationship between FVII, its activated form (FVIIa), and cardiovascular disease. This interest stems from the initial observation, made in the Northwick Park Heart study, that elevated levels of FVII coagulant activity were associated with coronary risk (Meade et al., 1986). Several subsequent studies have, however, either failed to confirm such an association (Smith et al., 1997) or have proven un-decisive (Junker et al., 1997). The extrinsic coagulation cascade, triggered by TF binding to FVIIa, is thought to be

responsible for the initiation of thrombus formation (Dahlback, 2000). As such FVII and its activated form could play a direct role in the thrombotic complications of atherosclerosis. Increased FVII coagulant activity may, however, denote an increased inflammatory state (Morrissey et al., 1993) (Nemerson, 1995) (Tapper and Herwald, 2000). As aforementioned, inflammatory cytokines, as well as bacterial products such as endotoxin, induce the expression of TF, which is known to enhance the enzymatic activity of FVIIa (Nawroth et al., 1986) (Moore et al., 1987) (Morrissey et al., 1993) (Nemerson, 1995) (Speiser et al., 2001).

The conversion of FXII to its active form, FXIIa, is thought to be the essential step initiating the activity of the contact phase of coagulation (Saito H, 1994). Contact with negatively charged surfaces is thought to lead to FXII activation (Morrison and Cochrane, 1974) (Hojima et al., 1984). FXIIa, through the activation of factors XI (Saito H, 1994) and VII (Seligsohn et al., 1979) of the coagulation cascade, could contribute to the propagation of coagulation, thus participating in the thrombogenicity of atherosclerotic lesions. Contradicting results have, however, been published regarding the association between elevated levels of FXIIa and CHD (Gordon et al., 1987) (Kelleher et al., 1992) (Kohler et al., 1998a). The independent association of FXIIa with several major CHD risk factors (Miller et al., 1997) (Kohler et al., 1998a), suggests that FXIIa levels may serve as a marker for an increased atherosclerotic state. Indeed atherosclerotic lesions are characterised by vascular endothelial damage, accumulation of ox-LDL and apoptotic cell death, processes which provide negatively charge phospholipids surfaces capable of enhancing FXII activation (Khrenov et al., 2002). This would lend weight to the suggestion that elevated concentrations of FXIIa are a consequence rather than a cause of underlying vascular disease.

As far as proteins involved in the fibrinolytic system are concerned, decreased t-PA activity might be expected to be associated with thrombotic risk, since it is involved in activation of the system (Collen, 1999) (Irigoyen et al., 1999). Interestingly two prospective studies (US Physicians Health study and the ECAT study) have found an increased risk of future MI in individuals with elevated t-PA levels (Ridker et al., 1994) (Thompson et al., 1995). The paradoxical association between elevated levels of t-PA and cardiovascular disease may reflect a positive correlation between circulating levels of t-PA and PAI-1, since the majority of t-PA is bound to PAI-1 in circulation (Reiner et al., 2001). Elevated levels of PAI-1, the major inhibitor of t-PA (Nordenhem and Wiman, 1998), have been associated with vascular disease fairly consistently (Hamsten et al., 1985) (Meade et al., 1993) (Margaglione et al., 1994) (Thogersen et al., 1998). Whether elevated PAI-1 levels play a direct role in the development of atherosclerosis or merely act as a marker for atherosclerosis remains to be determined. Elevated PAI-1 levels have been found to cluster with other vascular risk markers particularly in the presence of insulin resistance with or without diabetes mellitus (Juhan-Vague et al., 1991) (Juhan-Vague et al., 1993) (Juhan-Vague et al., 1996) (Mansfield et al., 1997), through which the observed association between PAI-1 levels and vascular disease may be mediated. In addition, PAI-1 expression and synthesis is enhanced by a number of inflammatory cytokines (Colucci et al., 1985) (Schleef et al., 1988) (Orbe et al., 1999), suggesting that PAI-1 might simply be denoting an enhanced inflammatory state. As far as TAFI, the recently identified inhibitor of fibrinolysis, is concerned, elevated plasma levels have been associated both with increased risk of coronary artery disease (Silveira et al., 2000) (Morange et al., 2003), as well as protection against MI (Juhan-Vague et al., 2002). Elevated plasma TAFI levels might be expected to be associated with increased risk, since its activated form TAFIa cleaves the carboxy-terminal lysine residues from the surface of partially degraded fibrin, thus preventing plasminogen and

t-PA binding and hindering clot lysis (Sakharov et al., 1997) (Mutch et al., 2003). The observation that TAFI antigen levels above the 90th percentile were protective against MI (Juhan-Vague et al., 2002) might be explained by the anti-inflammatory properties attributed to TAFIa, which has been shown to inactivate the complement-derived anaphylatoxin C5a (Campbell et al., 2001) (Campbell et al., 2002).

Thrombin is the pivotal enzyme of the coagulation cascade. In addition to promoting clot formation (Gailani and Broze, Jr., 1991) (Brummel et al., 1999), it acts as a mitogen for lymphocytes, fibroblasts and vascular SMCs (Chen and Buchanan, 1975), stimulates human platelet activation and aggregation (Bizios et al., 1986) (Harmon and Jamieson, 1986) (Huang and Detwiler, 1987), is chemotactic to monocytes (Bar-Shavit et al., 1983a) (Bar-Shavit et al., 1983b) and neutrophils (Sehnal and Slany, 2002), and induces the expression of leukocyte adhesion molecules and cytokines on the surface of vascular endothelial cells (Colotta et al., 1994) (Ueno et al., 1996). These cellular responses are in part mediated through the activation of G-protein-coupled protease activated receptors (PAR), namely PAR1 and PAR4 (Chung et al., 1990) (Rasmussen et al., 1991) (Vu et al., 1991). By triggering these inflammatory and proliferative processes, which are central to the development of atherosclerosis, thrombin is likely to play a central role in development of arterial thrombosis (Coughlin et al., 1992).

The roles of the anti-coagulant proteins AT, PC and PS have received some attention in relation to their deficient states. Whereas deficiencies of these anti-coagulant proteins have consistently been associated with increased incidence of venous thrombosis, tendency towards arterial thrombosis is not as common (Lane et al., 1996a) (Lane et al., 1996b) (Laffan and Tuddenham, 1997) (Simioni, 1999). AT deficiency has been reported in arterial thrombosis (Coller et al., 1987) (Johnson et al., 1990), particularly in

individuals who are homozygous for defects in the heparin-binding region (Lane et al., 1994). No clear association with arterial thrombosis has, however, been demonstrated for either PC or PS deficiency states. Of the proteins involved in the regulation of coagulation, Tm and EPCR have not been extensively investigated due to their relative inaccessibility on the endothelial cell surface. Variations in the genes encoding these proteins, leading to protein dysfunction, are candidate risk factors for arterial thrombosis. Tm in relation to CHD will be discussed in section 1.4.6.

1.3.3.2 CHD and polymorphisms in haemostatic factors

Genetic abnormalities that compromise the production, function, metabolism and availability of the aforementioned haemostatic factors may alter the physiological balance, thus favouring thrombosis and contributing to thromboembolic and atherothrombotic events.

Polymorphisms in the genes coding for the membrane GPs used by platelets to adhere and aggregate have been proposed to be associated with CHD (Mbopi-Keou et al., 2000). The Leu33Pro polymorphism in Gp IIb/IIIa has been extensively studied with regards to cardiovascular disease. An intial study reported an association of the 33Pro allele with risk of acute coronary thrombosis (Weiss et al., 1996). Subsequent studies have proven inconsistent, with large studies either being negative (Herrmann et al., 1997) (Ridker et al., 1997b), or positive only in some subgroups, such as young patients who were also smokers (Carter et al., 1997). In the gene encoding the GP Ibα subunit a variable number tandem repeat polymorphism (Lopez et al., 1992) (Ishida et al., 1996), in strong linkage disequilibrium with a Thr145Met substitution (Simsek et al., 1994), has been shown to alter the the structure of this platelet adhesion receptor (Lopez,

1994). Inconsistent results have, however, been reported regarding the relationship between these variant sites and cardiovascular disease (Gonzalez-Conejero et al., 1998) (Ardissino et al., 1999) (Douglas et al., 2002). In the gene encoding the GP Ibα subunit, a cpmponent of the GP Ib-IX-V complex, a -5T/C Kozak polymorphism has been of interest (Kaski et al., 1996). *In vitro* studies have suggested that this Kozak sequence may affect the surface expression of the GP Ib/IX/V complex on the platelet surface (Afshar-Kharghan et al., 1999). Inconsistent results have, however, been reported regarding the relationship between this variant site and cardiovascular disease (Corral et al., 2000b) (Croft et al., 2000) (Frank et al., 2001) (Douglas et al., 2002).

The fibrinogen protein, comprising two globular D domains and a central smaller globular E domain, is encoded by three genes denoted alpha, beta and gamma (Chung et al., 1990). A number of polymorphisms have been identified in the three genes encoding the protein: Thr312Ala and *Taq 1* polymorphisms in the alpha chain, and the -455G>A, -148C>T and Arg448Lys polymorphisms in the beta chain (Humphries et al., 1987) (Humphries et al., 1992) (Behague et al., 1996). Although these polymorphisms have consistently been associated with plasma levels of fibrinogen, inconsistent results have been reported regarding the relationship between these variant sites and cardiovascular disease (Lane and Grant, 2000).

Numerous polymorphisms have been identified in the genes encoding proteins of the coagulation cascade. As is the case for fibrinogen, polymorphic sites have been identified in the FVII gene, which have been associated with circulating levels of FVII (Bernardi et al., 1996). These include a decanucleotide insertion at position -323 (Marchetti et al., 1993), in strong linkage disequilibrium (LD) with Arg353Gln in the coding region of the gene (Green et al., 1991), a G to C substitution at position -401

(-401G>C), a G to A substitution at position -402 (-402G>A) (van 't Hooft et al., 1999) and an intronic length variant in the hypervariable region 4 of intron 7 (Pinotti et al., 2000). *In vitro* experiments have demonstrated lower secretion for the coding region variant (Hunault et al., 1997), whereas the promoter variants have been associated with altered transcription activity (van 't Hooft et al., 1999). Despite these findings, the relationship between FVII genotype and CHD risk has proven inconsistent (Lane and Grant, 2000).

A C to T change at position 46 of the FXII gene (46C>T) has been associated with low plasma levels of FXIIa (Kanaji et al., 1998) (Kohler et al., 1999) (Zito et al., 2000). This change has been shown to impair mRNA translation (AUG, methionine) *in vitro*, due to the introduction of a novel translational start site upstream of the correct start site, resulting in reduced FXIIa levels (Kanaji et al., 1998).

FV Leiden (FV Arg506Gln), which renders FVa resistant to APC cleavage, has been identified as the genetic defect responsible for most (90%) APC-resistant cases (Bertina et al., 1994) (Greengard et al., 1994) (Voorberg et al., 1994) (Dahlback, 1999). This results in increased thrombin generation due both to impaired downregulation of FVa pro-coagulant activity and altered FV anti-coagulant activity (cleavage at position 506 is required for FV to act as a cofactor to APC in the downregulation of FVIIIa) (Shen and Dahlback, 1994) (Varadi et al., 1996) (Thorelli et al., 1999). A prothrombin variant, 20210G>A, in the 3' untranslated region of the gene, has been shown, *in vitro*, to increase both the efficiency of mRNA processing and mRNA stability (Gehring et al., 2001) (Carter et al., 2002) and has been associated with increased prothrombin levels (Poort et al., 1996). Increased levels of prothrombin may enhance thrombin generation and thus lead to the formation of occlusive thrombi. Whereas both the FV Leiden and

the prothrombin 20210G>A mutations have consistently been associated with venous thrombosis, the role of either variant in arterial thrombosis remains controversial (Ridker et al., 1995) (Ferraresi et al., 1997) (Ridker et al., 1999) (Juul et al., 2002). Positive associations have mainly been found in studies where the two variants are present together (Rosendaal et al., 1997) (Doggen et al., 1998a).

FXIII plays a key role in determining fibrin clot structure. Once activated, by thrombin, FXIIIa cross-links the insoluble fibrin network giving rise to a stable clot that is more resistant to fibrinolysis (Gerth et al., 1974) (Roberts et al., 1974) (Lorand et al., 1980) (Naski et al., 1991). A common polymorphism in the FXIIIa gene (Val34Leu), which occurs three amino acids from the thrombin cleavage site, is thought to increase sensitivity to thrombin-mediated activation, leading to increased turn-over of active enzyme and enhancing the rate of fibrin cross-linking (Ariens et al., 2000) (Wartiovaara et al., 2000). Whereas two studies have suggested that the Val34Leu polymorphism is associated with reduced risk of MI (Kohler et al., 1998b) (Wartiovaara et al., 1999) other studies have failed to identify such an association (Canavy et al., 2000) (Corral et al., 2000a). Possible interactions between FXIII Val34Leu and other thrombotic risk factors may alter its effects on thrombotic risk (Kohler et al., 1998b) (Schroder and Kohler, 2000).

Of the proteins involved in the fibrinolytic cascade, a 311 bp Alu insertion/deletion in intron 8 of t-PA is the most studied nucleotide sequence, with respect to cardiovascular disease (Ludwig et al., 1992). Whereas an initial study suggested an association between the number of Alu repeats and arterial thrombosis (van der Bom et al., 1997), subsequent studies failed to confirm such an association (Ridker et al., 1997a). In addition, t-PA expression did not seem to be influenced by the Alu polymorphism

(Eijnden-Schrauwen et al., 1995). In a separate study, subjects homozygous for the Alu insertion were shown to have higher forearm vascular release rates of t-PA than those heretozygous and homozygous for the Alu deletion (Jern et al., 1999). Further analysis of the t-PA gene identified 8 novel polymorphisms, 3 of which (-7351C>T in the upstream enhancer, 20099T>C in exon 6, and 27445 T>A in intron 10) were in tight linkage disequlibrium with the Alu polymorphism (Ladenvall et al., 2000). The -7351 C>T polymorphism, which lies within an Sp1 transcription factor binding site, has been associated with reduced t-PA release and increased risk of MI (Ladenvall et al., 2000) (Ladenvall et al., 2002).

Several polymorphic loci have been identified in the PAI-1 gene to date, including a 3' *HindIII* site, a CA(n) dinucleotide repeat in intron 3 (Dawson et al., 1991) and a 4G/5G insertion/deletion at position -675 of the promoter (Dawson et al., 1993). Of these, the 4G/5G insertion/deletion polymorphism has been most frequently studied, with the 4G allele having been correlated with elevated plasma levels of PAI-1, an effect that was more pronounced in subjects with elevated triglyceride levels (Panahloo et al., 1995). A triglyceride responsive element has been identified close to the 4G/5G site (Eriksson et al., 1998), thought to explain the observed interaction with serum triglyceride concentrations. Conflicting data on the strength of the relationship between the 4G/5G insertion/deletion PAI-1 polymorphism and vascular disease have, however, been reported; reviewed by (Simmonds et al., 2001). Two separate meta-analyses have yielded an overall marginal increased risk of MI associated with the 4G allele (Iacoviello et al., 1998) (Boekholdt et al., 2001).

Several polymorphic sites have also been identified in the TAFI gene and account for much of the variability in protein levels. Of these, the Ala147Thr and 1542C>G

polymorphisms, in combination, were found to account for >60% of TAFI level variability, though the mechanism of action has yet to be determined (Henry et al., 2001) (Tregouet et al., 2001). Despite this strong genetic control on plasma TAFI antigen levels, there are inconsistent results from studies examining the relationship between TAFI sequence variations and disease (Juhan-Vague et al., 2002) (Morange et al., 2003).

Variants in the Tm gene in association with CHD will be discussed in section 1.4.7.

1.4 Thrombomodulin (Tm)

Tm was discovered by Esmon and Owen following an elegant and imaginative experiment. They showed that the perfused microvasculature of an isolated rabbit heart was able to accelerate the thrombin-mediated activation of PC by 20,000-fold (Esmon and Owen, 1981) (Esmon and Owen, 2004), which suggested the presence of an endothelial cell receptor that acts as a cofactor for thrombin. This cofactor was successfully isolated from rabbit lung homogenates (Esmon et al., 1982b) and termed 'thrombomodulin' because it was found to alter thrombin substrate specificity, converting thrombin enzyme activity from pro- to anti-coagulant (Esmon et al., 1982a) (Esmon et al., 1983).

1.4.1 The Tm molecule

The human protein has an apparent molecular weight of 75 kDa, following analysis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The apparent molecular weight exhibits a shift to 105 kDa, upon reduction of disulfide bonds, due to secondary structure involving many cysteine bridges (Esmon et al., 1982b). Structurally, Tm is organised into domains that resemble those of the LDL-receptor (Jackman et al., 1986). The translated protein consists of 575 amino acids. An 18-residue signal peptide domain, seemingly not involved in the localization of the protein, is cleaved, giving rise to the mature Tm protein. The mature Tm protein is 557 amino acids in length and consists of a large overall hydrophobic amino-terminal domain, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr rich domain, a transmembrane domain and a short cytoplasmic tail (Suzuki et al., 1987), illustrated schematically in Figure 1.3.

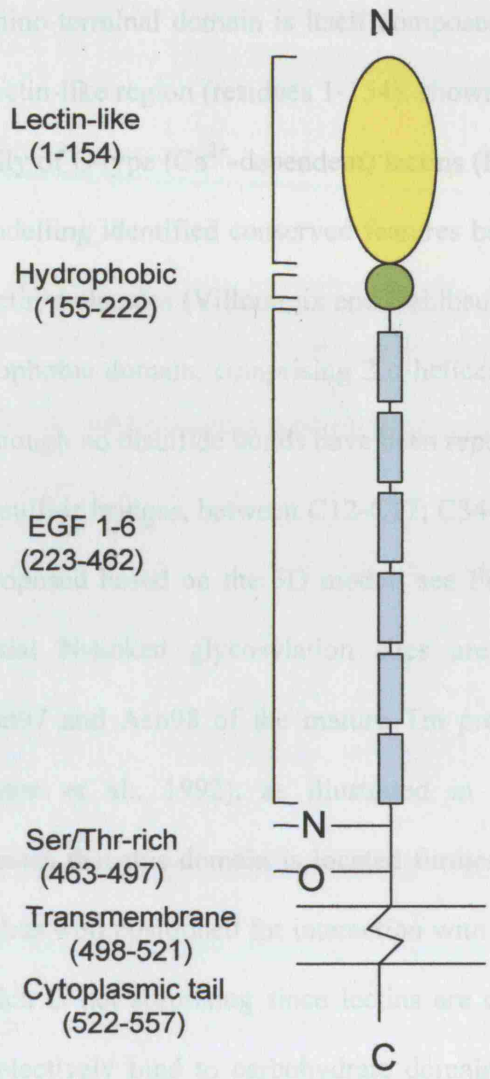


Figure 1.3 Schematic representation of the Tm protein.
Tm is a multidomain membrane-spanning protein with an extracellular amino-terminus. From its amino-terminus, Tm contains a lectin-like domain, a hydrophobic region, six epidermal growth factor-like (EGF) repeats, a serine/threonine rich region, a transmembrane domain and a cytoplasmic tail. The numbers represent the corresponding amino acids (numbering as in the mature protein) (adapted from (Weiler and Isermann, 2003)).

The large hydrophobic amino-terminal domain is itself composed of two subdomains. The first subdomain is a lectin-like region (residues 1-154), shown to have some degree of homology with the family of C-type (Ca^{2+}-dependent) lectins (Petersen, 1988). Three dimensional molecular modelling identified conserved features between the Tm lectin-like domain and C-type lectin molecules (Villoutreix and Dahlback, 1998). This domain folds into a globular hydrophobic domain, comprising 2 α-helices and 2 antiparallel β-sheets; see Figure 1.4. Although no disulfide bonds have been reported in the Tm lectin-like domain, 4 potential disulfide bridges, between C12-C17; C34-C149; C78-C115 and C119-C140, have been proposed based on the 3D model; see Figure 1.4. In addition, three of the five potential N-linked glycosylation sites are located within this subdomain, at Asn29, Asn97 and Asn98 of the mature Tm protein (Villoutreix and Dahlback, 1998) (Parkinson et al., 1992), as illustrated in Figure 1.4. Electron microscopic analysis indicates that this domain is located furthest from the surface of the cell membrane and is thus well positioned for interaction with other proteins or cells (Weisel et al., 1996), which is not surprising since lectins are considered to be non-enzymatic proteins that selectively bind to carbohydrate domains (Drickamer, 1988). The lectin-like domain does not appear to be required for Tm to function as a cofactor in the thrombin-mediated activation of PC (Conway et al., 1997) (Conway et al., 2002). Although questions still remain with regards to the biological function of the Tm lectin-like domain, a number of studies have suggested that this domain provides Tm with properties distinct from its established role as an anti-coagulant. As such, it is postulated that this domain may mediate the internalization of the molecule (Conway et al., 1997), regulate cell proliferation and tumour growth (Zhang et al., 1998), mediate cell adhesion in a Ca^{2+}-dependent manner (Huang et al., 2003) and regulate endothelial function in inflammation (Conway et al., 2002). The second subdomain is a largely hydrophobic region (residues 155-222), which lies adjacent to the EGF-like domain. Large areas of

identity have been reported between the human and mouse cDNAs in this region, which would imply an important function (Sadler, 1997). The biological function of this domain has, however, yet to be determined.

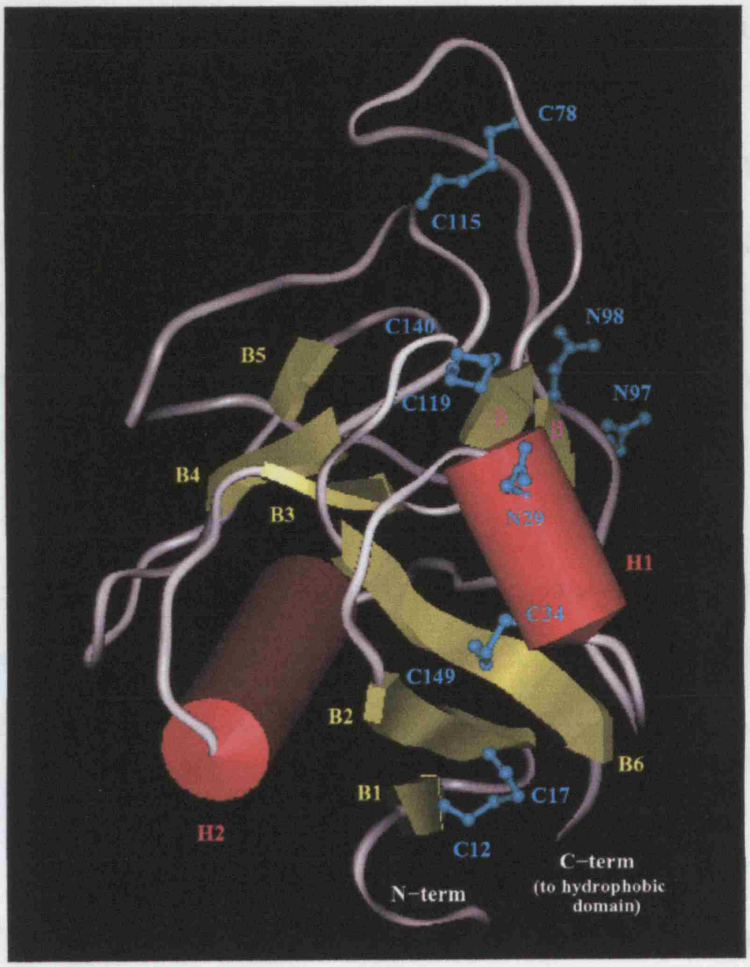


Figure 1.4 Tm lectin-like domain three dimensional structure.
A-helices (red), β-sheets (yellow), Disulfide bridges (light green), asparagine residues glycosilated (dark green). Reproduced from (Villoutreix and Dahlback, 1998) with permission.

EGF structural motifs are relatively common in receptors and coagulation proteins, occurring in multiple copies that form extended structures (Campbell and Bork, 1993). Tm contains six tandemly repeated EGF-like domains (residues 223-462). Thrombin binding is mediated via the 5th and 6th EGF-like domains (Kurosawa et al., 1988) (Ye et al., 1992). EGF4-6 are required for Tm to accelerate the thrombin-mediated activation of PC (Stearns et al., 1989). Recently, it has been determined that the 3rd EGF-like repeat is necessary for the activation of TAFI (Kokame et al., 1998). The Tm EGF-like repeats are thought to participate in post-translational modification of the protein, since a consensus sequence (CXD/NXXXXF/YXCXC) required for the hydroxylation of Asp or Asn residues has been identified in two of the six EGF-like regions of bovine Tm (344-346, EGF-3; 687-689, EGF-6) (Stenflo et al., 1988).

The structural characteristics of EGF 4, 5 and 6 have been studied by NMR (Adler et al., 1995) (Sampoli Benitez et al., 1997), as well as X-ray crystallography (Fuentes-Prior et al., 2000). The detailed secondary structure of these three domains varies considerably, probably due to the necessity of adaptation to different ligands and function. The 4th and 6th EGF-like Tm domains fold similarly to other EGF-like domains. EGF-like domains typically contain six cysteine residues that exhibit disulfide pairing between the 1st and 3rd, the 2nd and 4th and the 5th and 6th cysteine residues and consist of a flat major and twisted minor β-sheet. They can be divided into two subdomains, each containing a single large loop. The amino-terminal subdomain contains disulfide pairings between the 1st and 3rd and the 2nd and 4th cysteine residues. The stretch of residues between the 3rd and 4th cysteine residues form the large amino-terminal subdomain loop (the B-loop). The carboxy-terminal subdomain contains a single disulfide bond between the 5th and 6th cysteine residues. The stretch of amino acids between these two cysteine residues form the carboxy-terminal subdomain loop

(the C-loop), which forms a short tri-stranded β-sheet (Sampoli Benitez et al., 1997). Despite the observation that both EGF4 and EGF6 follow the classical scheme of EGF-like domains, they differ considerably in detail. In EGF4, residues Val371-Phe389 (the C-loop) constitute the initiation site for folding. Hydrophobic interactions centered on Phe376 and Ile379, stabilize the two β-turns between Ala373-Phe376 and Ile379-Glu382. A third, less defined turn occurs between Glu382-Arg385. The segment connecting the 4th and 5th EGF-like domains (Gln387-Phe389) is clamped to EGF4 by hydrogen bonds and to EGF5 through major hydrophobic interactions. Of particular importance is Met388, which slots into a hydrophobic groove spanned by the disulfide bridges formed between the 1st and 2nd and the 5th and 6th cysteine residues (see below) of EGF5, thus anchoring EGF4 to EGF5 and orientating EGF4 almost perpendicular to the linear EGF56 tandem (Fuentes-Prior et al., 2000). The importance of this loop in juxtaposing the thrombin binding EGF-like repeats of Tm (EGF5 & 6) with EGF4 is underlined by the observation that Tm anti-coagulant activity is drastically reduced upon oxidation of Met388 (Glaser et al., 1992) (Clarke et al., 1993). The 5th EGF-like repeat exhibits a unique non-crossed disulfide bonding pattern, with disulfide pairings occurring between the 1st and 2nd, the 3rd and 4th, and the 5th and 6th cysteine residues. This structural feature induces a bend bringing the amino- and carboxy-terminal loops closer together. This results in the concentration and exposure of most of the residues involved in thrombin binding on one face of the molecule. Thrombin affinity of this disulfide isomer (1-2, 3-4 and 5-6 pairing) is higher that that of the common one (1-3, 2-4 and 5-6 pairing) (Sampoli Benitez et al., 1997). Cardinal to the overall domain folding is a calcium ion with octahedral coordination as predicted by mutagenesis (Nagashima et al., 1993) and calcium binding studies (Light et al., 1999); see Figure 1.5. Two potential N-linked glycosylation sites have been identified in the 4th and 5th EGF-like

domains, of the mature Tm protein, at Asn364 and Asn391, respectively (Parkinson et al., 1992).

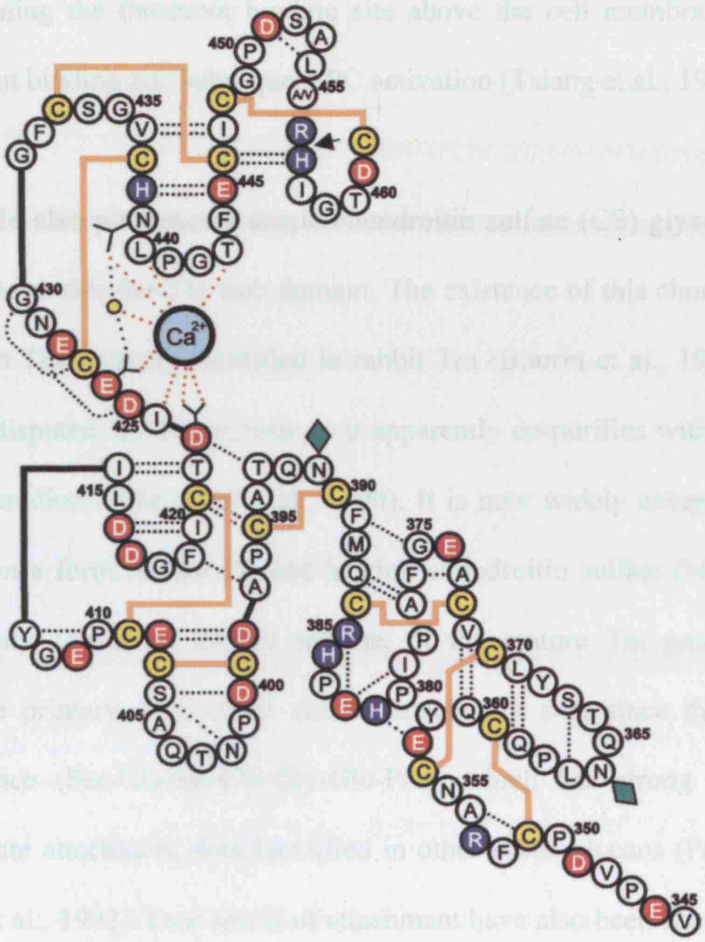


Figure 1.5 Sequence and secondary structure of the 4th, 5th and 6th EGF-like repeats of human Tm.
Acidic residues; red, basic residues; blue. Protein-protein hydrogen bonds; black dotted lines, those made with the bound calcium ion; red dotted lines. Disulfide bridges; orange. Glycosylation sites, green diamonds. H_2O molecule, Yellow circle. Reproduced from (Fuentes-Prior et al., 2000) with permission.

A Ser/Thr rich domain (residues 463-497) follows the EGF-like repeats. This domain is the major site of O-linked glycosylation, with three of the seven potential O-linked glycosylation sites located here (Suzuki et al., 1987). This region is thought to act as a 'spacer', positioning the thrombin binding site above the cell membrane, facilitating efficient thrombin binding and subsequent PC activation (Tsiang et al., 1992).

The Tm molecule also possesses a single chondroitin sulfate (CS) glycosaminoglycan molecule attached to this Ser/Thr rich domain. The existence of this chondroitin sulfate moiety in human Tm, initially identified in rabbit Tm (Bourin et al., 1988) (Bourin et al., 1990), was disputed for a long time as it apparently co-purifies with vitronectin in human Tm preparations (Preissner et al., 1990). It is now widely accepted that Tm is expressed both in a form containing and lacking chondroitin sulfate (Nadanaka et al., 1998). The highly conserved Ser474 residue, of the mature Tm protein, has been identified as the primary chondroitin sulfate attachment site, since this residue lies within a sequence (Ser-Gly-Ser474-Gly-Glu-Pro), which has strong similarities to chondroitin sulfate attachment sites identified in other proteoglycans (Parkinson et al., 1992) (Gerlitz et al., 1993). Low levels of attachment have also been shown for Ser472. The overall degree of attachment can be improved by mutating Ser 472 to Glu, which alters the sequence surrounding Ser474 so that it conforms to the xylosyltransferase acceptor consensus sequence acidic-Gly-Ser-Gly-acidic. A potential acceptor consensus sequence overlap at Ser474 suggests that the mechanism behind glycosaminoglycan attachment, to the Tm molecule, may involve a competition for substrate between xylosyltransferase (the enzyme that initiates O-linked glycosaminoglycan attachment) and N-acetylgalactosaminyltransferase (the enzyme that initiates the attachment of simple polysaccharides to the hydroxyl groups of serine/threonine residues) (Gerlitz et al., 1993).

The Ser/Thr rich domain is followed by a 24 amino acid (residues 498-521), highly hydrophobic, transmembrane domain, responsible for membrane insertion (Suzuki et al., 1987). This region is highly conserved between species. Like Tm, the transmembrane domain of the platelet derived growth factor receptor (PDGF), demonstrated to participate in transmembrane signaling, is highly conserved between species (Escobedo et al., 1988). This observation might suggest a signaling role for the Tm transmembrane domain, though this has yet to be demonstrated experimentally.

The last domain of the Tm molecule, consists of 36 amino acids (residues 522-557) and is located on the cytoplasmic side of the plasma membrane (Suzuki et al., 1987). The cytoplasmic tail of Tm contains potential phosphorylation sites namely serine, threonine and tyrosine residues. Phosphorylated regions have been associated with increased endocytosis and degradation (Dittman et al., 1988).

1.4.2 Tm location

The major site of Tm expression is the endothelial cell surface, where about 100,000 molecules of Tm are expressed per cell (Maruyama et al., 1985a). Tm has been identified also in a number of different cell types including platelets (60 molecules/cell) (Suzuki et al., 1988), monocytes (50,000-100,000 molecules/cell) (McCachren et al., 1991), neutrophils (5,000 molecules/cell) (Conway et al., 1992b), mesothelial cells (Collins et al., 1992), syncitiotrophoblasts of the placenta, adherent synovial cells (> 50,000 molecules/cell) (Conway and Nowakowski, 1993) and SMC in culture (Soff et al., 1991).

Tm concentration has been found to vary with vessel size. Effective Tm concentration increases as the surface area to blood volume ratio rises (surface area/blood volume) (Esmon, 1989). This ratio has been shown to change more than 1000-fold, moving from major vessels to the microvasculature (Busch et al., 1982). Assuming there are around 100,000 Tm molecules per endothelial cell (Maruyama et al., 1985a), major vessels would have an effective Tm concentration of 0.15 nM, whereas the microvasculature would have a Tm concentration of 500 nM (Esmon, 1989). As such, capillaries, which comprise more than 99% of endothelial surface area, contain the majority of Tm.

Although the prime physiological site for Tm is thought to be on the surface of endothelial cells, smaller heterogeneous soluble Tm fragments, collectively termed soluble Tm (sTm), have been detected in plasma and urine of healthy individuals (Ishii and Majerus, 1985). Four (64, 60, 52 & 47 kDa) (Takano et al., 1990), six (105, 85, 80, 56, 31, 28 kDa) (Ishii et al., 1990), or seven (94, 74, 48, 36, 27, 14, 12 kDa) (Uehara et al., 2001) molecular subspecies of plasma Tm fragments have been reported. The structure of sTm is not known. Part of the soluble form of Tm is, however, smaller than the cellular form, presumably because it represent a cleaved form of the surface associated Tm, with loss of part of the Ser/Thr rich domain, the transmembrane domain and the cytoplasmic tail (Ishii and Majerus, 1985). In endothelial cell culture, activated leukocytes and hydrogen peroxide release sTm in the culture media, suggesting that sTm results from cellular damage, and supporting the thesis that sTm represents a cleaved form of membrane associated Tm (Ishii et al., 1991).

sTm circulates at a concentration of around 20 ng/ml (Takano et al., 1990), but elevated levels have been detected in various clinical conditions including cardiovascular disease and diabetes (Ishii et al., 1991) (Inukai et al., 1996). Consequently, sTm is traditionally

thought to serve as a marker of endothelial cell damage (Ishii and Majerus, 1985) (Takano et al., 1990) (Ishii et al., 1991) (Borawski et al., 2001). A number of studies have, however, demonstrated that the variously sized Tm molecular subspecies, produced from the endothelial cell surface during the degradation process, retain Tm functional activity (Hosaka et al., 1998) (Takahashi et al., 1998), though the range of cofactor activity may vary (Ishii et al., 1990) (Uehara et al., 2001). Additionally, recent findings suggest that sTm may possess anti-inflammatory and anti-coagulant properties (Uchiba et al., 1996) (Mohri et al., 1998) (Conway et al., 2002), thereby conferring protection against atherosclerosis and its complications. It has been suggested that plasma sTm levels in healthy individuals may result from the constitutive cleavage of membrane bound Tm, and may thus reflect the levels of Tm synthesized and expressed in endothelial cells. A large prospective study, the Atherosclerosis Risk in Communities (ARIC) study, demonstrated a reduced risk of CHD in healthy individuals with elevated plasma sTm levels (Salomaa et al., 1999).

1.4.3 Tm function

Tm is found mainly on the surface of vascular endothelial cells, where it serves as a major anti-coagulant. Recently additional functions have been identified for Tm, independent of its anti-coagulant activity, including a role in development, fibrinolysis and inflammation.

1.4.3.1 Tm involvement in neonatal development

Tm-deficient (Tm -/-) mouse embryos do not survive past embryonic day 8.5, due to an overall retardation in growth, and are completely resorbed 24 hours thereafter (Healy et al., 1995). At this developmental stage, Tm is expressed in the giant trophoblast cells and the parietal endoderm cells, which come into direct contact with maternal blood (Isermann et al., 2001). The subsequent observations that removal of Tm deficient mouse embryos from the maternal deciduas (Healy et al., 1995), and the selective expression of Tm in the placenta (namely the trophoblast and parietal endoderm cells) (Isermann et al., 2001), prevented early embryonic lethality, led to the conclusion that loss of Tm function from the placenta is what causes the abortion of Tm -/- embryos (Healy et al., 1995). It was initially postulated that loss of Tm in embryonic trophoblasts would lead to increased thrombin deposition at the maternal-embryonic interface, resulting in blood clot formation and placental infarction, thus contributing to the Tm -/- lethal phenotype (Healy et al., 1995) (Rosenberg, 1997). Tm -/- embryos, however, failed to show increased fibrin deposition in the placenta (Weiler-Guettler et al., 1998). Loss of Tm function was, however, found to correlate with a complete loss of diploid trophoblast cell proliferation and apoptotic cell death of giant trophoblast cells (Isermann et al., 2003). In a subsequent set of experiments, Tm -/- mice were shown to survive to birth and early adulthood when crossed with TF deficient mouse embryos or animals expressing minute TF activity (Isermann et al., 2003). TF co-localises with Tm in giant trophoblast cells (Isermann et al., 2003) and is a potent initiator of the blood coagulation cascade (Bevilacqua et al., 1984) (Broze, Jr. et al., 1990) (Mann, 1999); see section 1.2.1. It is now thought that the developmental arrest of Tm-null embryos is TF dependent and involves two distinct mechanisms (Isermann et al., 2003). TF causes death of giant trophoblast cells, indirectly, through the generation of thrombin, the

conversion of fibrinogen to fibrin and the subsequent generation of fibrin split products. Tm is able to prevent trophoblast death by suppressing thrombin formation and limiting fibrin split product generation through the activation of TAFI; see section 1.2.2.6. On the other hand, TF is thought to induce growth retardation of diploid trophoblast cells (the precursor pool to giant trophoblast cells) through thrombin mediated activation of PAR2 or PAR4, since thrombin as well as PAR2 and PAR4 agonist peptides were able to inhibit proliferation of cultured diploid trophoblasts. The observation that PAR1 activation stimulates cell proliferation led to the suggestion that the Tm-PC system may help maintain placental growth. It has been shown that sustained thrombin generation, through the constitutive expression of TF at the trophoblast surface, leads to APC generation by the Tm-thrombin complex and subsequent activation of PAR1 by the APC-EPCR complex (Isermann et al., 2003). Thus it appears that the critical function of Tm in embryogenesis is to control the activation of the coagulation and fibrinolytic cascades, as is the case on the vascular endothelial cells surface. The observation that the cytoplasmic domain of Tm does not appear to play a role in embryonic development, since mice lacking this domain developed normally (Conway et al., 1999), would support this notion. Although the importance of Tm in neonatal development has been demonstrated in mouse models, the conserved expression of Tm (Fazel et al., 1998) and PAR1 (Even-Ram et al., 1998) in trophoblasts of the human placenta and a similar structural organisation of the placenta that places the fetal trophoblast in direct contact with the mother's blood (Cross et al., 1994), suggest that Tm is relevant and important to human reproduction.

1.4.3.2 Tm anti-coagulant activity

Tm exerts its anti-coagulant function through interactions with thrombin and PC. Tm, expressed on the surface of vascular endothelial cells, forms a high affinity 1:1 complex with thrombin (Owen and Esmon, 1981), which alters thrombin substrate specificity (Musci et al., 1988) (Ye et al., 1991) (Ye et al., 1992), converting thrombin enzyme activity from pro- to anti-coagulant (Esmon et al., 1982a) (Esmon et al., 1982b) (Esmon et al., 1983). As such, thrombin in contact with Tm is no longer able to clot fibrinogen (Esmon et al., 1982a), activate factors V (Esmon et al., 1982a) and XIII (Polgar et al., 1986) or platelets (Esmon et al., 1983). In addition to its direct anti-coagulant properties, Tm acts as a cofactor for thrombin in the activation of PC, greatly accelerating the rate of activation (Esmon et al., 1982b). Finally, Tm is also capable of enhancing the action of AT in inhibiting thrombin, through an attached chondroitin sulfate moiety (Koyama et al., 1991).

The 5^{th} and 6^{th} EGF-like repeats of Tm are required to bind thrombin (Kurosawa et al., 1988) (Ye et al., 1992). Tm is, however, able to bind a second thrombin molecule via the attached chondroitin sulfate moiety (Ye et al., 1993). Alanine scanning mutagenesis identified amino acid residues Glu408, Tyr413, Ile414, Leu415, Asp416, Asp417, Asp423, Ile424, Asp425 and Glu426, within EGF5 and 6 of the mature Tm molecule, as critical for thrombin binding. Asp398, Asp400, Asn402 and Asn429 in EGF 5, as well as Asp461 in EGF6 were also critical (Nagashima et al., 1993). Thrombin binding appears to be mediated via two discrete regions within EGF5 and 6 that make up the thrombin binding site on Tm. Two synthetic peptides corresponding to the entire third loop of the 5^{th} EGF-like domain (ECPEGYILDDGFICTDIDE; residues Glu408 to Glu426 of the mature Tm molecule) and parts of the second and third loops of the 6^{th}

EGF-like domain (residues Pro442 to Cys456 of the mature Tm molecule) (Hayashi et al., 1990) (Tsiang et al., 1992), respectively, competitively inhibited the binding of thrombin to Tm. The acidic tetrapeptide Asp-Ile-Asp-Glu that links the 5^{th} and 6^{th} EGF-like domains also participates in thrombin binding, since it was shown to contribute to the inhibitory activity of the first synthetic peptide (Tsiang et al., 1992). Tm binds to anion-binding exosite I of thrombin, a cluster of basic (Lys and Arg) residues distant from the active site residues Ser195, His57 and Asp102 (Kurosawa et al., 1988) (Bode et al., 1989) (Ye et al., 1992) (Mathews et al., 1994b).

The recent elucidation of the crystal structure of human α-thrombin bound to the smallest functional Tm fragment, composed of the last three EGF-like repeats, at a 2.3 Å resolution, revealed important details for the interaction of thrombin, Tm and PC in the activation complex (Fuentes-Prior et al., 2000). It provided a framework for the interpretation of the results of previous mutagenesis studies, which helped identify the critical amino acid residues required for cofactor activity (Fuentes-Prior et al., 2000) (Nagashima et al., 1993). In the stoichiometric complex formed between human α-thrombin and this EFG456 Tm fragment, an angular Y-shaped Tm fragment, in which EGF4 is anchored nearly perpendicular to the linear EGF5 and 6 tandem, is bound to an ellipsoidal α-thrombin molecule; see Figure 1.6. EGF5 and part of EGF6 bind to anion-binding exosite I of thrombin, whereas EGF4 and most of EGF6 protrude from the thrombin surface (Fuentes-Prior et al., 2000).

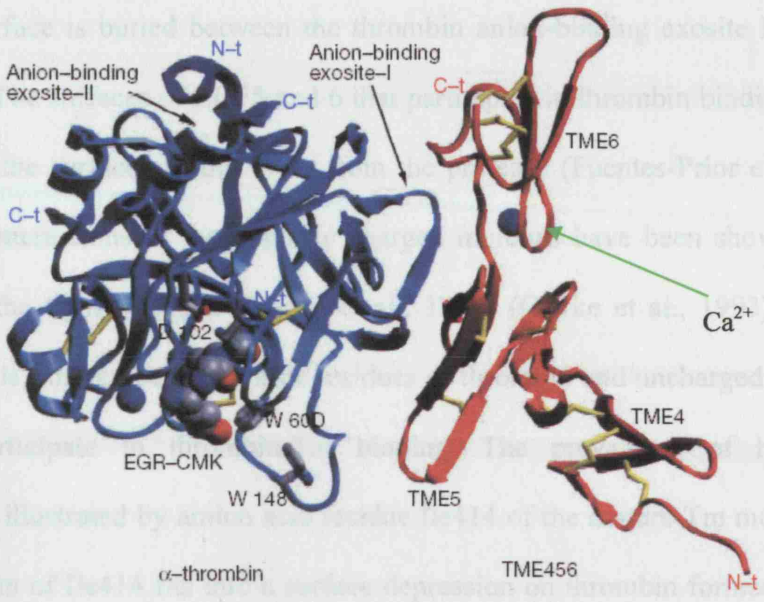


Figure 1.6 Ribbon model of complex formed between human α-thrombin and the EFG456 Tm fragment.
α-thrombin in blue, Tm fragment in red, disulfide bridges in yellow. The irreversible inhibitor L-Glu-Gly-Arg chloromethyl ketone (EGR-CMK) mimics the acidic P3 residue (see below) of the protein C activation peptide and is shown as a space-filling model with atoms color-coded (carbon, grey; nitrogen, blue; oxygen, red). The amino- and carboxy termini of all three chains are labeled. The buried calcium ion is shown as a blue sphere (indicated by green arrow). Reproduced from (Fuentes-Prior et al., 2000) with permission.

A 900 Å interface is buried between the thrombin anion-binding exosite I and EGF5 and 6 of Tm. The surfaces of EGF5 and 6 that participate in thrombin binding are more negative than the surfaces facing away from the protease (Fuentes-Prior et al., 2000). Electrostatic interactions of the opposite charged moieties have been shown to guide formation of the tight complex (Tsiang et al., 1992) (Clarke et al., 1993). However, hydrogen bonds, formed between basic residues of thrombin and uncharged residues on Tm, also participate in thrombin-Tm binding. The prevalence of hydrophobic interactions is illustrated by amino acid residue Ile414 of the mature Tm molecule. The alkyl side chain of Ile414 fits into a surface depression on thrombin formed by Phe34, Tyr76 and Ile82 with amino acid residues Leu65 of thrombin and Ile424 of Tm completing the hydrophobic patch (Fuentes-Prior et al., 2000). Both of these Tm residues have been shown to be essential for thrombin binding in previous mutagenesis studies (Nagashima et al., 1993). Finally, Ca^{2+} binding, to regions within the 3rd (amino acid residues Asp307 to Ala338 of the mature Tm molecule) and 6th EGF-like repeats (amino acid residues Asp423 to Ala453 of the mature Tm molecule) (Light et al., 1999), stabilizes the 5th and 6th EGF-like domains of Tm to form a region complementary to anion-binding exosite I of thrombin, increasing the binding affinity (Fuentes-Prior et al., 2000), and explaining the reduced cofactor activity of mutants of calcium ligands Asp423, Glu426 and Asn439 (Nagashima et al., 1993).

Tm binding alters thrombin specificity and enhances the rate of thrombin mediated PC and TAFI activation by at least three orders of magnitude (Esmon, 1995) (Bajzar et al., 1996a). Evidence has been presented suggesting that Tm binding causes allosteric changes that alter the conformation of the thrombin active site, thus enhancing thrombin-mediated activation of PC (Musci et al., 1988) (Ye et al., 1991) (Ye et al., 1992) (Yang et al., 2003). Acidic residues surrounding the thrombin active site were

also shown to play a key role in protein C activation (Gibbs et al., 1995). As a result, an additional model through which Tm could enhance thrombin-mediated PC activation was put forward. In this model, Asp residues in both the P3 and P'3 sites of PC are postulated to inhibit the activation of PC by thrombin, in the absence of Tm [P and P' refer to the distance from the Arg (P1) cleavage site moving towards the amino- and carboxy-terminus, respectively] (Le Bonniec and Esmon, 1991) (Le Bonniec et al., 1991). The replacement of Asp with Gly (neutral amino acid) in both the P3 and P'3 sites enhanced PC activation by free thrombin, an effect which was lost upon Tm binding (Ehrlich et al., 1990). These studies hint at the presence of key residues in thrombin that repel acidic residues at the P3 and P'3 sites of PC. Indeed, mutagenesis of the Glu residues at positions 192 and 39 of thrombin, to Gln and Lys, respectively, overcame the inhibitory influence (Le Bonniec and Esmon, 1991) (Le Bonniec et al., 1991).

The crystallographic studies of Fuentes-Prior *et al.*, suggest yet another mechanism to explain the Tm-induced switch in thrombin specificity. A high degree of conformational conservation was noted between the active site residues of thrombin (Ser195, His57 and Asp102; chymotrypsinogen numbering) and the surrounding acidic residues (Glu217, Glu192 and Glu39; chymotrypsinogen numbering), when comparing thrombin in the Tm complex with free thrombin (Bode et al., 1989) (Fuentes-Prior et al., 2000). Therefore, a conformational change in the active site and extended binding pocket of thrombin did not appear to be the main mechanism through which the complex enhances the PC activation. The fundamental change in thrombin substrate specificity is now thought to be due to the structure of the 4^{th} and 5^{th} EGF-like repeats of Tm. The outer surface of Tm EGF4 is noticeably hydrophobic, as illustrated by amino acid residues Leu363, Leu369 and Pro380 of the mature Tm molecule. In contrast, a number

of polar and charged side chains (Arg385, His384, Glu382, Glu357, Asn355 and more distally Asp349 and Glu346) as well as aromatic residues (Phe352, Tyr358 and Phe376) are present on the surface of the 4th EGF-like repeat extending towards the thrombin active site groove. The side chains of Asp398, Asn402, Thr403, Gln404 and Glu408 of the 5th EGF-like repeat also project towards the active site of thrombin (Fuentes-Prior et al., 2000). All of these residues are critical for Tm cofactor activity (Nagashima et al., 1993) and it has been postulated that the clustering of these critical residues on the Tm EGF45 surface represents the interacting region of the thrombin-Tm region with PC. Docking experiments, in which a Gla-domainless activated PC molecule was docked to the thrombin-Tm EGF456 complex, satisfied important specific requirements of thrombin and identified important interactions between the thrombin-Tm activation complex and PC. Numerous interactions were observed between PC and EGF4 and 5 of Tm (Fuentes-Prior et al., 2000). The highly conserved PC Lys37-Lys38-Lys39 triplet (Gerlitz and Grinnell, 1996) projects towards the 4th EGF-like repeat and contacts Glu382 (Fuentes-Prior et al., 2000). In addition, the critical PC side chain of Arg74 (Vincenot et al., 1995) is clamped between the carboxylates of Asp398 and Glu357 in an electrostatic "grip" (Fuentes-Prior et al., 2000). Interactions between the calcium binding site of PC and the 4th and the 5th EGF-like repeats of Tm are anticipated by the model (Fuentes-Prior et al., 2000), in agreement with previous reports (Yang and Rezaie, 2003). Direct contacts between EGF4 side chains and PC residues (Arg385-Glu80) that coordinate Ca^{2+} have also been predicted, and might explain the unique Ca^{2+} dependence of thrombin-Tm mediated activation of PC. The above mentioned interactions, between PC and Tm, would allow the 4th and 5th EGF-like repeats of Tm to align PC in such a way that its scissile peptide bond (Arg15-Ile16) is presented to thrombin's active site residues, thus facilitating activation (Fuentes-Prior et al., 2000).

1.4.3.3 Tm and fibrinolysis

Paradoxically, Tm appears to exert opposite effects on the activation of the fibrinolytic cascade, since it possesses both pro- and anti-fibrinolytic properties.

As already mentioned (see section 1.2.2.6), fibrinolysis is initiated by the binding of plasminogen and t-PA to the carboxy-terminal lysine residues on the surface of partially degraded fibrin, resulting in efficient plasmin formation; reviewed in (Booth, 1999). TAFI, a newly identified inhibitor of fibrinolysis, circulates as a zymogen and is activated to TAFIa by trypsin, plasmin and thrombin through proteolytic cleavage at Arg92 (Eaton et al., 1991) (Bajzar et al., 1995) (Tan and Eaton, 1995) (Marx et al., 2002). TAFIa then catalyzes the removal of carboxy-terminal lysine residues from the surface of partially degraded fibrin, thus hindering clot lysis (Sakharov et al., 1997). Tm accelerates the activation of TAFI by thrombin (Bajzar et al., 1996a) (Bajzar et al., 1998), and in doing so is able to, indirectly, suppress plasminogen activation and fibrinolysis. Tm enhances activation of PC and TAFI via distinct EGF-like domains (Stearns et al., 1989) (Kokame et al., 1998). Unlike PC activation (see section 1.4.3.2), residues within the c-loop of the 3rd EGF-like repeat and the interdomain connecting it to the 4th EGF-like domain of Tm are important for TAFI activation. Mutagenesis studies identified residues Val340, Asp341, Glu343 and Asp349 of the mature Tm molecule as essential for TAFI activation, with residues Tyr337, Asp338, Leu339, Val345, Glu346 and Val348 also playing an important role (Wang et al., 2000). Despite these differences in elements of Tm required for TAFI and PC activation, Tm is thought to accelerate the thrombin-mediated activation of TAFI, not through allosteric alteration of the thrombin active site, but rather by optimally orientating the thrombin active site

residues with the activation peptide of TAFI, as is the case for PC (Schneider et al., 2002); see section 1.4.3.2.

The pro-fibrinolytic activity of Tm is mediated, indirectly, through the generation of APC. Tm greatly accelerates the rate of PC activation by thrombin (Esmon et al., 1982b). Once generated, APC is capable of inactivating coagulation factors Va and VIIIa, by proteolytic cleavage, attenuating thrombin generation (Kisiel et al., 1977), which should decrease the extent of TAFI activation, thus stimulating fibrinolysis (Bajzar et al., 1995) (Bajzar et al., 1996b). In addition, APC has been shown to form a tight inhibitory complex with PAI-1, protecting t-PA from PAI-1 mediated inactivation and promoting the fibrinolytic process (Sakata et al., 1986).

Tm concentration has been identified as the factor that determines whether fibrinolysis promotion or suppression, by the aforementioned processes, predominates. Low concentrations of Tm (5 nM) were found to favour TAFI activation, prolonging lysis time. In contrast, the activation of TAFI was decreased at higher concentrations of Tm (10 nM). The reduction of TAFI activation at high concentrations of Tm was shown to be dependent on the ability of APC to attenuate thrombin generation (Mosnier et al., 2001). It thus appears that Tm acts as an anti-fibrinolytic agent at low concentrations and a pro-fibrinolytic agent at high concentrations.

1.4.3.4 Tm anti-inflammatory activity

Tm functions as an anti-inflammatory agent by direct and indirect mechanisms of action. As aforementioned, thrombin contributes to inflammation by triggering a range of cellular responses, partly through the activation of PARs, namely PAR1 and PAR4 (Chung et al., 1990) (Rasmussen et al., 1991) (Vu et al., 1991). Thrombin recognises and binds an acidic sequence in the amino terminal exo-domain of PAR1, via anion-binding exosite I (Mathews et al., 1994a). Thrombin subsequently cleaves the scissile peptide bond (between residues Arg-41 and Ser-42) to unmask a new amino terminus, which binds to the body of the receptor, intra-molecularly, to promote trans-membrane signalling (Coughlin, 1999). Both Tm and PAR recognise the same site (anion-binding exosite I) on the surface of thrombin (Stubbs and Bode, 1995). As such Tm is able to prevent thrombin from activating PARs, through competitive inhibition (Ye et al., 1992).

Tm bound to thrombin accelerates the thrombin-mediated activation of TAFI into a carboxypeptidase B-like enzyme, termed TAFIa (Bajzar et al., 1996a) (Bajzar et al., 1998). This carboxypeptidase, through the removal of arginine residues from C5a octapeptide, is able to inactivate the complement derived anaphylatoxin C5a (Campbell et al., 2001) (Campbell et al., 2002), providing another indirect mechanism through which Tm may control inflammatory responses.

In addition to its role in limiting coagulation response to injury, the PC pathway also participates in regulating inflammatory processes by downregulating pro-inflammatory cytokines, blocking neutrophil interactions with selectins and suppressing expression of leukocyte adhesion molecules (Grey et al., 1994) (Hirose et al., 2000) (Joyce et al.,

2001) (Esmon, 2002). Generation of APC is central to these functions. APC bound to EPCR has been shown to cleave the PAR1 receptor in endothelial cells. In doing so, the APC-EPCR complex is thought to trigger a range of cellular responses related to anti-apoptotic and anti-inflammatory activities (Riewald et al., 2002). By accelerating the thrombin mediated activation of PC, Tm triggers the conversion of the inactive PC-EPCR complex into the signaling competent APC-EPCR complex, thus indirectly regulating inflammatory responses. Whether or not APC-EPCR mediated activation of PAR1 is of physiological relevance is still a matter of debate (Esmon, 2005). When compared to thrombin, APC was 10^4-fold less potent at activating PAR1, with 100-times higher concentrations of APC required for PAR1 activation. Furthermore, co-incubation of thrombin and PC on endothelial cells resulted in APC generation as expected, since thrombin is required for PC activation; see section 1.2.2.5. The locally generated APC was, however, not able to potentiate PAR1 activation, above and beyond that seen with thrombin alone (Ludeman et al., 2005). These observations led to the suggestions that at physiological relevant concentrations APC is unlikely to contribute to PAR1 activation (Esmon, 2005) (Ludeman et al., 2005).

A recent study, suggests that Tm regulates inflammation through a direct mechanism independent of thrombin, TAFI and APC (Conway et al., 2002). Conway *et al.* demonstrated that mice lacking the Tm lectin-like domain exhibited reduced survival in response to endotoxin. These mice had elevated cytokine levels (TNF-α & IL-1β), increased leucocyte accumulation in their lungs, and increased neutrophil adhesion to endothelial cells as a result of increased intracellular adhesion molecule-1 (ICAM-1) expression. In response to LPS, mice lacking the lectin-like domain exhibited augmented activation of MAP kinase $ERK_{1/2}$, a signaling pathway implicated in the regulation of pro-inflammatory and adhesion molecules. This observation would

suggest that the lectin-like domain of Tm triggers intracellular pathways, which alter gene expression. Interestingly, a soluble, recombinant form of the Tm molecule, comprising the lectin-like structure, was able to restore normal leukocyte adhesion to endothelial cells by inhibiting the TNF-α induced activation of $ERK_{1/2}$ (Conway et al., 2002). The molecular interactions by which the Tm lectin-like domain exerts its anti-inflammatory effects are still unknown.

Recently, a novel mechanism through which Tm may function in thrombosis, inflammation and atherosclerosis has been suggested (David-Dufilho et al., 2005). These authors have shown that at low concentrations, thrombin binding to Tm activates NO production, in human endothelial cells, through the epidermal growth factor receptor (EGFR) kinase and calmodium kinase II (CaMKII). Tm is thought to activate a receptor tyrosine kinase analogous to that of EGFR, leading to the phosphorylation of phosphatidylinositol-3 kinase (PI3K) and phospholipase Cγ. CaMKII is subsequently activated through the generation of inosito 1,3,5-triphosphate (IP3) and Ca^{2+} release, leading to the formation of Ca-calmodium complex (*Ca-CaM*). *Ca-CaM* binding and PI3K- and CaMKII-dependent phosphorylation, activate NOS3 (nitric oxide synthase 3), resulting in NO synthesis. The thrombin-Tm complex also induced long-lasting *Src* family kinase phosphorylation, and modulated G-protein coupled receptor (GPCR)-induced Ca^{2+} signaling (David-Dufilho et al., 2005). Both *Src* family kinase phosphorylation and Ca^{2+} signaling are key regulators of cell proliferation through the MAP-ERK kinase signaling cascade (Berridge et al., 2000) (Stork and Schmitt, 2002) (Bromann et al., 2004). By activating NO synthesis and modulating the MAP-ERK kinase signaling cascade, Tm may regulate coagulation, fibrinolysis, inflammation, and cell proliferation (David-Dufilho et al., 2005).

1.4.4 Molecular biology of Tm

The Tm gene has been localized to the short arm of chromosome 20 (20p 11.2) (Espinosa, III et al., 1989); see Figure 1.7.

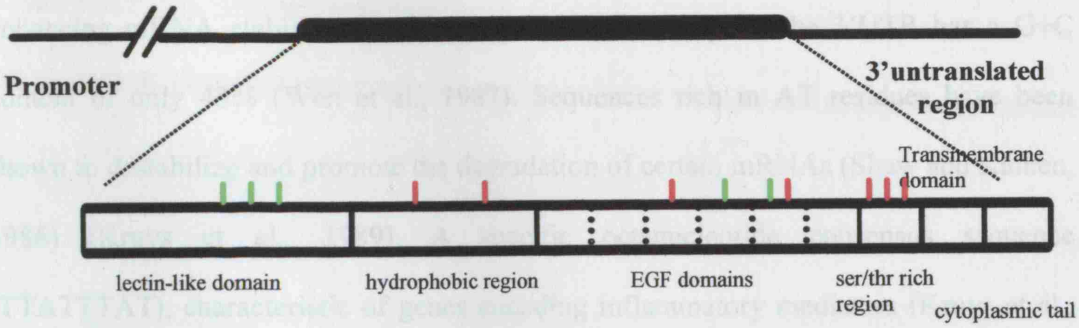


Figure 1.7 Cartoon of TM gene and encoded protein.
The Tm gene has been mapped to chromosome 20 p11.2, is 3.7 kb in length and contains no introns (Wen et al., 1987) (Jackman et al., 1987) (Shirai et al., 1988) (Espinosa, III et al., 1989). Green lines represents sites of N-linked glycosylation. Pink lines represent sites of O-linked glycosylation. Features of each domain are described in the text.

The human Tm cDNA is 3693 bp long (Wen et al., 1987). The transcription start site has been mapped to two positions 158 and 163 bp upstream of the ATG initiation codon (Jackman et al., 1987) (Yu et al., 1992). The coding region of 1725 nucleotides has an extremely large GC content (68%), resulting from long runs of Gs and Cs (Wen et al., 1987). Interestingly, the human Tm gene is intron depleted (Jackman et al., 1987). Genes encoding proteins of known structure that contain several functional domains, appear to almost always position the information coding for these domains on separate exons. Intronless genes are uncommon, though other genes, besides Tm, which completely lack introns have been described and include the human platelet GPs Ib α, Ib β, V and IX (Yagi et al., 1995), hamster β-adrenergic receptor, mammalian α- and β-interferons, angiogenin, histones and mammalian heat-shock proteins. The reason for

the lack of introns is unknown, but it has been suggested that the lack of introns could facilitate protein synthesis under conditions detrimental to RNA splicing (Jackman et al., 1987).

A 3' untranslated region (3'UTR) of 1779 bp, is followed by a 40 bp poly-A tail, enhancing mRNA stability. In contrast to the coding region, the 3'UTR has a G+C content of only 43% (Wen et al., 1987). Sequences rich in AT residues have been shown to destabilize and promote the degradation of certain mRNAs (Shaw and Kamen, 1986) (Kruys et al., 1989). A specific octanucleotide consensus sequence (TTATTTAT), characteristic of genes encoding inflammatory mediators (Kruys et al., 1989), is present in two overlapping copies of the 3'UTR (Wen et al., 1987). This particular consensus sequence has been associated with reduced translation efficiency, selective mRNA degradation and short mRNA half-life, for example, in the interferon and c-myc genes (Kruys et al., 1989).

A series of sequentially deleted fragments from the 5' flanking region of the Tm gene have been used to drive expression of a reporter gene. Key regulatory elements necessary for expression of the Tm gene were identified in endothelial cells (Yu et al., 1992) (Tazawa et al., 1993); see Figure 1.8. For clarity nucleotides are numbered according to (Yu et al., 1992).

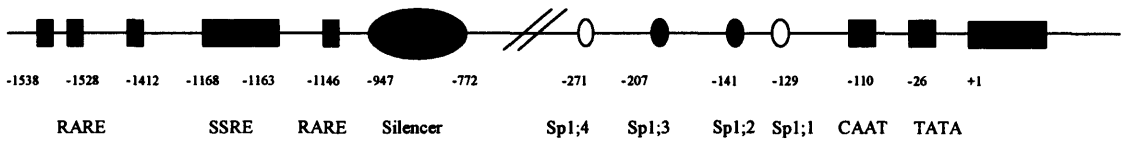


Figure 1.8 Schematic representation of TM promoter region.
Important regulatory elements depicted. Proximal promoter: TATA-box (-21 to -26), CAAT box (-106 to -110), four Sp1 transcription factor binding sites (-129, -141, -207 and -271) (Tazawa et al., 1993). Distal promoter: silencer element (-772 to -947) (Tazawa et al., 1993), SSRE (-1163 to -1168) (Takada et al., 1994), four RARE (-1141 to -1146, -1407 to -1412, -1523 to -1528 and -1533 to -1538) (Dittman et al., 1994). Abbreviations: **SSRE**: Shear Stress Response Element; **RARE**: Retinoic Acid Response Elements; +1: Coding Region. Numbering according to (Yu et al., 1992).

The region extending from -290 to -33 of the 5' flanking sequence was found to be required for full expression of the chimeric gene (Tazawa et al., 1993). This region was found to contain four potential Sp1 transcription factor binding sites, at positions -271, -207, -141 and -129, and a potential CAAT box sequence element GCAATC, at position -110, very similar to the typical CAAT motif (CCAATC) . Mutations were introduced into the core sequence of each potential Sp1 binding site and the potential CAAT box to determine their role in the Tm promoter activity. Site directed mutagenesis of the CAAT box decreased promoter activity, suggesting that this element is important for the full activity of the Tm promoter. Two of the four potential Sp1 sites, at –207 and -141, were identified as positive acting elements, since promoter activity decreased following site directed mutagenesis. Transcriptional activity was completely abolished upon deletion of the region extending from -32 to -11, a region which contains a functioning TATA-box at position -26 (Tazawa et al., 1993) (Yu et al., 1992). Progressive deletions of the region from -290 to -32 decreased transcriptional activity, with the exception of the region between -130 to -100, which when deleted increased promoter activity probably due to the presence of a negative acting element. A further negative acting element was located in the region spanning from position -947 to

-772 of the Tm 5' flanking sequence (Tazawa et al., 1993). Several consensus sequences have been identified within both the proximal (nucleotides -1 to -299, (Le Flem et al., 2001)) and distal (nucleotides -300 to -2052, (Le Flem et al., 2001)) promoter regions of the Tm gene, which represent putative responsive elements to regulatory influences. These include potential retinoic acid responsive elements (RARE; GGTCA/GTGAA) in the region between nucleotides -1538 and -1146, with the direct repeats of RARE separated by 4 base spacer sequences (DR4; 1531 to 1516) (Dittman et al., 1994), a putative shear stress responsive element (SSRE; GAGACC) in the region -1163 to -1168 (Tazawa et al., 1993), and the heat shock responsive element nGAAn present in multiple tandem copies in the -80 to -30 region (Conway et al., 1994).

1.4.5 Regulation of Tm cell surface expression

A variety of mechanisms seem to control the expression of the Tm gene. Altered transcription and translation rates together with alterations in the amount of cell surface expressed Tm contribute to the regulation of Tm expression. A large number of studies have focused on the effects of various effectors on Tm expression on the cell surface, with contradicting results in some cases; see Table 1.1. Regulatory response varies by cell type, which may, in part, account for differences in outcome, since some studies have been conducted on cultured cell lines, whereas others have been carried out on primary endothelial cells. Numerous physiological, biochemical and biomechanical factors have been shown to alter Tm expression (see Table 1.1), and will be the focus of this section.

Stimuli	Cell Type	Tm mRNA levels	Total Tm protein levels	Surface Tm antigen levels	Tm cofactor activity	Ref.
Thrombin	HSVEC	Increased	-	Decreased	-	(Dittman et al., 1989) (Bartha et al., 1993)
	BASMC	Increased	-	-	-	(Ma et al., 1997)
Inflammatory Cytokines						
TNF-α	HUVEC	Decreased	Decreased	Decreased	Decreased	(Lentz et al., 1991) (Nan et al., 2005)
	BAEC	-	Decreased	Decreased	Decreased	(Moore et al., 1989)
	EC	-	-	Decreased	-	(Speiser et al., 2001)
	THP-1	Unaffected	-	-	Unaffected	(Grey et al., 1998)
IL-1	HSVEC	-	-	-	Decreased	(Archipoff et al., 1991)
	EC	-	-	-	Decreased	(Nawroth et al., 1986)
Endotoxin	HUVEC	-	-	-	Decreased	(Moore et al., 1987)
Growth Factors						
TGF-β	HUVEC	Decreased	-	Decreased	Decreased	(Ohji et al., 1995)
VEGF	HAEC	Increased	-	Increased	Increased	(Calnek and Grinnell, 1998)
Atherosclerotic mediators						
Ox-LDL	HUVEC	Decreased	-	Decreased	Decreased	(Ishii et al., 1996) (Ishii et al., 2003)
	THP-1	Increased	-	Increased	Increased	(Oida et al., 1997)
Homocystein	HUVEC	Increased	Increased	-	Decreased	(Lentz and Sadler, 1991) (Hayashi et al., 1992)
Vitamins						
Vitamin D	THP-1	Increased	-	Increased	-	(Ohsawa et al., 2000)
RA	HUVEC	Increased	-	Increased	-	(Horie et al., 1992)
Biomechanical stimuli						
Shear Stress	HUVEC					(Takada et al., 1994)
	(15 dynes/cm^2)	Increased	-	Increased	-	
	(2 dynes/cm^2)	Unaffected	-	Unaffected	-	
	BAEC					(Malek et al., 1994)
	(15 dynes/cm^2)	Transient increase followed by decrease	-	-	-	
	(4 dynes/cm^2)	Unaffected	-	-	-	
	HSVEC					(Gosling et al., 1999)
	Arterial flow (100mmHg,90cpm 200 mL.min)	-	-	Decreased	Decreased	
Wall Tension	RVG	Decreased	Decreased	-	Decreased	(Kim et al., 2002) (Sperry et al., 2003)

Table 1.1 Factors regulating Tm expression.
RA: Retinoic acid; **Ox-LDL**: oxidized low-density lipoprotein; **TNF-α**: Tumour necrosis factor α; **IL-1**: Interleukin 1; **TGF-β**: Transforming growth factor β; **cAMP**: cyclic adenosine monophosphate; **VEGF**: Vascular endothelial growth factor. **HSVEC**: Human saphenous vein endothelial cells; **BASMC**: Bovine aortic smooth muscle cells; **HUVEC**: Human umbilical vein endothelial cells; **BAEC**: Bovine aortic endothelial cells; **EC**: Endothelial cells; **THP-1**: monocytic cell line; **HAEC**: Human aortic endothelial cells; **RVG**: Rabbit jugular vein grafts.

Treatment of mouse haemangioma cells as well as human saphenous vein endothelial cells (HSVECs) with thrombin increased Tm mRNA levels to at least double the initial value after a period of 2 hours (Dittman et al., 1989) (Bartha et al., 1993). Similarly, thrombin upregulated Tm mRNA in bovine aortic smooth muscle cells (BASMC), a response most probably mediated by PARs (Ma et al., 1997). However, the increase in Tm mRNA levels does not result in a net gain in the number of Tm receptors at the cell surface, as it is counteracted by loss of the Tm from the cell membrane, as shown by a two-fold increase in sTm antigen levels in the culture medium over a 24 hour period (Bartha et al., 1993). Tm may also be subjected to regulation by internalization, though the process remains unclear and controversial. On the one hand thrombin binding to Tm is thought to induce the internalization of the complex in the endothelial cell (HUVECs), with thrombin being degraded in lysosomes and Tm returning to the cell surface within 30 minutes (Maruyama and Majerus, 1985). This concept is supported by ultrastructural evidence, demonstrating internalization within 10 minutes mainly in small clusters in non coated pits, with bound thrombin transcytosed across the endothelium by plasmalemmal vesicles (Conway et al., 1992a) (Horvat and Palade, 1993). PC, but not APC, is capable of inhibiting thrombin-Tm internalization, thereby regulating the extent of its own activation and thrombin clearance from the circulation (Maruyama and Majerus, 1987). On the other hand, data have been presented demonstrating that the thrombin-Tm complex is not subjected to endocytosis on the surface of HSVECs and EA.hy 926 cells (Beretz et al., 1989). These contradictory findings may be due to limitations in experimental design or may reflect differences in function among various cell types.

Inflammatory mediators have a profound effect on Tm expression. Granulocyte lysosomal proteases (granulocyte elastase, cathepsin G) and hydrogen peroxide have been shown to cleave Tm from the surface of cultured HUVECs, releasing a 43 kDa fragment into the culture medium (Abe et al., 1994). *In vitro* experiments have demonstrated that exposure of endothelial cells to TNF-α caused a dose- and time-dependent decrease in Tm antigen and activity levels, with maximal reduction in antigen levels observed after a 24 hour incubation in HUVECs (Lentz et al., 1991) (Nan et al., 2005). Evidence has been presented, suggesting that TNF-α mediates its effect by downregulating transcription (Lentz et al., 1991). TNF-α sensitivity of the Tm gene, in endothelial cells, is in part dependent on three Ets core motifs (GGAA) located within a distinct region in the Tm promoter, just upstream of the TATA-box (-76 to -30; relative to the transcription start site) (von der Ahe et al., 1993). The observed detrimental effect on transcription is thought to be mediated by activation of nuclear factor κβ (NF-κβ) (Sohn et al., 2005). *In vivo*, Tm expression on the surface of endothelial cells was depleted following intra-dermal application of TNF-α (Speiser et al., 2001). This could be due to increased shedding of Tm from the endothelial cell surface, a concept supported by the observation that recombinant TNF-α therapy results in increased levels of sTm as a result of the concerted action of TNF-α and neutrophil proteases (Boehme et al., 1996). Alternatively, TNF-α may decrease Tm expression on the cell surface by inducing the internalization and degradation of Tm, as demonstrated in bovine aortic endothelial cells (BAECs) (Moore et al., 1989). Conversely, Tm expression levels were unaffected in THP-1 cells (monocytic-like cell line) treated with TNF-α (Grey et al., 1998). This suggests that Tm expression in monocytes or macrophages is regulated differently to the endothelium, with respect to inflammatory stimuli, and that extravascular mononuclear phagocytes may promote local production of APC. Other cytokines are also able to decrease Tm activity on the surface of endothelial cell. *In*

vitro, IL-1 induces a dose- and time- dependent decrease in Tm activity on the cell surface, with a 63% decrease in Tm cofactor activity after 24 hours (Archipoff et al., 1991). Intravenous infusion of IL-1 into rabbits also caused a dose- and time- dependent decrease in Tm cell surface co-factor activity, assessed as a measure of thrombin-mediated PC activation, with maximal (72%) reduction at 5h (Nawroth et al., 1986). Endotoxin, which is thought to trigger the host response to gram-negative septicemia, also caused a dose- and time- dependent decrease in Tm cofactor activity, with a 45% decrease at 24 hours that remained depressed at 48 hours (Moore et al., 1987). As such, inflammatory cytokines secreted by macrophages underlying the atherosclerotic plaque (Lusis, 2000) may alter the anti-thrombotic character of the endothelium, thus contributing to increased thrombogenicity of atherosclerotic lesions.

Growth factors can contribute to Tm regulation in various ways. In HUVECs, transforming growth factor beta (TGF-β), which is capable of stimulating growth of many types of cells, downregulates surface Tm activity and antigen levels time- and dose- dependently. Tm mRNA levels were also reduced in response to treatment. Maximal surface Tm activity and antigen reduction was observed at 12 hours and maximal reduction of mRNA levels was observed several hours earlier (3 and 6 hours). This suggests that TGF-β regulates Tm expression at the levels of transcription. These data would suggest that TGF-β, released by platelets, SMC and monocytes may contribute to thrombogenesis at the site of plaque rupture due to downregulatory effects on Tm (Ohji et al., 1995). Treatment of human aortic endothelial cells (HAECs) with vascular endothelial growth factor (VEGF), a mitogen for vascular endothelial cells, induced a 2.5-fold increase in both total cellular Tm antigen and total cellular Tm RNA levels, again suggesting that most if not all of the regulation was at the messenger level. VEGF was also able to block IL-1, TGF-β and LPS induced suppression of Tm surface

antigen and mRNA. These findings suggest that VEGF regulation of Tm may contribute to mechanisms that would maintain local haemostasis during angiogenesis and revascularization and could play a role in minimizing loss of vessel anti-coagulant function during inflammatory processes (Calnek and Grinnell, 1998).

Contributors to the atherosclerotic process have also been shown to regulate Tm expression, altering the anti-thrombotic character of the endothelium, and promoting thrombosis of atherosclerotic lesions. Ox-LDL (25-200 μg/ml), but not native or acetylated LDL, reduced Tm cofactor activity in parallel with its antigen levels in HUVECs. This was shown to be oxidation-, concentration- and time- dependent. The reduction in Tm antigen and activity levels, induced by ox-LDL, was preceded by a reduction in Tm mRNA, to 50% of the control levels at 3 hours after treatment. When the lysosomal degradation of ox-LDL was inhibited, ox-LDL mediated downregulation of Tm mRNA and cell surface antigen levels was prevented (Ishii et al., 1996). In addition, exposure of HUVECs to phospholipids extracts, but not to free cholesterol, triglyceride, or cholesterol ester isolated from ox-LDL, downregulated Tm mRNA levels to almost the same extent as intact ox-LDL (Ishii et al., 2003). These results suggest that ox-LDL, following endocytosis, releases oxidised phospholipids through a lysosomal pathway and that the released oxidised phospholipids downregulate Tm expression (Ishii et al., 1996) (Ishii et al., 2003). Oxidized phospholipids in ox-LDL are thought to elicit their effect on Tm expression by transcriptional activation via retinoid receptor β (RARβ). Specifically, exposure of HUVECs to oxidized phospholipids was shown to suppress the nuclear expression levels of RARβ, RXRα, Sp1 and Sp3. This led to decreased binding of the RXRα-RARβ retinoic acid receptor heterodimer and Sp transcription factors to their respective DNA recognition sequences in the Tm promoter, reducing transcription of Tm in HUVECs (Ishii et al., 2003). In contrast, Tm antigen,

activity and mRNA levels were increased in THP-1 cells (monocytic-like cell line) treated with ox-LDL, an effect not seen following treatment with native or acetylated LDL. This would suggest that Tm expression is regulated differently in blood cells to the endothelial cells, though the mechanism has yet to be elucidated (Oida et al., 1997). Homocysteine, a metabolic factor linked with vascular disease, has been shown to impair intracellular processing of Tm, and reduce cofactor activity by reducing the disulphide bonds (Lentz and Sadler, 1991) (Ye et al., 1993). Conversely, infusion of animals with HDL, believed to be an anti-atherogenic factor (Mertens and Holvoet, 2001), caused a 2-fold increase in Tm expression, in aortic atherosclerotic lesions (Nicholls et al., 2005). Thus, infusing small amounts of HDL may help reduce the thrombogenicity of atherosclerotic lesions.

As far as vitamins are concerned, retinoic acid (RA), a vitamin A derivative also referred to as 'vitamin A acid', increased surface Tm levels on HUVECs in a time- and dose-dependent manner. Antigen levels began to increase 3 hours after the addition of physiologically relevant levels of RA (10 μM), reaching a maximum level (2.5 times greater than the untreated control) at 24 hours. This level was maintained for a further 12 hours after which it gradually decreased. Tm mRNA levels were also increased in response to RA treatment, suggesting that the increased surface Tm levels in RA treated cells results from enhanced transcription (Horie et al., 1992). Furthermore, co-incubation of HUVECs with both TNF-α and RA prevented TNF-α induced (10 units/ml) reduction in surface Tm antigen, cofactor activity and mRNA, dose-dependently (Ishii et al., 1992). It has subsequently been demonstrated that the acceleration of Tm gene transcription by RA is mediated by nuclear retinoic acid receptor dimers and the nuclear Sp1 transcription factor interacting with two different sequences in the 5'-flanking region of the human Tm gene. Specifically, the RAR-

RXRα heterodimer interacts with the DR4 site between -1531 and -1516, and the Sp1 transcription factor binds to the 1st and 2nd Sp1 binding sites (-141 to -129) to augment Tm gene expression in response to RA (Horie et al., 2001). Similarly, a hormonally active form of vitamin D was shown to upregulate Tm antigen levels on the surface of a monocytic cell line in a dose-dependent manner, with maximal increase at 0.1 μmol/l. The increase in Tm antigen levels was paralleled with an increase in Tm mRNA levels, suggesting control at the level of transcription. These results imply that vitamin derivatives may serve as novel anti-thrombotic agents in treatment of atherosclerotic diseases (Ohsawa et al., 2000).

Heat shock has been investigated in relation to Tm expression. Cell surface expression enhanced 3.2 and 6.7-fold in HUVECs and A549 cells (lung carcinoma cell line), respectively, in response to prolonged (24 hours) heat shock stress (42°C) in culture. The mechanism is transcriptional and depends on heat shock responsive elements (nGAAn), present in multiple tandem copies in the -80 to -30 region of the Tm promoter. It is plausible that the augmentation of Tm expression, mediated by the binding of heat shock factors to these heat shock responsive elements, may serve to protect the vascular endothelium during numerous stresses, such as infection or inflammation (Conway et al., 1994).

Conflicting results have been published concerning the effect of shear stress on Tm expression. Application of fluid stress in the physiological range of magnitude (15 and 36 dynes/cm^2) on bovine aortic endothelial cells (BAECs) caused a short lived increase followed by a decrease in Tm mRNA levels (Malek et al., 1994). In contrast, exposure of HUVECs to moderate shear stress levels (15 dynes/cm^2) caused a dramatic increase in Tm mRNA levels, which preceded the increase in surface Tm antigen levels,

suggesting transcriptional control (Takada et al., 1994). Despite the discrepancies between these studies, the results suggest that haemodynamic forces may be critical factors in the regulation of thrombosis.

When excised saphenous vein fragments were exposed to arterial shear rate, a decrease in Tm expression occurred, reflected by a consequent decrease in the ability of the cells to promote PC activation, an effect thought to be due to the increased flow or shear stress. This result would suggest that shear stress may contribute to the loss of cell surface Tm antigen and activity in vein bypass graft, contributing to occlusion and failure of vein grafts in patients undergoing arterial bypass procedures (Gosling et al., 1999). Similarly, endothelial expression of Tm in rabbit vein graft sections was dramatically decreased (<90%) immediately after implantation into the carotid circulation, with a gradual recovery by 6 weeks. The loss of Tm expression, within two weeks of implantation, was associated with a 95% decreased capacity of grafts to activate PC, resulting in increased thrombin generation (Kim et al., 2002). Subsequent experiments comparing the effects of external stenting with surgical manipulations that decreased blood flow demonstrated that wall tension is an important regulator of Tm expression in vein grafts (Sperry et al., 2003). These results suggest that wall tension is involved in the regulation of Tm expression and may contribute to altered Tm expression in atherosclerotic arteries, as well as reduced thromboresistance in vein bypass grafts, leading to thrombotic occlusions (Lentz, 2003).

1.4.6 Evidence supporting a role for reduced Tm in CHD risk

A number of studies have supported a role for reduced Tm as risk for CHD. Weiller-Guettler *et al.* (1998) used site directed mutagenesis to eliminate the ability of Tm to drive *in vitro* thrombin-dependent PC activation in a mouse model. Mice homozygous for this mutation were characterized by a dramatic increase in fibrin deposition in the heart and lungs. In another animal study, local over-expression of Tm was found to reduce *in vivo* thrombus formation in isolated rabbit arterial segments, which suggests that high Tm levels help maintain a thromboresistant state (Waugh et al., 1999). In human subjects, it has been demonstrated that Tm is downregulated on the surface of endothelial cells overlaying atherosclerotic plaques in coronary arteries, which may favour local thrombus formation (Laszik et al., 2001). Furthermore, a large prospective study (ARIC), found a reduced risk of CHD as levels of sTm increased (healthy individuals at the time of study) (Salomaa et al., 1999). Since sTm in plasma is thought to reflect endothelial cell injury, increased levels of sTm may be expected to be associated with CHD in patients post event (Ishii et al., 1991). However, the finding of lower plasma levels in individuals who subsequently developed CHD, suggests that variants may be present that reduce Tm expression (Salomaa et al., 1999). Collectively, these studies suggest that reduced expression of Tm is associated with increased fibrin deposition and increased incidence of heart disease. Tm is likely to have a role in protecting endothelium both from inflammatory affects leading to atherosclerosis and against the clinical outcome associated with clot formation.

1.4.7 Tm gene variants

1.4.7.1 Biological importance of gene variants

Gene structure is not constant across populations, with variations resulting from permanent change in the DNA base sequence occurring frequently. A substantial number of variations are the result of single base-pair substitutions. Sequence changes that alter the function of the gene, usually in a detrimental way, are described as mutations.

Gene variants that occur with a population frequency of at least 1% are referred to as polymorphisms. Some variants may affect the structure of the transcribed protein, or the quantity of protein transcription (promoter mutations). Sequence changes that have this effect are referred to as 'functional gene variants' and may have important biological consequences. A single base substitution may alter a codon so as to produce an altered amino acid altering the protein structure, a so called missense or non-synonymous mutation, or it may change the codon that specified an amino acid to one of the termination codons, a so called nonsense mutation. Introduction of a nonsense mutation terminates protein synthesis, since translation of the mRNA transcribed from this gene will stop prematurely, to produce a truncated protein. Other single base substitutions may cause no change in their product and can only be detected by sequencing, so called silent or synonymous mutation. Variation that alters the RNA-slice sites may cause mis-splicing in the intron or exon. This can result in the synthesis of a protein lacking part or all of an exon, or having additional amino acids, again altering the sequence of the protein product. The addition or removal of base pairs from the DNA of a gene may shift the reading frame of the gene. As a result, the mRNA will be translated into a new

group of codons altering the structure of the gene product. Such frameshifts may also create new termination codons and thus generate nonsense mutations.

In addition to changes in the structure of the gene product, variation may impact on the quantity of protein synthesised. This occurs if there are changes in the rate of gene transcription or mRNA stability. The amount of gene product may be affected if mRNA stability is influenced by variations in RNA processing and translation. The rate of gene transcription may be altered by variation in gene regulatory sequences, such as the promoter region. The promoter region sets the location and direction of transcription on a DNA template. Initiation of transcription involves the binding of RNA polymerase to the promoter, which requires the formation of a transcription initiation complex with several general transcription factors. Transcription factors recognize and bind to sequences within the promoter and interact with one another and with the general transcription factors to regulate the rate of transcription from a particular gene. As such, variants located within the promoter region are of particular interest.

When two polymorphic sites are in complete (or strong) allelic association, caution is needed before assigning an altered biological activity to any one variant site. Allelic association, also called linkage disequilibrium (LD), is non-random association between two or more alleles. LD is a population-based concept and describes a situation in which a certain allele at one locus occurs in the presence of a specific allele at a second locus more frequently than would be expected by chance alone. As such, the gene variant under investigation may simply act as a marker for the second variant. It is usually measured as delta (Δ) or D', with values ranging from -1 (complete negative association) to +1 (complete positive association). A value of 0 represents little or no

association. D' differs from Δ, in that the calculation considers the difference in allele frequency between the two alleles under investigation.

1.4.7.2 Genetic variation in the Tm gene

A total of 125 variants are listed for Tm on the CHIP Bioinformatics database (http://snpper.chip.org), but not all have been validated. 51 are within the promoter region (27 validated), 22 are within the Tm exon (12 validated) and 52 are within the 3' UTR (31 validated). Numerous Tm gene variants have been associated with risk of CHD.

Eight sequence variants in the Tm distal promoter region were studied in venous thrombosis (Le Flem et al., 2001). Of these, –1166G>A, -1208/09TT>delTT and -1748G>C appeared to have functional consequences. The –1166G>A variant, which occurs in about 1% of the population, alters the core binding sequence of a putative SSRE. The common –1748G>C variant (21% of the general population) creates a putative Sp1-like transcription factor binding site with only one mismatch relative to the consensus sequence. The –1748C allele has also been reported to be associated with higher plasma D-dimer levels, which would be expected if this allele is associated with reduced Tm levels leading to increased fibrin production. The –1208/09TT>delTT variant, which occurs in 10% of the population, does not affect a known regulatory element. However, a 2-fold higher risk of varicose veins has been suggested for the delTT allele. None of these distal promoter variants have been studied in CHD.

Of the variants identified in the proximal promoter region of Tm, a C to A substitution at position -133 (-133C>A), which lies in close proximity to transcription factor binding sites (Sp1) shown to be important for Tm transcription, has been identified in a small

number of patients with a personal history of MI (Ireland et al., 1997). *In vitro*, this substitution caused a significant decrease in reporter gene expression and impaired nuclear protein binding. By reducing the binding of nuclear proteins, the -133C>A promoter mutation may reduce expression of the Tm gene (Nakazawa et al., 2002). A G to A substitution at position -33 (-33G>A) was initially identified in an Indian Asian population (Ireland et al., 1997). This variant lies in close proximity to a consensus regulatory element (TATA-box) (Yu et al., 1992) (Tazawa et al., 1993). Its frequency is extremely low in the Caucasian population, but has since been identified at much higher frequency in Korean and Taiwanese populations (Li et al., 2002). The -33A allele has been associated with risk of CHD and MI in Taiwanese populations (Li et al., 2000) (Li et al., 2002). *In vitro* analysis has shown that the -33A allele causes a 20% reduction in reporter gene expression, suggesting that the -33G>A variant is responsible for reduced protein expression (Li et al., 2001) (Nakazawa et al., 2002). A dinucleotide mutation (-9/-10GG>AT), which also lies in close proximity to the TATA-box, has been identified in patients with a personal history of MI (Ireland et al., 1997). *In vitro* analysis has shown that the -9/-10GG>AT mutation has no effect on gene expression (Nakazawa et al., 2002). It is worth noting that while the 5' untranslated and promoter region has now been extensively studied to identify variants, the 3' untranslated region has not.

Variants in the coding region of the Tm gene have also been associated with risk of CHD. A low frequency variant (127G>A; Ala25Thr) has been identified in the Tm lectin-like domain (Ireland et al., 1997) (Norlund et al., 1997b), a region implicated in constitutive endocytosis of Tm (Conway et al., 1997) and thought to possess anti-inflammatory properties (Conway et al., 2002). This variant has been associated with a 2-fold increase risk of MI in a case-control study (SMILE), which increased 4-fold in

patients with metabolic syndrome and 9-fold in smokers (Doggen et al., 1998b). An 11 bp deletion (del791-801), identified in a 22-year-old male with pulmonary embolism, changes the reading frame of the Tm protein from Arg246, resulting in a premature stop codon at position 306 within the 2nd EGF-like repeat. *In vitro* this mutation abolished Tm expression on the cell surface and was associated with reduced Tm plasma levels in carriers (Kunz et al., 2002). The Arg385Ser variant, in the 4th EGF-like repeat, caused a 50% reduction in Tm expression and 4-fold reduction in Tm cofactor activity (Kunz et al., 2002). This particular amino acid is adjacent to the short 3 amino acid loop connecting the 4th and 5th Tm EGF-like domains, a region shown to be essential for Tm cofactor activity (Clarke et al., 1993). A common variant site (A455V; 1418C>T) has been identified in the 6th EGF domain of Tm (van der Velden et al., 1991), a region with a key role in thrombin binding and activation of protein C (Hayashi et al., 1990) (Tsiang et al., 1992). However, the functional consequences of the A455V variant are unknown. There are contradictory reports for CHD risk, with both or neither allele having been associated with risk in different populations (van der Velden et al., 1991) (Norlund et al., 1997a) (Wu et al., 2001) (Aleksic et al., 2002). A G to T substitution at nucleotide position 1456 (1456 G>T; Asp468Tyr) was the first mutation to be reported in the Tm gene. This variant site was identified in a 45-year-old man with pulmonary embolism who had low plasma Tm levels (Ohlin and Marlar, 1995). Although the Asp468Tyr variant lies within the Ser/Thr rich domain of the mature Tm molecule, in close proximity to the chondroitin sulfate attachment site (Gerlitz et al., 1993) (Lin et al., 1994), variant Tm expression and PC cofactor activity remained unaltered (Nakazawa et al., 1999). A frameshift mutation (insT 1689), which predicts an elongated protein due to the substitution of 12 carboxy-terminal amino acids with 61 abnormal residues, was identified in the Tm gene of a patient with MI and was associated with significantly reduced sTm levels in the plasma of family members carrying the mutation. *In vitro*

functional work demonstrated accumulation of Tm protein in a peri-nuclear fashion rather than, as shown for wild-type, on the surface of the cells (Kunz et al., 2000).

Although the presence of mutants in Tm in individuals and families with CHD suggests a possible contribution to the disease, the numbers of individuals who could be affected is unknown. Similarly, whether high frequency polymorphisms can affect the levels or function of Tm sufficiently to contribute to disease is also unknown.

1.5 Aims of thesis

1.5.1 Hypothesis

Genetic variants in the Tm gene, which cause reduced protein expression, a dysfunctional protein, or increased shedding from the endothelial surface collectively contribute to heart disease.

1.5.2 Aims

Aim 1: To examine the association between known potentially functional variants in the Tm gene and risk for CHD, and the consequences of these variants upon thrombin generation and inflammation.

Aim 2: To determine whether the overall contribution to heart disease, by dysfunctional variants, can be estimated by measurement of sTm levels.

Aim 3: To assess the *in vitro* function of any variants shown to contribute to CHD risk.

CHAPTER TWO

METHODS

2. Methods

2.1 Reagents and commonly used stock solutions

2.1.1 Materials and Suppliers

Amersham Biosciences (UK) Ltd. Buckinghamshire, UK.

GFX PCR DNA and Gel Bans Purification Kit, $pd(N)_6$ Random Hexamer, ExoSAP-IT.

Appleton Woods Laboratory Equipment & Consumables. Birmingham, UK.

96-well Clear Polycarbonate Plates.

Applied Biosystems. CA, USA.

Blue Dextran/EDTA loading buffer, Big Dye Sequencing Kit.

BDH Laboratory Supplies. Poole, UK.

Dimethyl Sulphoxide (DMSO), Formamide solution, Ethanol, Chlorophorm, Industrial Methylated Spirit (IMS), Ammonium Persulphate (APS), NNN'N'-Tetramethylethylendediamine (TEMED) and commonly used chemicals unless otherwise specified.

Becton-Dickinson Labware Europe. Le Pont de Claix, France.

Falcon tissue culture dishes

Bibby Sterilin Ltd. Staffordshire, UK.

Gamma Irradiated 96-well Microtitre Plates.

Bioline Ltd. London, UK.

Taq Polymerase.

Biontex Laboratories GmbH. Munich, Germany.

METAFECTENE™

Cambrex BioScience Inc. MD, USA.

Endothelial Cell Basal Growth Medium (EBM^{R}), EGM^{TM} Single Quots.

Diagnostica Stago Inc. Asnieres, France.

Asserachrom^{R} Thrombomodulin (Enzyme Immunoassay of Thrombomodulin)

Gibco-BRL Ltd. Paisley, UK.

Escherichia coli (*E.coli*) strain DH5α^{TM} competent cells, Lipofectin®, LipofecAMINE^{TM}, Lipofectamine^{TM} 2000, 1 kb ladder, TRIzol reagent, SUPERSCRIPT^{TM} II reverse transcriptase, Platinum^{R} Pfx DNA Polymerase, Opti-MEM I tissue culture medium, Heat-inactivated foetal bovine serum (FBS), Trypsin-EDTA, Oligonucleotide primers.

National Diagnostics (UK) Ltd. Hull, UK.

Ultra-Pure SequaGel^{R} Complete Buffer Reagent, SequaGel^{R} XR.

New England Biolabs (UK) Ltd. Hertfordshire, UK.

Restriction endonucleases (*Aci I, Ban II, Bbs I, BstU I, Hind IIMsp I, Pme I*), NEBuffer 2, 3 & 4, Purified Bovine Serum Albumin (BSA), T4 DNA ligase, Ligase buffer.

Pharmacia Biosystems Ltd. Milton Keynes, UK.

dNTPs.

Promega Co. Madison, WI, USA.

pGL-3-Basic reporter vector, pGL-3-Control vector, pRL-TK *Renilla* Luciferase vector, *Renilla*-Luciferase vector dual *luciferase* assay kit, passive lysis buffer.

PromoCell GmbH. Heidelberg, Germany.

Human Umbilical Vein Endothelial Cells (HUVEC, pellets & cryopreserved vials).

Qiagen Ltd. Crawley, West Sussex, UK.

Qiagen Spin Miniprep Kit, RNeasy Mini Kit, RNase-free DNase set.

R&D Systems Inc. MN, USA.

Recombinant Human TNF-α (10 ng/ml), Recombinant Human Il-1β (5 ng/ml).

Severn Biotech Ltd. Worcestershire, UK.

Tris-Borate EDTA, 19:1 acrylamide:bisacrylamide ratio and 30% acrylamide.

Sigma-Aldrich Company Ltd. Dorset, UK.

Gene Elute^{TM} HP Plasmid Maxiprep Kit, Glutaraldehyde solution, β-mercaptoenthanol (BME), Low melting point agarose, Molecular biology grade agarose, Ampicillin, Bacto-Tryptone, Bacto-Yeast, Bacto-Agar, X-gal, Bromophenol blue, Xylene Cyanol Wide Range, Glycerol, 2% gelatine.

Stratagene. CA, USA.

QuickChange^{R} Site-Directed Mutageneis Kit, *E.coli* strain XL1-Blue competent cells.

CJ Edgell. Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA.

Ea.hy 926 cell line.

2.1.2 Equipment and Reagents

Glass and plastic-ware were sterilised by autoclaving. Solutions were either sterilised by autoclaving or filtration through a 0.2 μm filter. Reagents were prepared using double-distilled deionised water.

2.1.3 Commonly used stock solutions

DNA Extraction:

1 M $MgCl_2$: 20.33 g $MgCl_2$ dissolved in 100 ml dH_2O.

1 M Tris pH 7.5: 12.11 g Tris, made up to 100 ml in dH_2O, correct pH to 7.5 and autoclaved.

Sucrose lysis mix: 109.5 g sucrose, 5 ml 1 M $MgCl_2$, 10 ml 1M Tris pH7.5, 10 ml Triton-X-100, made up to 1000 ml in dH_2O and stored at 4°C.

0.5 M Na_2EDTA: 37.22 g EDTA (pH 8.0, with NaOH), made up to 200 ml in dH_2O.

10% SDS: 10 g Sodium dodecyl sulphate, made up to 100 ml in dH_2O.

Nuclear lysis mix: 1 ml 1 M Tris-HCl pH 8.2, 2.34 g NaCl, 0.4 ml 0.5M Na_2EDTA pH 8.0, 10 ml 10% SDS, made up to 90 ml in dH_2O.

5 M Sodium perchlorate: 70.24 g sodium perchlorate, made up to 100 ml in dH_2O.

TE buffer pH 7.6: 1.21 g Tris, 0.37 g EDTA, made up to 1000 ml in dH_2O.

Bacterial work:

L-Agar: 1000 ml dH_2O, 10 g Bacto-Tryptone, 5 g Bacto-Yeast, 5 g NaCl, 15 g Bacto-Agar.

L-Broth: 1000 ml dH_2O, 20 g Bacto-Tryptone, 10 g Bacto-Yeast, 10 g NaCl, 4 g Glucose.

PCR buffer mixes:

DMSO Pol Mix buffer: 166 mM $(NH_4)_2$ SO_4, 670 mM Tris-HCl (pH 8.8), 67 mM $MgCl_2$, 100 mM BME, 67 µM EDTA, 1.7 mg/ml BSA, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 2 mM dCTP.

Pol Mix buffer: 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 0.01% gelatine, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 2 mM dCTP.

NH_4 Pol Mix buffer: 16 mM $(NH_4)_2$ SO_2, 67 mM Tris-HCl (pH 8.4), 0,01% Tween 20, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 2 mM dCTP.

Microtitre Array Diagonal Gel Electrophoresis (MADGE) gels (7.5%):

For each gel a solution was made up containing:-

Per gel: 5 ml of 10x TBE, 12.2 ml 30% acrylamide (19:1 acrylamide-bisacrylamide ratio), 32.5 ml of distilled dH_2O, 150 µl TEMED, 150 µl 25% APS.

Loading Buffer: 98% formamide, 10 mmol/l EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol.

Sticky saline: 0.5% v/v glacial acetic acid, 0.5% v/v γ methacryloxy-propyl-trymethoxy-silane.

10x TBE (Tris-Boric acid-Ethylenediaminetetraacetic acid) buffer: 0.9 M Tris, 0.9 M Ortho-boric acid, 0.2 M ethylenediaminetetraacetic acid.

2.2 Methods

2.2.1 Biochemical assays

Blood had been collected into tri-sodium citrate and centrifuged to prepare platelet poor plasma. Each plasma sample was removed, snap frozen on dry-ice in aliquots and stored at -80°C.

2.2.1.1 Plasma sTm (commercial assay)

Plasma sTm was measured using a commercially available Enzyme Linked ImmunoSorbent Assay (ELISA) from Diagnostica Stago (DS), France (Asserachrom^{R} Thrombomodulin). This is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants in the Tm molecule. During incubation, sTm in the sample reacts with a mouse anti-human anti-Tm monoclonal antibody bound to each microtitre well. After washing to remove non-reactive plasma components, a peroxidase conjugated mouse anti-human anti-Tm monoclonal antibody recognizes the Tm bound to the solid phase. After a second incubation and a second wash that removes the unbound enzyme labeled antibody, the bound conjugate is detected by its oxidative action on the substrate ortho-phenylenediamine (OPD) in the presence of urea peroxide. The reaction is stopped with sulphuric acid to give a colorimetric endpoint that is read spectrophotometrically at 492 nm. The observed optical density is directly proportional to the concentration of Tm. A plasma sample for which sTm concentration was predetermined and known to be at a concentration of approximately 40 ng/ml was used as a control. This 'control' plasma sample was used to determine both inter- and intra-

assay variability. The inter-assay CV was 22%, and the intra-assay CV was 2.5%. sTm levels using the Asserachrom^{R} Thrombomodulin assay were measured in a total of 590 samples. A single batch of kits (Asserachrom^{R} Thrombomodulin lot number: 2318B45) was used to measure sTm levels in the 590 samples. Reference Tm (Lot number: 116C16), provided with the kit, was reconstituted with 2 ml dilution buffer giving a concentration of 110 ng/ml. Four doubling dilutions were then carried out to generate the assay calibrators 110, 55, 27.5, 13.75, 6.875 ng/ml (final concentration).

2.2.1.2 sTm cofactor activity (in-house assay)

Plasma sTm cofactor activity for PC activation was measured by Ohlin *et al.* (Ohlin et al., 2005). Briefly, a monoclonal antibody against human Tm (MAb Tm43b) (Clarke et al., 1993) was coupled to a microtitre plate according to the manufacturer's instructions (100 µl, 10 µg/ml MAb Tm 43b/well; Nunc A/S DK-4000 Roskilde, Denmark).

Solulin (Glaser et al., 1992), a recombinant truncated soluble form of Tm, consisting of the entire extracellular part of the Tm molecule, was used as a standard in this assay. 1.0 ng Solulin antigen level or activity was defined as 1 Seq. A stock solution of Solulin (500 ng/ml) was diluted with phosphate buffer saline (PBS), containing 0.1% dry milk and 0.1% Tween, and added to normal pooled citrated plasma to generate the following assay calibrators 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0.08 ng/ml (final concentration). Plasma samples were also diluted 1 in 4 with the same buffer.

During the antigen immobilization step, 200 µl blank (PBS with 0.1% dry milk and 0.1% Tween), standard or test plasma was added to a microtitre plate coated with MAb Tm43b. The plate was incubated for 2 hours at room temperature under rotation at 600

rpm. The plate was then subjected to 3 successive washes with 300 µl PBS containing 0.1% dry milk and 0.1% Tween. 50 µl 8.8 nM thrombin (in 20 mM Hepes, 0.15 M NaCl, 2 mM $CaCl_2$, 0.1% Tween 20; pH 7.4) and 50 µl 0.4 µM human PC (in 20 mM Hepes, 0.15 Nacl, 2 mM $CaCl_2$, 0.1% Tween; pH 7.4) were subsequently added to the wells and the plates incubated for 3 hours at 37°C under rotation at 280 rpm. Following PC activation, thrombin activity was inhibited by adding 10 µl Hirudin (Refludan®) at 132 IU/mL (in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl2, 0.1% Tween; pH 7.4) and incubating the plate for 10 minutes at 37°C. In the final step, 75 µl S-2366 (2 mM; Chromogenix, Goteborg, Sweden) in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl2, 0.1% Tween; pH 7.4 was added to each well and absorbance monitored at 405 nm for 30 minutes. Activity measured in the blank sample was subtracted from all standard and plasma sample measurements. Inter- and intra- assay coefficients of variation for the in-house Tm activity assay were <15% and <8%, respectively, as previously reported (Ohlin et al., 2005).

A 1000-fold molar excess of an anti-Tm polyclonal antibody or an unrelated polyclonal antibody (anti-human Cystatin C) was added to the standards and three normal plasma samples, to confirm the measured activity was Tm specific.

2.2.1.3 Plasma sTm (in-house assay)

Palsma sTm was determined by Ohlin *et al.* (Ohlin et al., 1996) (Ohlin et al., 2005) using an in-house developed "sandwich" ELISA, using two monoclonal antibodies, Tm 43b and Tm 531. In brief, 96-well microtitre plates were coated with a monoclonal anti-human Tm antibody (Tm 43b) and incubated overnight at 4°C. The wells were then washed three times with washing solution (50 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween

20, pH 8.0). Plasma samples (100 ml/well) were added to the coated wells, following dilution (1:5) in washing buffer with 0.1% dry non-fat milk, and incubated for 1 hour at 37°C. After washing to remove non-reactive plasma components, a biotinylated monoclonal anti-human Tm antibody, Tm 531 (4 mg/ml in washing buffer with 0.1% dry non-fat milk), was added and incubated for 1 hour at 37°C. After a second wash, 100 ml of streptavidine-alkaline-phosphatase (Dakopatts, Alvsjo, Sweden; diluted 1:2000 in washing solution) was added and the plate incubated for a further hour at 37°C. Following yet another wash, the bound phosphatase was revealed by its action on the para-nitrophenyl-phosphate (pNPP) substrate (100 ml) (Sigma, Chemicon, Malmo, Sweden; 1 tablet was dissolved to 2 mg/ml in 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4). The absorbance was measured spectrophotometrically at 450 nm, following a 1.5 hour incubation at 37°C, to allow sufficient development of the enzyme-substrate reaction.

Solulin (Glaser et al., 1992) was used as a standard. Solulin was diluted in washing buffer to give concentrations ranging between 0.062 ng/ml and 2.0 ng/ml. sTm concentration in individual plasma samples was calculated by comparing the absorbance from wells to the standard curve.

Inter- and intra-assay coefficients of variation were <6% and 5% for the in-house assay, as previously reported (Ohlin et al., 1996). Both Tm activity and Tm antigen levels determined using the in house assays, were expressed as SEq/ml (Solulin equivalents), and the detection limit of the assays was 0.03 SEq/ml.

2.2.2 DNA extraction

2.2.2.1 DNA extraction from blood using the "salting out method"

DNA was extracted from potassium-EDTA or citrate anti-coagulated peripheral blood using a salting out method (Miller *et al.* 1988), performed in batches of 24 or 36 samples. This DNA extraction method involves several steps, cellular and nuclear lysis, followed by deproteinisation, extraction and ethanol precipitation of DNA.

(i) Cell and nuclear lysis

10 ml blood was thawed and transferred to a 30 ml polypropylene tube. 12 ml cold sucrose lysis buffer (4°C) was added and each tube inverted by hand. Tubes were centrifuged at 4°C for 10 minutes at 1300g (Sorvall RC5 centrifuge using rotor SA-600). The supernatant was discarded (without disturbing the pellet) and the pellets re-suspended in 20 ml sucrose lysis buffer by pipetting. Samples were subsequently centrifuged at 1300g for a further 10 minutes. Following centrifugation, the supernatant was discarded, and 2 ml of nuclear lysis buffer was added and the pellet re-suspended by pipetting.

(ii) Deproteinisation

1 ml of 5M sodium perchlorate was added and the samples mixed by inversion. Samples were then left on a shaker for at least 10 minutes.

(iii) DNA extraction

2 ml cold (-20°C) chloroform was added and the samples inverted several times by hand to ensure mixing. Samples were subsequently centrifuged at 1300g for 3 minutes at room temperature. Following centrifugation the upper aqueous phase (containing the DNA from each sample) was transferred to a fresh 30 ml polypropylene tube making sure not to disrupt the organic phase.

(iv) Precipitation and washing

10 ml cold (-20°C) 100% ethanol was gently added onto the aqueous phase and mixed by inverting. DNA was 'spooled' using a Pasteur pipette, washed in 70% ethanol and transferred to a sterile microtube containing 1 ml Tris-EDTA (TE) buffer.

(v) Dissolving DNA

Microtubes containing the spooled DNA in TE buffer were incubated at 37°C overnight, before being stored at 4°C. They were left for a period of at least four weeks to allow complete dissolution of DNA.

(vi) DNA Array generation

DNA concentrations were measured by diluting 10 µl DNA in 100 µl dH_2O. Absorbance was measured using a spectrophotometer, at 260 and 280 nm. Stock DNA was diluted in dH_2O to a concentration of 20 ng/µl and aliquots placed in a 96-well, deep array. These 'Stock Arrays' were stored at -20°C. Working arrays were generated by transferring 100 µl of each sample from the stock array, to another 96-well array. These 'Working Arrays' were stored at 4°C.

2.2.2.2 DNA extraction from bacterial cells

i) Small scale preparation of plasmid DNA 'miniprep'

DNA was extracted from bacterial cells using the QIAprep® Spin Miniprep kit (Qiagen Ltd. Crawley, West Sussex, UK), according to the manufacturers instructions. This kit is designed for the rapid, small scale (up to 20 µg DNA) preparation of high purity plasmid DNA.

Briefly, the bacterial pellet was prepared as described in section 2.2.6.2 and resuspended in 250 µl of glucose resuspension solution (Buffer P1). 250 µl alkaline lysis solution (Buffer P2) was added and mixed gently by inverting 4-6 times to ensure lysis. Neutralizing solution (Buffer N3; 350 µl) was added to the cell lysate and mixed gently by inversion. The samples were centrifuged for 10 minutes at 18000g to precipitate and remove any protein contaminants. The supernatants were applied to the QIAprep spin column and centrifuged at 18000g for 1 minute to ensure binding of DNA to the silica membrane (anion-exchange resin). The silica membrane, of each column, was washed with 750 µl medium-salt wash solution (PE Buffer) by centrifugation at 11000g for 1 minute. DNA was eluted from the column by addition of 30 µl high salt elution buffer (EB Buffer) and centrifugation at 18000g for 1 minute.

ii) Large scale preparation of plasmid DNA 'maxiprep'

DNA was extracted from bacterial cells using the Gene Elute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich Company Ltd. Dorset, UK), following the manufacturers protocol. This kit is designed for the rapid, large scale (100 to 500 µg DNA) preparation of high purity plasmid DNA.

In brief, the bacterial pellet was prepared as described in section 2.2.6.2 and resuspended in 12 ml of glucose resuspension solution. Alkaline lysis solution (12 ml) was added, mixed gently by inverting 6-8 times and allowed to clear by incubating at room temperature for 5 minutes. Neutralizing solution (Buffer P; 12 ml) was added to the cell lysate and mixed gently by inversion, followed by the addition of binding solution (Buffer G; 9 ml). The mix was immediately added to the barrel of the filter syringe and allowed to sit for 5 minutes. A binding column was placed into a collection tube, column preparation solution (12 ml) was added and the column and tube centrifuged at 3000g for 2 minutes. Following the 5 minute incubation, the cleared lysate (in the filter syringe) was expelled into the binding column. The filtered solution was centrifuged at 3000g for 2 minutes ensuring adsorption of the DNA onto the silica membrane of the binding column. Medium salt buffer wash solution (12 ml) was added to the column and centrifuged at 3000g for 5 minutes. DNA was eluted from the column by adding 3 ml high salt elution buffer and centrifugation at 3000g, for 5 minutes.

The purified plasmid DNA was precipitated by adding 1:10 volume NaAc (3 M, pH 5.6) and 2 x volume ethanol (100%), and incubated at -20°C overnight. The mix was then centrifuged at 10000 rpm for 30 minutes at 4°C. The supernatant was carefully removed and the DNA pellet washed with 200 µl ethanol (70%). The supernatant was again carefully removed, the pellet allowed to air-dry for 5-10 minutes, and re-dissolved in 300 µl high salt elution buffer.

2.2.2.3 DNA concentration calculation

The concentration of the mini- and maxi- preparations of plasmid DNA and following genomic extraction was calculated from the OD measured at 260 nm using a spectrophotometer. The formula used was: OD_{260} x 50 (dsDNA molar extinction coefficient) x dilution factor = ng/µl. The purity was calculated as the OD_{260}/OD_{280} ratio. Only preparations with a ratio above 1.5 were used for sequencing (section 2.2.5), cloning (section 2.2.6) and transfections (section 2.4.3).

2.2.3 Methods used for the identification of genetic variants or mutants

2.2.3.1 Polymerase chain reaction (PCR)

The first step of a polymerase chain reaction (PCR) involves a short period of high temperature, to denature the DNA. The reaction is subsequently cooled in the presence of oligonucleotide primers, complementary to the DNA (typically 20 bp in length), flanking the DNA sequence of interest. The primers anneal, and DNA polymerase adds deoxynucleoside triphosphates (dNTPs) base by base, copying the target and thereby replicating the DNA. Typically, the polymerase used is derived from the bacterium *Thermus aquaticus* (*Taq*) and is not denatured by the fluctuating temperatures of automated PCR. Typically, 30 temperature cycles are used to provide sufficient DNA for analysis. After the first cycle newly formed strands become the target DNA, the product size then becomes defined by the position of the oligonucleotide primers, see Figure 2.1.

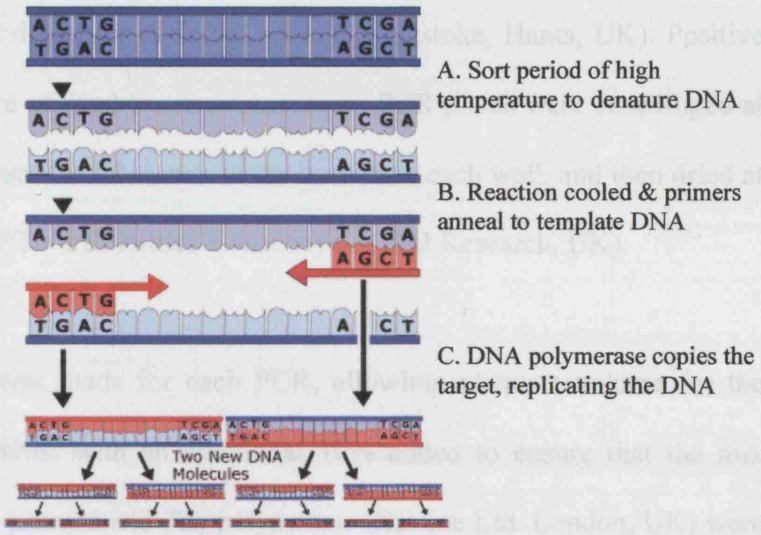


Figure 2.1 PCR schematic.
Reproduced from www.oceanexplorer.noaa.gov with permission.

Typically, 100-150 ng DNA was used for each reaction. Standard PCR reactions were carried out in a total volume of 20 µl. Each reaction contained buffer [166 mM ammonium sulphate, 670 mM tris-HCl (P.H 8.8), 6.7 mM MgCl, 100 mM BME, 67 µM EDTA, 1.7 mg/ml BSA], 8 pmol of each primer, 0.2 units *Taq* polymerase (Bioline Ltd. London, UK). Reactions were overlaid with 20 µl mineral oil to prevent evaporation and sealed with a clear sticky plastic lid. Plates were centrifuged at 200g for 1 minute to ensure all PCR reagents were below the oil layer. PCR amplification was performed on a PTC-225 Peltier Thermocycler (MJ Research, UK).

Genomic DNA samples were prepared for PCR by centrifuging the DNA working array at 1000g for 1 minute. This was done to ensure that all the DNA samples were at the bottom of their respective wells, thus reducing the possibility of cross-well contamination when the array lid was removed. 2.5 µl of each DNA sample (50 ng/µl of DNA) was transferred from a 96-well working array into a standard 96-well PCR plate (Appleton Woods Laboratory Equipment & Consumables. Birmingham, UK) using a

Finnipipette multichannel dispenser (Life Sciences, Basingstoke, Hants, UK). Positive and negative controls were utilized to ensure accuracy. PCR plates were centrifuged at 1000g for 1 minute to ensure the DNA was at the bottom of each well, and then dried at 80°C for 10 minutes on a PTC-225 Peltier Thermocycler (MJ Research, UK).

A bulk-mix of reagents was made for each PCR, allowing adequate volume for the planned number of reactions, with an additional 10% added to ensure that the mix would not run short. PCR primers and *Taq* polymerase (Bioline Ltd. London, UK) were kept on ice and added last. Standard PCR reactions were carried out in a total volume of 20 µl. Each reaction contained buffer [166 mM ammonium sulphate, 670 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 100 mM BME, 67 µM EDTA, 1.7 mg/ml BSA], 8 pmol of each primer, 0.2 units *Taq* polymerase (Bioline Ltd. London, UK).

The PCR mix was added to each well of the PCR plate using an automatic Biohit repeating dispenser (Alpha Laboratories, UK). Reactions were overlaid with 20 µl mineral oil to prevent evaporation and sealed with a clear sticky plastic lid. Plates were centrifuged at 200g for 1 minute to ensure all PCR reagents were below the oil layer. PCR amplification was performed on a PTC-225 Peltier Thermocycler (MJ Research, UK). The primer sequences and PCR conditions used to study variants within the Tm gene are shown in Tables 2.1 and 2.2, respectively.

Variant	Primer Pairs *(Forward/Reverse)*	PCR Product size (bp)
-1748G>C	5' $^{-2073}$CAATGGACCCCACATTTTCAC^{-2053} 3'	609
	5' $^{-1465}$GCCACGTGCGACAGACAC^{-1482} 3'	
-1208/09TT>delTT	5' $^{-1580}$ACAGTCGTGCTTGCCTTTTTC^{-1560} 3'	604
	5' $^{-977}$TTCCATTTCGTCCAGAGTCC^{-996} 3'	
-1166G>A	5' $^{-1580}$ACAGTCGTGCTTGCCTTTTTC^{-1560} 3'	604
	5' $^{-977}$TTCCATTTCGTCCAGAGTCC^{-996} 3'	
A455V	5' $^{+1570(+1400)}$GCCCGACTCGGCCCTTT***T***$^{+1588(+1418)}$ 3'	236
	5' $^{+1570(+1400)}$GCCCGACTCGGCCCTTC***C***$^{+1588(+1418)}$3'	
	5' $^{+1806(+1636)}$CGGCGCCCTGCTTCTTGC$^{+1788(+1618)}$ 3'	

Table 2.1 Primer pairs for variants analysed.
Nucleotides are numbered from the transcription start site mapped to 163 bp upstream of the ATG initiation codon (Jackman et al., 1987) (Yu et al., 1992). For non-synonymous variants within the coding region are numbered from the site of translation (numbers are given in parenthesis. For the 1418C>T (A455V) substitution, ARMS primers with destabilising mismatches were designed according to Kwok *et al.* (Kwok et al., 1990) and O'Dell *et al.* (O'Dell et al., 2000), for allele specific amplification of either the A or the V allele. The underlined base represents the destabilising mismatch base, whereas the base in ***bold italics*** matches the specific allele. A common reverse primer was used. The primers used to genotype for the -1166G>A variant were used to generate a second Tm fragment that was used as an internal control for each ARMS PCR reaction. Primers were designed using the 'Primer 3' primer design software.

Variant	Step 1	Step 2	Step 3	Step 5	Number of cycles (Steps 2-5)	Termination Step
-1748G>C	95°C 5 min	95°C 1 min	58°C 1 min	72°C 1 min	35	72°C 5 min
-1208/09TT>delTT	95°C 5 min	95°C 1 min	58°C 1 min	72°C 1 min	35	72°C 5 min
-1166G>A	95°C 5 min	95°C 1 min	58°C 1 min	72°C 1 min	35	72°C 5 min
A455V	95°C 5 min	95°C 30 sec	58°C 30 sec	72°C 1 min	30	72°C 5 min

Table 2.2 PCR conditions for variants being studied.

2.2.3.2 Restriction digestion

Restriction endonucleases are derived from bacteria, and cleave double stranded DNA into defined and reproducible fragments. The restriction endonuclease recognises a short symmetrical sequence of DNA and cuts the DNA molecule at a particular site within that recognition sequence. Restriction endonucleases are sensitive even to a single base change in the recognition sequence, which enables them to be used in the detection of point mutations and single base polymorphisms.

Most restriction digests were performed using an overnight incubation period at 37°C. A bulk mix of restriction enzyme digest mix was made up for each batch, containing sufficient enzyme to digest the PCR products in each well of the PCR plate. In a typical digest mix for a 96 well plate, 130 µl of buffer was added to 200 units of enzyme and made up to 500 µl with distilled water. 5 µl of digestion mix was added to 8 µl of each reaction product using a repeater pipette as for the PCR mix. Each plate was centrifuged at 1000g for thirty seconds to ensure that the PCR product and restriction enzyme were mixed. The specific details for each PCR product and digestion mix are detailed in Table 2.3.

Variant	PCR Products (bp)	Restriction Enzyme	Restriction Pattern	
			Common Allele (bp)	Rare Allele (bp)
-1748G>C	609	*Ban II* (0.5 U)	434, 175	327, 175, 107
-1208/09 TT>delTT	604	*Pme I* (3 U)	604	371, 231
-1166G>A	604	*Bbs I* (0.75 U)	604	413, 191
A455V	236	-	-	-

Table 2.3 Restriction profiles of variants being studied.
In a characteristic reaction, 8 µl of each PCR product were added to 5 µl of digestion mix containing 0.5-3 U of enzyme and 1.3 µl of 1x appropriate restriction buffer in a final volume of 13 µl.

2.2.4 Detection of PCR products, variant sites or mutations

2.2.4.1 Agarose gel electrophoresis

Gels consisting of 1-2% agarose were used to analyse PCR products to determine that the PCR had worked and the product was the anticipated size. An appropriate mass of agarose was melted in 1x TBE buffer. EtBr was added to cool molten agarose, to a final concentration of 0.3 mg/l. The agarose was poured into a gel tray with an appropriate comb. When the gel had set, it was transferred to an electrophoretic tank and submerged in 1x TBE buffer. Samples were mixed with 1x formamide dye (95% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cynol) and loaded into wells. A 1 kb DNA ladder (Gibco-BRL Ltd. Paisley, UK) was used to determine the size of the DNA fragments. Gels were electrophoresed at 100-120 V, following which, DNA fragments were visualised under UV light and photographed using a Syngene Trans-illuminator and the Gene-Snap Syngene software (Syngene, Cambridge, UK). A picture of a typical agarose gel is presented in Figure 2.2.

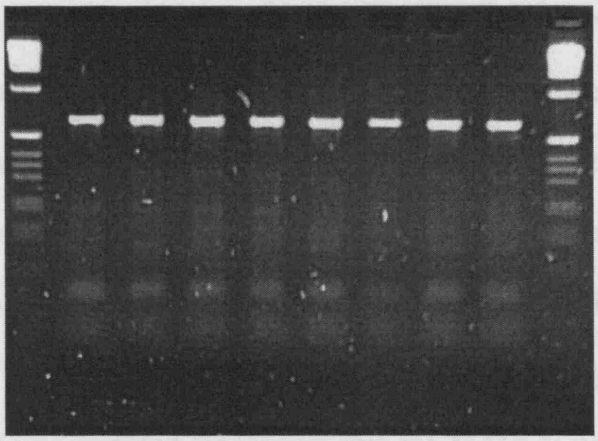


Figure 2.2 Agarose gel.
Agarose gel of 8 samples amplified for the -1748G>C Tm gene variant. Columns 1 & 9: 1 kb ladder (Gibco-BRL Ltd. Paisley, UK).

2.2.4.2 MADGE for analysis of genomic variants

The DNA fragments produced following restriction enzyme digest of PCR products were separated using electrophoresis on a non-denaturing polyacrylamide gel, Microtitre Array Diagonal Gel Electrophoresis; MADGE. This technique allows all 96 samples of a standard PCR plate to be electrophoresed on a single gel, thereby allowing medium throughput screening of the PCR products. 2 mm deep wells are arranged at an angle of 71.2° to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used. As a result the maximum track length is 26.5 mm, which allows sufficient travel for genotype resolution.

Typically, glass plates (160 x 100 x 2 mm) were cleaned with detergent, rinsed with dH_2O and ethanol, and dried. Once dry, one side was coated with γ-methacryloxypropyltrimethoxysilane ('sticky' saline) to facilitate adhesion to the glass plate. 7.5% MADGE gels (50 ml/plate) were prepared from a 30% stock polyacrylamide solution (19:1 acrylamide bis-acrylamide) containing 1x TBE buffer.

Polymerisation was initiated by the addition of 150 µl of both TEMED and 25% APS. The gel solution was mixed and poured into a gel former. The coated glass plate was gently placed on top, coated side down, and a small weight placed on top to ensure pressure between the former and the glass plate. This was then left for fifteen minutes to set. Following polymerisation, the glass plate and attached gel were then prised away from the former, excess gel removed and gels stored in 1x TBE buffer at 4°C.

PCR-digest product was prepared for loading onto the MADGE gel. 2.5 µl of loading buffer was mixed with 10 µl of digested sample. Prior to loading, the digested PCR products, each gel was soaked in EtBr (100 ml of 1x TBE and 10 µl EtBr) for 20 minutes. The MADGE gel was transferred to an electrophoretic tank and submerged in 1x TBE buffer and 7.5 µl of the digested DNA loaded onto the gel in columns of eight using a multichannel pipette. The gel was electrophoresed at 150 V. Following electrophoresis DNA fragments were visualised under UV light, due to staining by EtBr, and photographed using a Syngene Trans-illuminator and the Gene-Snap Syngene software (Syngene, Cambridge, UK). A picture of a typical MADGE gel is presented in Figure 2.3.

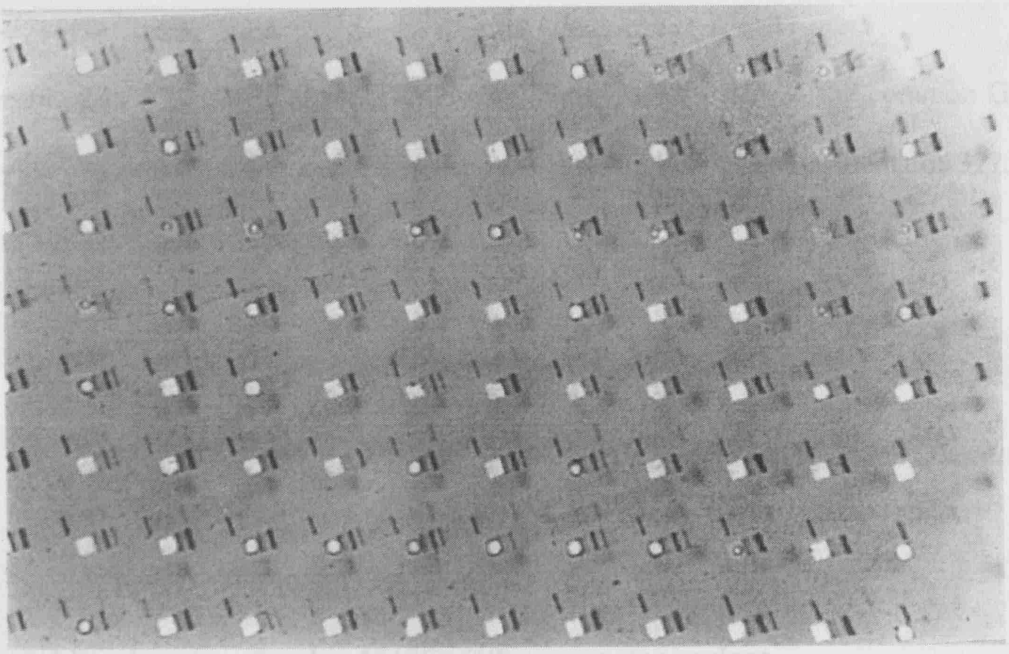


Figure 2.3 MADGE gel for the Tm -1748G>C gene variant.
Madge gel of digest in 93 samples with 3 blank controls.

2.2.4.2.1 Genotyping for polymorphisms in the Tm gene

PCR conditions were optimized in each case to yield a strong, specific product with no non-specific amplification. The Tm gene has a high overall GC content, resulting from long runs of Gs and Cs. These form hairpin-like secondary structures, which impede amplification. Inclusion of DMSO is the reaction mix was necessary for all fragments, to resolve problems related to secondary structure of the gene. Magnesium chloride was kept constant as optimization of the PCR was possible by adjusting the annealing temperature. Primer sequences, PCR conditions and restriction enzyme digestion conditions for the Tm variants under investigation are summarised in Tables 2.1-2.3.

i) Tm -1748G>C

PCR resulted in a 609 bp fragment. Following digestion with *Ban II*, the common G allele gave fragments of 434 and 175 bp and the rare C allele gave fragments of 327, 175 and 107 bp, as shown in figure 2.4.

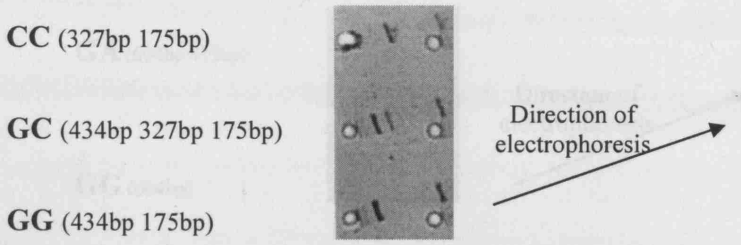


Figure 2.4 Band pattern for the -1748G>C Tm promoter variant.

ii) Tm -1208/09TT>delTT

PCR resulted in a 604 bp fragment. Following digestion with *Pme I*, the common TT allele gave a relevant fragment of 604 bp and the rare delTT allele gave fragments of 371 and 231 bp, as shown in figure 2.5.

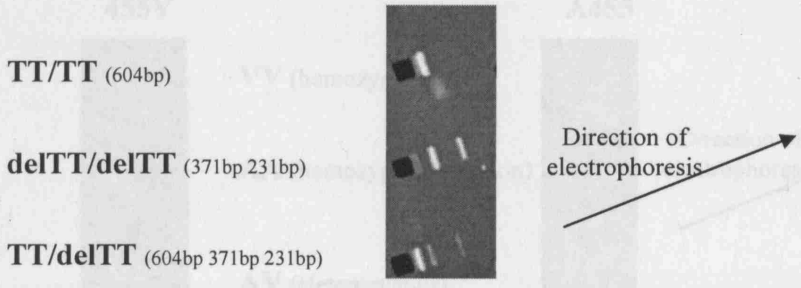


Figure 2.5 Band pattern for the -1208/09 TT>delTT Tm promoter variant.

iii) Tm -1166G>A

PCR resulted in a 604 bp fragment. Following digestion with *Bbs I*, the common G allele gave a fragment of 604 bp, whereas the rare A allele gave fragments of 413 and 191 bp, as shown in figure 2.6.

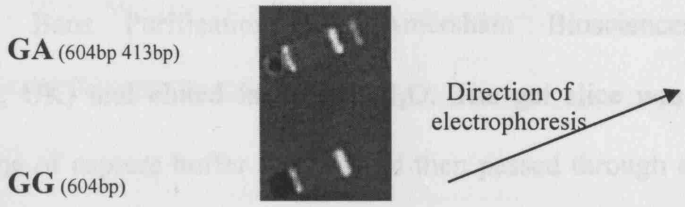


Figure 2.6 Band pattern for the -1166G>A Tm promoter variant.

iv) Tm 1418C>T (A455V)

ARMS primers with destabilising mismatches were designed according to Kwok *et al.* (Kwok et al., 1990) and O'Dell *et al.* (O'Dell et al., 2000), for allele specific amplification of either the A or V allele; see Figure 2.7.

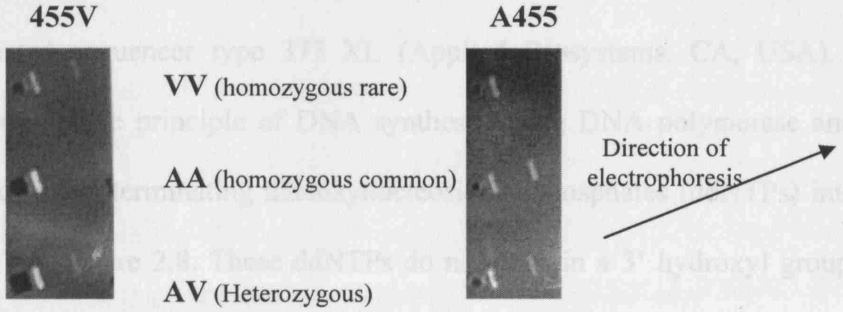


Figure 2.7 Band pattern for the 1418C>T (A455V) Tm coding region variant.
Underlined base represents the destabilizing mismatch base. Bold italics base matches the specific allele. Same DNA samples amplified with 455V F and A455 F. F = Forward; R = Reverse. The primers used to genotype for the -1166G>A variant were used as an internal control. First band: Control (604 bp); Second band: Allele specific PCR product (236 bp).

2.2.4.3 Purification of DNA fragments for cloning

PCR products or restriction reaction mix were electrophoresed on a 1% preparatory grade, low melting point agarose (Sigma-Aldrich Company Ltd. Dorset, UK). The desired fragments were excised and purified from the gel slice using the GFX PCR DNA and Gel Bans Purification Kit (Amersham Biosciences (UK) Ltd. Buckinghamshire, UK) and eluted in 30 µl dH_2O. The gel slice was melted in the appropriate volume of capture buffer at 60°C and then passed through a DNA binding GFX column. The column was washed with a high-salt solution and the DNA eluted in dH_2O. This procedure ensures that primers and other reagents used in the PCR are removed.

2.2.5 DNA sequencing either for genomic or plasmid DNA

Direct sequencing was done by the chain termination sequencing method (Sanger and Coulson, 1975), using the ABI Big Dye Terminator v 3.1 Cycle Sequencing reagents and an automated sequencer type 377 XL (Applied Biosystems. CA, USA). This method is based on the principle of DNA synthesis using DNA polymerase and the incorporation of chain terminating dideoxynucleoside triphosphates (ddNTPs) into the nascent DNA; see Figure 2.8. These ddNTPs do not contain a 3' hydroxyl group and the chain cannot be extended further, resulting in termination at positions where they become incorporated. During the amplification reaction, molecules are terminated at random, stopping at every nucleotide position along the newly formed DNA strand. The sequencing reaction is then subjected to electrophoresis. The ddNTPs, labeled with a different dichlororthodamine fluorescent dye (dR110, dR6G, dTAMRA and dROX) for

each base, allow the sequencing equipment to identify the different label colours and give sequence information in a single reaction mixture.

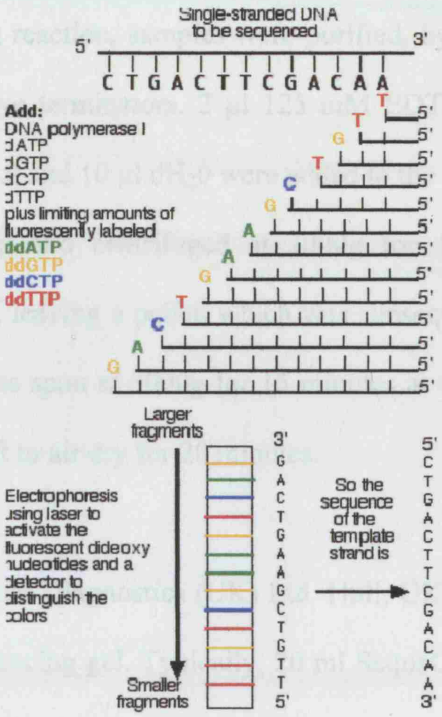


Figure 2.8 Schematic representation of the chain termination sequencing method. (Sanger and Coulson, 1975). Reproduced from http://users.rcn.com with permission.

DNA fragments to be sequenced were amplified by PCR in a 50 µl reaction and run on a 1% low melting point agarose, stained with EtBr, to make sure the correct fragment size was obtained. Samples were purified using the GFX PCR DNA and Gel Bans Purification Kit (Amersham Biosciences (UK) Ltd. Buckinghamshire, UK), according to the manufacturer's protocol; see section 2.2.4.3.

The sequencing reaction was performed using 2 µl 5x BigDye sequencing buffer, 2 µl terminator ready reaction mix, 30-100 ng gel purified PCR product or 1 µg plasmid DNA, 3.2 pmol appropriate primer (forward or reverse) made up to a final volume of 10 µl with distilled water. The sequencing cycling conditions employed were: 96°C for 1

minute x 1 cycle followed by (96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes) x 25 cycles.

Following the sequencing reaction, samples were purified, by ethanol precipitation, to remove unincorporated dye terminators. 2 µl 125 mM EDTA, 2 µl 3 M ammonium acetate, 10 µl 100% ethanol and 10 µl dH_20 were added to the 10 µl sequencing reaction mix, mixed by vortexing and centrifuged at 3000g for 45 minutes at 4°C. The supernatant was removed, leaving a pellet, which was subsequently washed with 50 µl 70% ethanol. The tube was spun at 3000g for 15 minutes at 4°C, the ethanol removed carefully and the pellet left to air dry for 20 minutes.

The SequaGel XR (National Diagnostics (UK) Ltd. Hull, UK) polyacrylamide solution was used to cast the sequencing gel. Typically, 20 ml SequaGel XR monomer solution (containing urea, acrylamide and acrylamide derivatives) and 5 ml SequaGel complete buffer solution (containing TBE and TEMED) were combined and 200 µl 10% APS added to induce polymerisation. The gel was pre-run at 65 W (1600 V, 80 A) in 1x TBE buffer for 30 minutes to warm the gel. The samples were re-suspended in 4 µl sequencing loading dye, denatured at 95°C for 2-3 minutes and placed on ice water immediately to prevent re-annealing. 2 µl of the re-suspended samples were loaded onto the polyacrylamide gel and run in 1x TBE buffer at 1600 V for 9 hours.

Sequencing strategy for genomic DNA

The coding region (1.7 kb) and approximately 1960 bp of the 5' untranslated region (from the transcription start site) of the Tm gene were amplified in 6 overlapping fragments, represented in Figure 2.9. The sequence and position of the primer pairs together with annealing temperatures for each primer pair and the size of the resulting

fragment is represented in Table 2.4. The fragments were subsequently sequenced using either the forward or reverse primer or both.

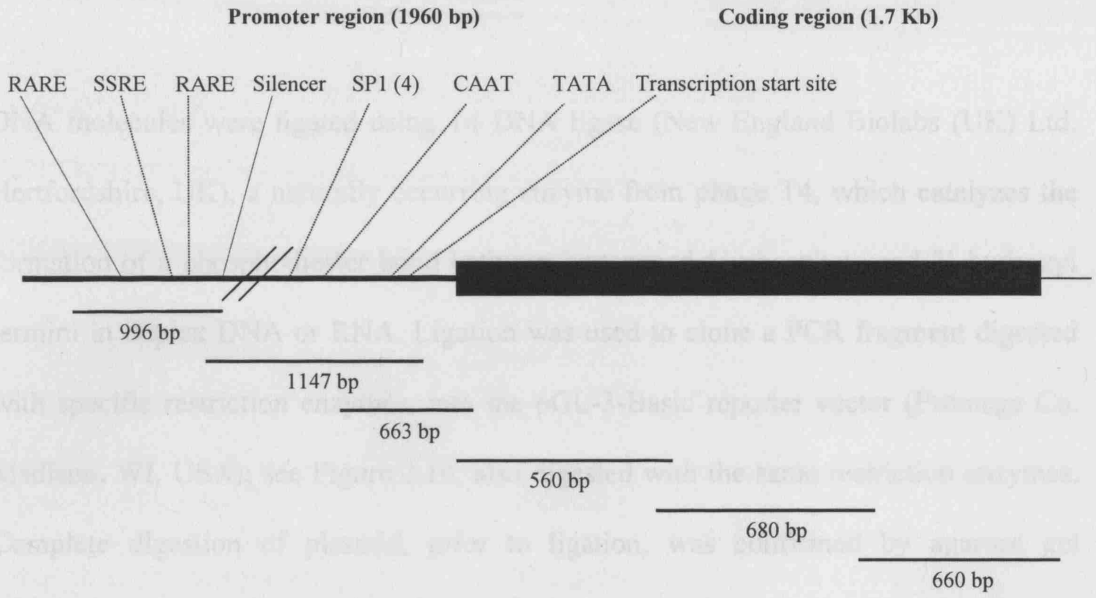


Figure 2.9 Schematic representation of the strategy used to sequence the Tm gene. Large amplification fragments were designed for sequencing. They covered 2 kb of the promoter region (encompassing the major regulatory elements), and the entire coding region (1.7 kb) in an overlapping fashion.

Primer Pairs (Forward/Reverse)	PCR Products (bp)	Annealing Temperature
5' $^{-1960}$CAGTGCCCCAACGAGGTTCC^{-1940} 3' 5' $^{-944}$CAGCTGATCCCACCCGG^{-961} 3'	996	67°C
5' $^{-1047}$AGTGCCCTTTGCCGAGC^{-1030} 3' 5' $^{+96}$GCACAGGACGCCGATGGC^{+78} 3'	1147	67°C
5' $^{-304}$GATGAAAGAGGGCTGCACG^{-285} 3' 5' $^{+328}$TCGCAGATCTGACTGGCATT^{+308} 3'	663	62°C
5' $^{+267}$ACGACTGCTTCGCGCTC^{+284} 3' 5' $^{+818}$AGCCACCGCGGCGGAGCT^{+800} 3'	560	66°C
5' $^{+727}$TGCCGCCGTCTCGATCACCT^{+747} 3' 5' $^{+1409}$ACAGGCAGTCTGGTTGCA^{+1391} 3'	680	66°C
5' $^{+1296}$ACCAGTGCCAGCCCCTGAAC^{+1316} 3' 5' $^{+1960}$CAAAGCTGGGGGTGAGG^{+1943} 3'	660	63°C

Table 2.4 Primer pairs used to amplify Tm fragments for sequencing. Nucleotides are numbered from the transcription start site. PCR thermal specific profiles: initial denaturation (95°C, 5 minutes), 30 cycles (95°C, 30 seconds + annealing temperature, 30 seconds + 72°C, 1 minute), final extension (72°C, 5 minutes).

2.2.6 Cloning

2.2.6.1 Ligation & Cloning

DNA molecules were ligated using T4 DNA ligase (New England Biolabs (UK) Ltd. Hertfordshire, UK), a naturally occurring enzyme from phage T4, which catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. Ligation was used to clone a PCR fragment digested with specific restriction enzymes, into the pGL-3-Basic reporter vector (Promega Co. Madison, WI, USA); see Figure 2.10, also digested with the same restriction enzymes. Complete digestion of plasmid, prior to ligation, was confirmed by agarose gel electrophoresis; see section 2.2.4.1. The PCR fragment for insertion into plasmid was electrophoresed and gel purified; see section 2.2.4.3. The relative concentration of the plasmid DNA and the target DNA were estimated after agarose gel electrophoresis. A molar ratio of 1:2-1:4 was used for ligation. Ligation of the target DNA and pGL-3-basic vector (Promega Co. Madison, WI, USA) was performed mixing 50-200 ng DNA, 1 µl T4 ligase and 1 µl ligase buffer in a final volume of 10 µl. All reactions were incubated at 4°C for 48 hours.

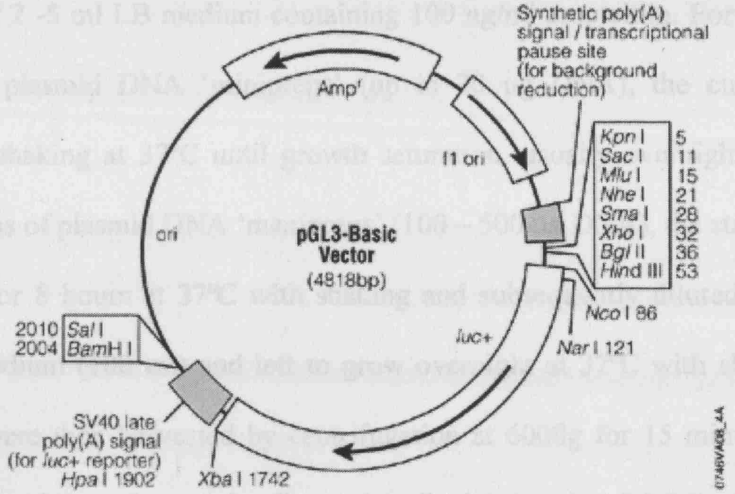


Figure 2.10 Schematic representation of pGL-3-Basic reporter vector containing the *luciferace* gene.
The ampicillin resistance gene enables selection on the basis that only colonies containing the transformed plasmid are able to grow in the presence of ampicillin.

2.2.6.2 Transformation of DH5α or XL1-Blue bacterial cells

Competent cells (DH5α or XL1-Blue) were thawed slowly on ice. Typically an aliquot of 50 µl was used per reaction. 3 µl of ligated target and plasmid DNA (prepared as described in section 2.2.6.1), 3 µl of circular plasmid or 1 µl of *Dpn I* treated DNA (prepared as described in section 2.4.2) were added. Following incubation on ice for 30 minutes, the reaction mixture was transferred to a water bath at 37°C for 1 minute. The cells were heat-shocked, 300 µl of LB medium added and the tubes incubated with shaking for 1 hour at 37°C, to allow expression of the ampicillin resistance encoded by the β-lactamase gene of the vector. 200 µl of the mixture were plated onto LB + agar plates containing a final concentration of 100 ng/ml ampicillin and incubated at 37°C overnight.

A single colony was picked from freshly streaked selective plates and inoculated in a starter culture of 2 -5 ml LB medium containing 100 ng/ml ampicillin. For small scale preparations of plasmid DNA 'minipreps' (up to 20 µg DNA), the cultures were incubated with shaking at 37°C until growth saturation, usually overnight. For large scale preparations of plasmid DNA 'maxipreps' (100 – 500 µg DNA), the starter culture was incubated for 8 hours at 37°C with shaking and subsequently diluted 1/500 into selective LB medium (100 ml) and left to grow overnight at 37°C with shaking. The bacterial cells were then harvested by centrifugation at 6000g for 15 minutes at 4°C. DNA was extracted from bacterial cells as described in section 2.2.2.2. A schematic representation of the cloning strategy is illustrated in Figure 2.11.

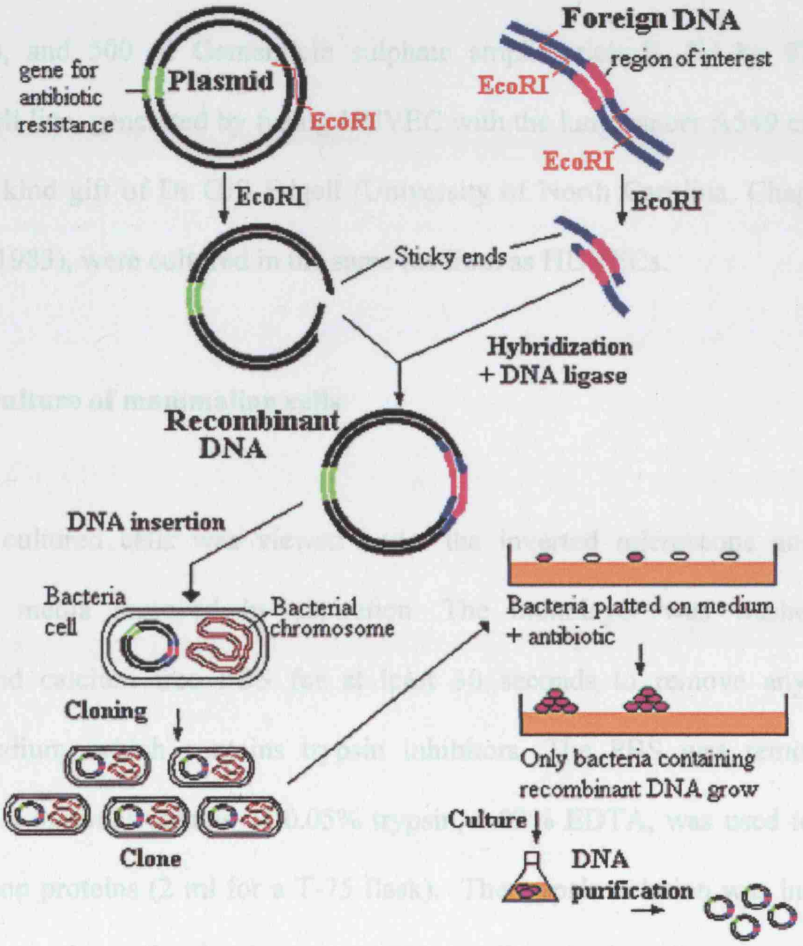


Figure 2.11 Cloning strategy schematic.
Reproduced from www.accessexcellence.org with permission.

2.3 Mammalian cell culture

2.3.1 Mammalian cells, equipment and medium

All cells were cultured in Falcon tissue culture dishes (Becton-Dickinson Labware Europe, Le Pont de Claix, France), and maintained at 37°C in 5% CO_2 atmosphere in humidified incubators (Wolf Laboratories). All equipment and reagents were sterilised either by filtration or autoclaving. Reagents from commercial sources were tested for tissue culture applications. HUVECs, obtained from PromoCell (PromoCell GmbH. Heidelberg, Germany), were typically cultured in EBM medium supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml bovine brain extract (BBE), and 500 µl Gentamicin sulphate amphotericin-B. EA.hy 926 cells (endothelial cell line, generated by fusing HUVEC with the lung cancer A549 cell line), which were a kind gift of Dr CJS Edgell (University of North Carolina, Chapel Hill) (Edgell et al., 1983), were cultured in the same medium as HUVECs.

2.3.2 Subculture of mammalian cells

The flask of cultured cells was viewed under the inverted microscope and when confluent the media removed by aspiration. The monolayer was washed with magnesium and calcium free PBS for at least 30 seconds to remove any serum containing medium, which contains trypsin inhibitors. The PBS was removed by aspiration and a minimal volume of 0.05% trypsin, 0.02% EDTA, was used to digest the cell adhesion proteins (2 ml for a T-75 flask). The trypsin solution was incubated with the cell monolayer for 5 minutes at 37°C, until a single cell suspension was obtained. Complete media was added (10 times the volume of trypsin used), the cell

suspension transferred to a sterile 15 ml conical centrifuge tube and centrifuged at 120g for 5 minutes in a tabletop centrifuge. The supernatant was discarded carefully, so as not to disturb the cell pellet, and the cells resuspended in 5 ml complete media. Cell number was calculated by making a 1 in 10 dilution of the cell suspension and placing 20 µl between a cover slip and the base of a haemocytometer. Cells were counted in 8 diagonal squares cells/ml = cells counted x dilution factor/8. The volume was adjusted if necessary and cells were subsequently seeded at the desired concentration. HUVECs were seeded in flasks or wells that had been coated with 1% gelatine solution (Sigma-Aldrich Company Ltd. Dorset, UK), to facilitate cell adhesion.

2.3.3 Freezing mammalian cells

Cells were frozen for long-term storage in order to avoid senescence and reduce the risk of contamination. The cryoprotective agent, dimethylsulfoxide (DMSO), was used in conjunction with complete medium in order to preserve cells at -70°C or lower. DMSO reduces the freezing point and allows a slower cooling rate, which reduces the risk of ice crystal formation and cell damage. Cells in log-phase were trypsinised and the cell pellet resuspended in growth medium containing 10% DMSO (v/v). Cells were mixed thoroughly and counted using a haemocytometer as described above. 1 ml aliquots of cell suspension (10^6-10^7 cells), in 2 ml cryovials, were placed at -70°C overnight and subsequently transferred to liquid nitrogen for long term storage.

2.4 Functional characterisation of promoter variants

Variants within the promoter region were studied to determine whether there was evidence for disruption of positive or negative regulatory elements for gene transcription. Genetic reporter systems, most frequently used as indicators of transcriptional activity in cells, have made a significant contribution to the study of eukaryotic gene expression and its regulation. Typically, in these experiments, a reporter gene is joined to a promoter sequence, containing the target sequence to be tested, in an expression vector. The vector is transfected into cells chosen to represent a cell-type known to express the protein of interest. Following transfection, the cells are assayed for the presence of the reporter gene by measuring either the amount of reporter mRNA, the reporter protein itself or the enzymatic activity of the reporter protein. The composition and organization of regulatory elements present in the target promoter sequence direct transcription of the reporter gene, which in turn controls the level of reporter gene product. Thus, the functional importance of a given sequence can be assessed by constructing different versions of the promoter region and comparing the relative efficiency of reporter gene expression. Co-transfection with a vector, which encodes a different reporter gene under the control of a ubiquitous promoter, is also advisable as an internal control to which the level of expression of the reporter gene of interest can be normalised.

2.4.1 Tm promoter-reporter gene fusion constructs

The Tm promoter region to be cloned into the fusion construct spanned the sequence between -1960 and +96 (nucleotides numbered from transcription start site). The region was amplified by PCR, using genomic DNA as template (see section 2.2.3.1). The upstream and downstream primers were 5' $^{-1960}$CGGGGTACCCAGTGCCCCAACGAGGTTCC^{-1940} 3' and 5' $^{+96}$CCCAAGCTTGCACAGGACGCCGATGGC^{+78} 3', respectively. These primers were modified to introduce restriction sites for *Acc65I* and *HindIII* (underlined), respectively, to facilitate cloning. 50-100 ng genomic DNA was amplified in a final volume of 50 µl, using polymerase buffer [500 mM KCl, 100 mM Tris (PH 8.3), 0.01% gelatine and 2 mM dNTPs], 5 pmol of each primer, 1.25 units *Pfx* platinum polymerase (Gibco-BRL Ltd. Paisley, UK) in a PTC-225 Peltier Thermocycler (MJ Research, UK). Cycling conditions consisted of an initial denaturation step, 95°C for 2 min; followed by 30 cycles of 95°C for 30 sec, 67°C for 30 sec, 72°C for 3 min; and a final extension of 5 min at 72°C. The fragment obtained was gel purified (see section 2.2.4.3), digested with *Acc65I* and *HindIII* and cloned into the corresponding sites of the reporter vector, see section 2.2.6.1. The resulting promoter-reporter gene fusion construct was designated pTm WT. The reporter vector used was pGL-3-Basic (Promega Co. Madison, WI, USA). The vector contains the gene coding for the *luciferase* gene cloned from the firefly *Photinus pyralis*. This gene is able to catalyse a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+}, resulting in light emission. A luminometer is used to measure light output, thereby quantitating the *luciferase* reaction; see section 2.4.5.

The pGL-3-Control vector (Promega Co. Madison, WI, USA) was used as a positive control to test whether transfection was successful; see Figure 2.12. This is a vector in which the *luciferase* gene is placed under the control of the SV40 promoter, and using the SV40 enhance element (de Wet et al., 1987). The pGL-3-Basic vector (Promega Co. Madison, WI, USA), which contains the *luciferase* gene without promoter or enhancer, was used to provide a reading to which experimental firefly *luciferase* activity could be normalised; see Figure 2.10. The pUC 19 vector was used as a negative control; see Figure 2.12.

The *Renilla* luciferase vector (pRL-TK) (Promega Co. Madison, WI, USA) was used as an internal control to assess transfection efficiency; see Figure 2.12. This family of vectors is designed to provide constitutive *Renilla* luciferase expression in transfected cells. pRL vectors may be used in combination with any experimental firefly *luciferase* vector to co-transfect mammalian cells. pRL vectors contain the cDNA encoding *Renilla* luciferase cloned from the anthozoan coelenterate *Renilla reniformis* (sea pansy). The herpes simplexvirus thymidine kinase promoter region provides low-level constitutive expression of pRL-TK *Renilla* luciferase (Stewart et al., 1987) (Wagner et al., 1985). The firefly and *Renilla* luciferases are of distinct evolutionary origins. As such they have different enzyme structures and substrate requirements, which enable their respective bioluminescent reactions to be distinguished. In an attempt to ensure independent genetic expression between experimental and control reporter genes, a high ratio of test vector to control was used (100:1). Relatively small quantities of a pRL control vector are needed to provide low-level, constitutive expression of *Renilla* luciferase control activity. Precise measurements of considerably different experimental and control luminescence values are possible due to the extreme sensitivity (subattomole range) of the firefly and *Renilla* luciferase assays (Pazzagli et al., 1992).

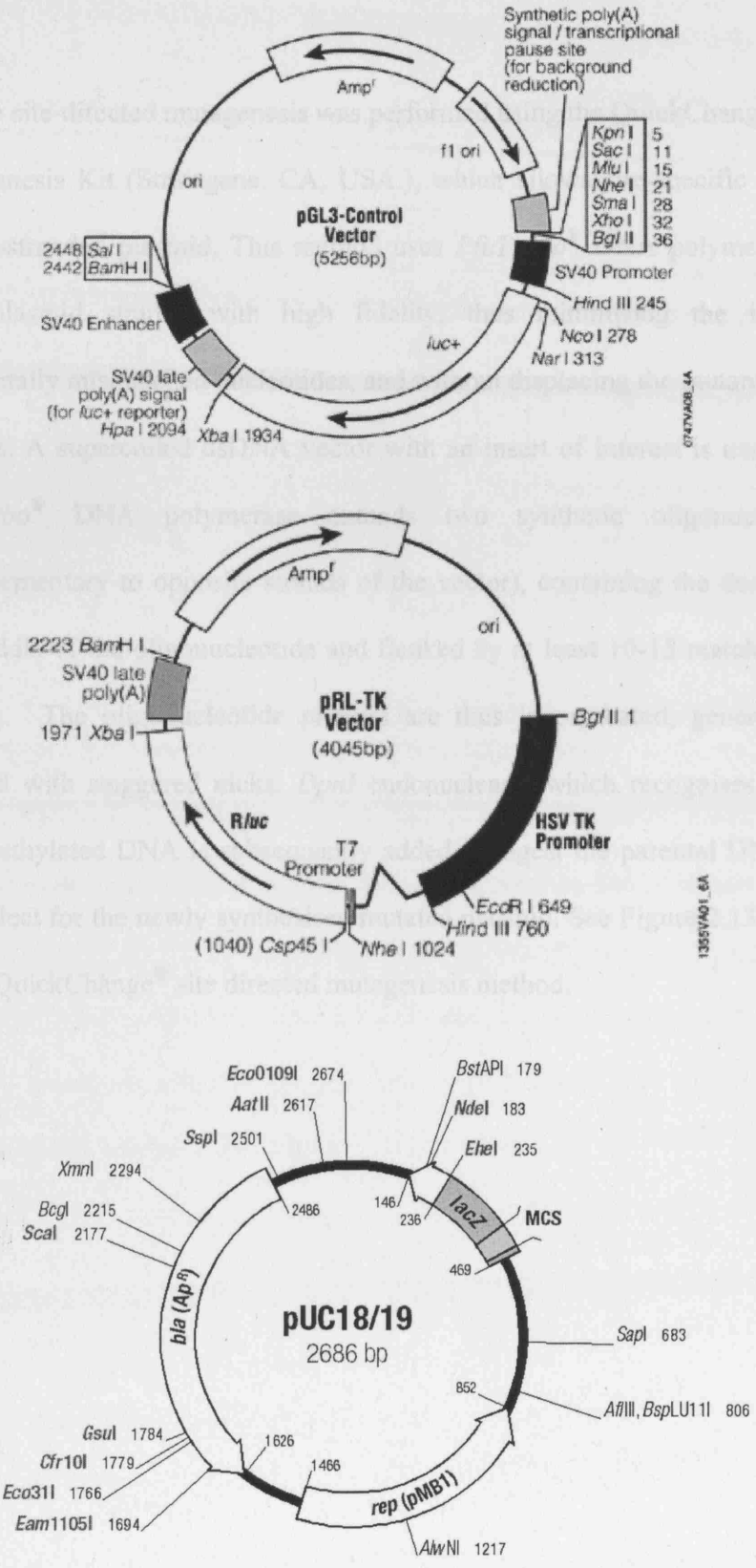


Figure 2.12 Schematic representation of vectors.
pGL-3-Control, pRL-TK and pUC19.

2.4.2 Site-directed mutagenesis

In vitro site-directed mutagenesis was performed using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene. CA, USA.), which allows site-specific mutation in any double-stranded plasmid. This method uses *PfuTurbo®* DNA polymerase to replicate both plasmid strands with high fidelity, thus minimising the incorporation of accidentally mismatched nucleotides, and without displacing the mutant oligonucleotide primers. A supercoiled dsDNA vector with an insert of interest is used as a template. *PfuTurbo®* DNA polymerase extends two synthetic oligonucleotide primers (complementary to opposite strands of the vector), containing the desired mutation in the middle of the oligonucleotide and flanked by at least 10-15 matched bases, during cycling. The oligonucleotide primers are thus incorporated, generating a mutated plasmid with staggered nicks. *DpnI* endonuclease, which recognises methylated and hemimethylated DNA is subsequently added to digest the parental DNA template and thus select for the newly synthesised mutated plasmid. See Figure 2.13 for an overview of the QuickChange® site directed mutagenesis method.

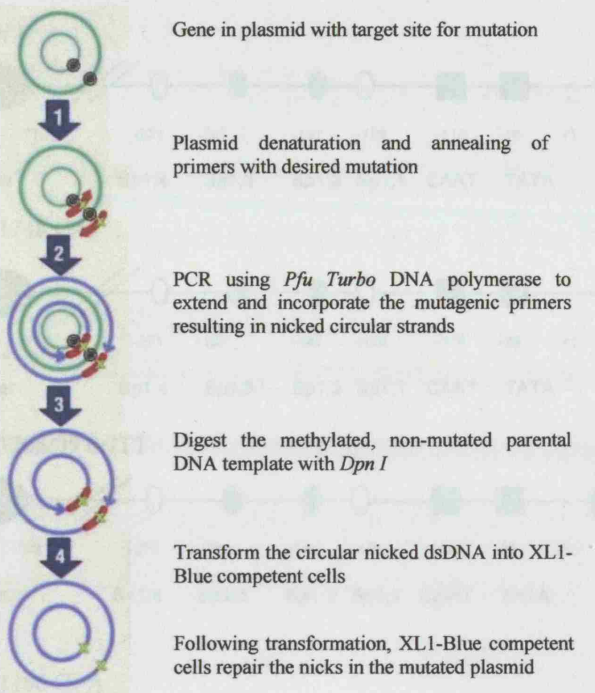


Figure 2.13 Overview of the QuickChange® site directed mutagenesis method. Reproduced from www.sciencemag.org with permission.

The pTm WT vector (see section 2.4.1) was used as the wild-type template for the introduction of all the desired single point mutations within the cloned Tm promoter fragment; see Figure 2.14. Table 2.5 indicates the primer pairs used to incorporate the desired mutations into the Tm promoter region cloned into the pGL-3-Basic vector (Promega Co. Madison, WI, USA).

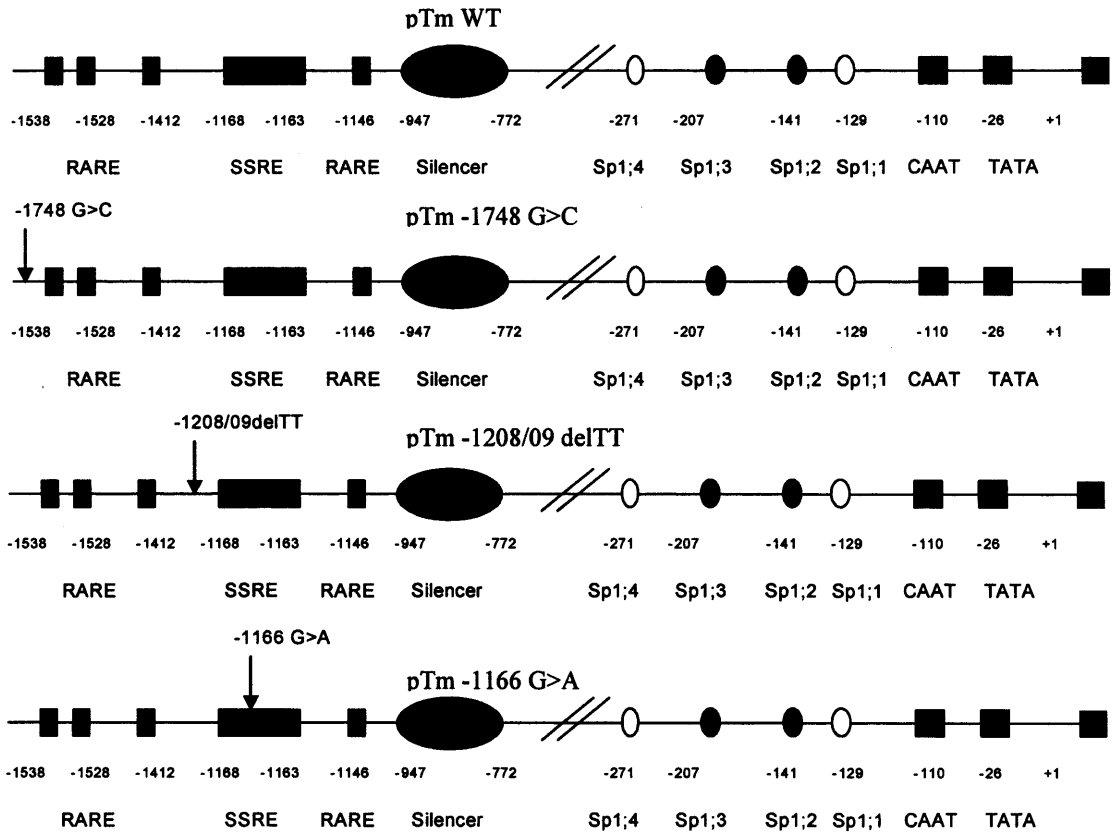


Figure 2.14 Schematic representation of the TM promoter fragment cloned into the pGL-3-Basic vector illustrating the different mutated constructs generated, the position of the point mutations introduced and their proximity to regulatory elements identified within the promoter region.
Regulatory elements: TATA-box (-21 to -26), CAAT box (-106 to -110) (Tazawa et al., 1993), four Sp1 transcription factor binding sites (-129, -141, -207 and -271) (Tazawa et al., 1993), silencer element (-772 to -947) (Tazawa et al., 1993), SSRE (-1163 to -1168) (Takada et al., 1994), four RARE (-1141 to -1146, -1407 to -1412, -1523 to -1528 and -1533 to -1538) (Dittman et al., 1994). Abbreviations: **SSRE**: Shear Stress Response Element; **RARE**: Retinoic Acid Response Elements; +1: Coding Region.

Primer Pairs (Forward/Reverse)	**Mutation introduced**
5' $^{-1761}$CTCTTCCCAGGGG**T**TCCGCGTCAGGGG^{-1735} 3'	-1748G>C
5' $^{-1735}$CCCCTGACGCGGA**A**CCCCTGGGAAGAG^{-1761} 3'	
5' $^{-1228}$GAGAGAGGCAAGGGTGTTT**tt**AAACAGTTTGCCTCTCACC^{-1189} 3'	-1209/09
5' $^{-1189}$GGTGAGAGGCAAACTGTTT**aa**AAACACCCTTGCCTCTCTC^{-1228} 3'	TT>delTT
5' $^{-1180}$GGCGACCCGAGGGG**A**AGACCCACTCTTCC^{-1152} 3'	-1166G>A
5' $^{-1152}$GGAAGAGTGGGTCT**T**CCCCTCGGGTCGCC^{-1180} 3'	

Table 2.5 Primers used to incorporate the desired mutations into the TM promoter region cloned into the pGL-3-Basic vector.
The **bold underlined** base represents the base modified during site directed mutagenesis.

PCR was performed in a total volume of 50 µl containing 125 ng of each oligonucleotide primer, 1x reaction buffer, 50 ng dsDNA template, 0.25 mM dNTP mix and 1 µl *PfuTurbo*® DNA polymerase (2.5 U/µl). The reaction mix was overlaid with 50 µl paraffin oil. The cycling profile consisted of an initial denaturation at 95°C for 30 seconds 5, followed by 16 cycles of 30 second denaturation at 95°C, 30 seconds annealing at 60°C temperature, and 14 minutes extension at 68°C. Following cycling, the reaction was placed on ice for 2 minutes to cool the reaction to 37°C. 1 µl of *DpnI* (10 U/µl) was added directly to each amplification reaction and incubated for 1 hour at 37°C.

Following digestion with *DpnI,* XL1-Blue supercompetent cells (Stratagene. CA, USA.) were heat shock transformed (see section 2.2.6.2), plasmid DNA extracted from bacterial cells (see section 2.2.2.2) and the Tm promoter fragment cloned into the pGL-3-Basic vector (Promega Co. Madison, WI, USA) sequenced (see section 2.2.5) to ensure the desired mutation had been incorporated successfully. The primers used to sequence the Tm promoter constructs are listed in Table 2.6.

Primer	Direction	Location
5' $^{-1047}$AGTGCCCTTTGCCGAGC^{-1030} 3'	Forward	Tm promoter
5' $^{-944}$CAGCTGATCCCACCCGG^{-961} 3'	Reverse	Tm promoter
5' CTAGCAAAATAGGCTGTCCC 3'	Forward	pGL-3-Basic 5' of MCS
5' CTTATGTTTTTGGCGTCTTCC 3'	Reverse	pGL-3-Basic 3' of MCS

Table 2.6 Primers used to amplify Tm promoter constructs.
Nucleotides are numbered from the transcription start site. PCR thermal specific profiles: initial denaturation (96°C, 1 minute), 25 cycles (96°C, 10 seconds + 50°C, 5 seconds, 30 seconds + 60°C, 4 minute).

2.4.3 Transfection

A range of techniques have been developed to deliver macromolecules into eukaryotic cells. Nucleic acids delivery into eukaryotic cells can be achieved by either infection or transfection. During infection, viruses carrying the gene of interest in their genome are used to infect cells. Transfection utilizes biochemical and physical methods to introduce the gene of interest into cells. Nucleic acid delivery by transfection is faster, simpler and safer than infection, and as such is a more popular method for DNA delivery. Transfection techniques have facilitated the study of eukaryotic gene expression and regulation. Techniques available for nucleic acid delivery into cells include calcium phosphate co-precipitation, electroporation, micro-injection, biolistic particle delivery and complex formation with DEAE-dextran or cationic lipid reagents.

In calcium phosphate co-precipitation transfection techniques, extremely small, insoluble particles of calcium phosphate containing condensed DNA are precipitated when calcium chloride, DNA and phosphate buffer are mixed (Chen and Okayama, 1987). The calcium phosphate-DNA complexes adhere to cell membranes and enter into the cell cytoplasm by phagocytosis (Loyter et al., 1982). During electroporation, cells are exposed to a brief electrical pulse. Exposing a cell suspension to an electrical pulse, induces a potential across the cell membrane, inducing temporary pores in the cell membrane and facilitating DNA delivery (Neumann et al., 1982) (Wong and Neumann, 1982). DEAE-dextran or polyprene positively charged polymers, complex with negatively charged DNA complexes, thus enabling DNA binding to the cell surface. These DNA complexes are then delivered into cells by osmotic shock using DMSO or glycerol (Vaheri and Pagano, 1965) (Kawai and Nishizawa, 1984). Micro-injection introduces nucleic acids or proteins into the cell cytoplasm or nucleus using a

fine needle (Capecchi, 1980), whereas biolistic particle delivery uses high velocity microprojectiles to introduce macromolecules into cells (Daniell et al., 1990).

Problems associated with transfection methods include low efficiency of DNA delivery, poor reproducibility and cell toxicity. Cationic lipid reagent mediated transfection, however, generates high transfection efficiency, is simple to perform, guarantees reproducible results and requires relatively small amounts of DNA. Cationic lipid reagents (e.g. Lipofectin®, LipofecAMINE™, METAFECTENE™ and Lipofecatamine™ 2000) are recently developed DNA delivery reagents, which consist of polycationic liposomes comprised of a positively charged lipid N-[1-(2,3-dioleyoxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoylphosphatidylethanolamine (DOPE) in water. The positively charged reagent spontaneously interacts with negatively charged DNA, forming DNA/lipid complex. The polycationic liposomes fuse with the cell membrane resulting in efficient uptake of DNA by the cells (Felgner et al., 1987) (Felgner and Ringold, 1989); see Figure 2.15.

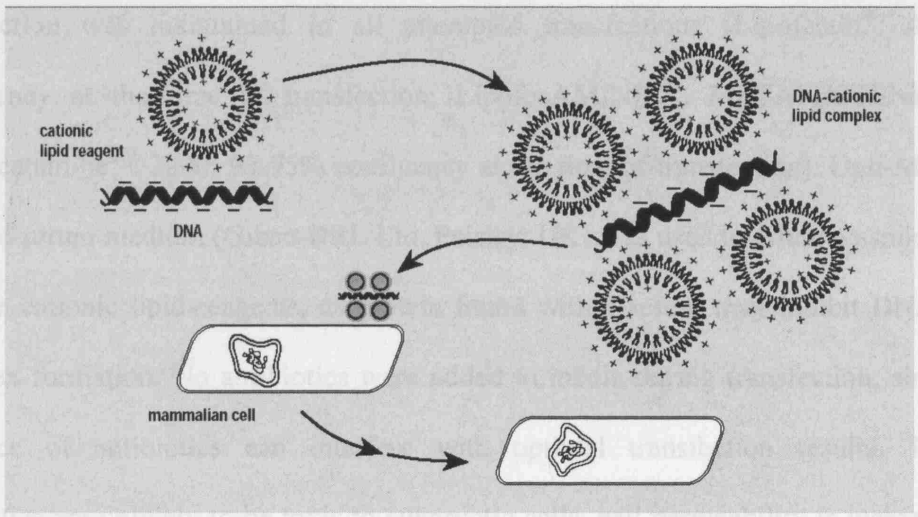


Figure 2.15 Model for cationic lipid reagent-mediated transfection.
Reproduced from www.ub.es. Positively charged reagent interacts with negatively charged DNA, forming DNA/lipid complex. The polycationic liposomes fuse with the cell membrane resulting in efficient uptake of DNA by the cells.

2.4.3.1 Transfection of HUVECs

Successful transfection of HUVEC primary endothelial cells, using cationic lipid reagents, has previously been reported, in experiments carried out to identify key regulatory elements necessary for expression of the Tm gene in endothelial cells and to characterize Tm gene mutations in the 5' regulatory region (Tazawa et al., 1993) (Matsumura et al., 1999) (Nakazawa et al., 2002). Unfortunately, efforts to transfect HUVEC primary endothelial cells in a 96-well plate format proved unsuccessful.

A range of cationic lipid reagents were used, including Lipofectin® (Gibco-BRL Ltd. Paisley, UK), LipofecAMINE™ (Gibco-BRL Ltd. Paisley, UK), METAFECTENE™ (Biontex Laboratories GmbH. Munich, Germany) and Lipofecatamine™ 2000 (Gibco-BRL Ltd. Paisley, UK). In trying to optimize this transfection fully, a set of specific guidelines supplied with each reagent were followed. Cell health was maintained prior to plating for transfection by following a routine subculturing procedure; see section 2.3.2. The appropriate cell density recommended for each reagent to obtain optimal transfection was maintained in all attempted transfections (Lipofectin®: 40-60% confluency at the time of transfection; LipofecAMINE™, METAFECTENE™ & Lipofecatamine™ 2000: 90-95% confluency at the time of transfection). Opti-MEM® I reduced serum medium (Gibco-BRL Ltd. Paisley, UK) was used to dilute plasmid DNA and the cationic lipid reagents, as protein found within serum may inhibit DNA/lipid complex formation. No antibiotics were added to media during transfection, since the presence of antibiotics can interfere with optimal transfection results. Though antibiotics are unlikely to be toxic to eukaryotic cells, cell permeability is increased by cationic lipid reagents, thus allowing delivery of antibiotics into the cells. This may decrease cell health and result in reduced transfection efficiency. Plasmid DNA quality

was checked against the conditions suggested by each protocol. Plasmid preparations with a concentration ≥ 1000 ng/µl and an OD260/OD280 ratio greater than 1.5 were used in transfection; see section 2.2.2.3. In addition, the concentration of plasmid DNA and the amount of cationic lipid reagent were altered to encompass a range of plasmid DNA:cationic lipid reagent ratios, ranging from 1 in 1 to 1 in 20, to determine the ratio at which efficient transfection is achieved and to determine the correct concentration of plasmid DNA to be added. For successful transfection, a slightly net positive charge of DNA/lipid complex is required to facilitate association of the complex with the negatively charged surface of the cell. The pGL-3-Control vector (Promega Co. Madison, WI, USA), was used in the optimization procedure, thus providing strong constitutive reporter gene expression (de Wet et al., 1987). Reporter gene activity was measured using the *luciferase* assay; see section 2.4.5.

2.4.3.2 Transfection of EA.hy 926 cells

EA.hy 926 is an endothelial cell line, generated by fusing HUVEC with the lung cancer A549 cell line (Edgell et al., 1983). Successful transfection of EA.hy 926 cells, using cationic lipid reagents, has previously been reporter (Le Flem et al., 1999) (Gu et al., 2000a). EA.hy 926 cells were chosen for these experiments because they have been previously used in experiments carried out to determine the transcriptional regulation of the Tm gene and are know to express Tm (Yu et al., 1992). In addition, transformed cell lines are easy to grow and maintain in culture and tend to be more amenable to transfection than primary cells.

The Lipofectamine™ 2000 method (Gibco-BRL Ltd. Paisley, UK) was used to introduce constructs into EA.hy 926 cells. The same optimization procedure, as the one employed to transfect HUVECs primary endothelial cells (see section 2.4.3.1), was used to optimize transfection efficiency of EA.hy 926 cells with the Lipofecatamine™ 2000 reagent (Gibco-BRL Ltd. Paisley, UK). As was the case for HUVECs, the pGL-3-Control vector (Promega Co. Madison, WI, USA) was used in the optimization procedure (de Wet et al., 1987) and reporter gene activity measured using the *luciferase* assay; see section 2.4.5. Efficient and successful transfection of EA.hy 926 cells was achieved when a 1:2.5 ratio of DNA (200 ng) to Lipofecatamine™ 2000 reagent (0.5 µl) was used.

Exponentially growing EA.hy 926 cells were seeded, in normal growth medium (EBM supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml BBE, and 500 µl Gentamicin sulphate amphotericin-B), in sterile 96-well culture plate, at a density of 4 x 10^4 cells/well, 24 hours before transfection (to give 90-95% confluency on the day of transfection). In a typical experiment, 0.5 µl Lipofectamine™ 2000 (Gibco-BRL Ltd. Paisley, UK) was diluted in 25 µl Opti-MEM® I (Gibco-BRL Ltd. Paisley, UK) reduced serum medium and incubated for 5 minutes. In the meantime, 200 ng DNA were diluted in 25 µl Opti-MEM containing 2 ng *Renilla* luciferase vector (pRL-TK) (Promega Co. Madison, WI, USA). The two solutions were subsequently combined and incubated for another 15-20 minutes, during which time the cells to be transfected were washed with Opti-MEM® I (150 µl). Finally the combined DNA-Lipofectamine 2000 complex (50 µl) was added directly to the wells containing the cells to be transfected. After 5-6 hour incubation in a 37°C, 5% CO_2 incubator (Wolf Laboratories), the serum free medium was replaced with 100 µl normal growth

medium (EBM supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml BBE, and 500 µl Gentamicin sulphate amphotericin-B).

2.4.4 Biochemical agonists/antagonists for cell culture

Cell cultures were also subjected to biochemical antagonists (TNF-α & IL-1β; R&D Systems Inc. MN, USA.) to assess how variant sites within the Tm gene promoter may affect expression. Exponentially growing EA.hy 926 cells were seeded and transfected as mentioned in section 2.4.3.2. Cells were exposed to the transfection reagents for 5-6 hour (37°C, 5% CO_2), after which point the serum free medium was removed and cells incubated with normal growth medium (EBM supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml BBE, and 500 µl Gentamicin sulphate amphotericin-B) for 24 hours to allow cells to recover before treatment. Transfected cells were subsequently treated by incubating them with normal growth medium supplemented with the following mediators. A dose- and time- response was initially carried out for both TNF-α and IL-1β, using a range of TNF-α (0 to 2 ng/ml) and IL-1β (0 to 1 ng/ml) concentrations and time points (0, 3, 6, 15, 24 hours), to determine the optimum experimental conditions. Subsequent experiments used 1 ng/ml TNF-α and 0.5 ng/ml IL-1β for 15 hours. *In vivo*, this would relate to the plasma concentration found in a poorly controlled subject with diabetes. Each construct was assessed in quadruplicate and the experiment repeated five times.

2.4.5 Measurement of *luciferase* expression

The expression of the *luciferase* gene in EA.hy 926 cells was assessed 36-48 hours after transfection using a dual *luciferase* assay. Both firefly and *Renilla* luciferase activities were measured sequentially from a single sample by means of a chemiluminescent assay (Luciferase Assay System; Promega Co. Madison, WI, USA). Cells were washed with 1x PBS (100 µl) and lysed in passive lysis buffer (20 µl; diluted 1:5) containing Triton-X. The lysed samples (10 µl) were used to measure *luciferase* activity in a 96-well plate by direct addition of the substrate. The firefly *luciferase* reporter was measured first by adding Luciferase Assay Reagent II (LAR II) to generate a "glow-type" luminescent signal. Following quantification of the firefly luminescence, this reaction was quenched and the concomitant activation of the *Renilla* luciferase initiated by adding Stop & Glo™ reagent to the sample tube. The Stop & Glo™ produces a "glow-type" signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. Light emission was measured on a CytoFluor Series 4000 fluorescence multi-well plate reader (Perspective Biosystems) using a single-injection TD-20/20 Luminometer in the "DLR" mode. TD 20/20 luminometer software permitted direct capture of the data stream over the 10 second integration of each *luciferase* reaction. This system allowed the quantification of both firefly and *Renilla* luciferase activities within 30 seconds. Promoter activity was taken as the ratio of firefly luminescence to *Renilla* luminescence, thus adjusting for transfection efficiency. This normalised ratio was then averaged to give a mean ratio for each transfected construct. The mean of each construct was then normalised against the pGL-3-Basic reading and expressed relative to the values obtained for the wild-type construct.

2.4.6 RNA extraction

Total RNA was extracted from mammalian cells (HUVECs & EA.hy 926) grown in twelve-well plates using TRIzol reagent (Gibco-BRL Ltd. Paisley, UK), following the manufacturer's protocol. TRIzol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. The addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase, with RNA remaining in the aqueous phase. RNA is subsequently recovered by precipitation with isopropyl alcohol. Once the aqueous phase has been removed, DNA and protein can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, whereas additional precipitation with isopropyl alcohol yields protein from the organic phase.

Briefly, 500 µl of TRIzol reagent was added directly to each well of a twelve-well plate, incubated for 5 minutes at room temperature and transferred to a 1.5 ml eppendorf tube. Chloroform (100 µl) was added, the sample mixed by shaking vigorously for 15 seconds and centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh clean eppendorf making sure not to transfer any of the interphase. RNA was precipitated from the aqueous phase by adding isopropanol (250 µl) and mixing by inverting. The sample was incubated for 10 minutes at room temperature and centrifuged at 12000g for 10 minutes at 4°C. The supernatant was removed, the RNA pellet washed with 70% ethanol (500 µl, made up in DEPC-treated water) and centrifuged at 7500g for 5 minutes at 4°C. The ethanol was removed and the pellet left to air-dry. The RNA pellet was resuspended in 40 µl RNase-free water and stored at -70°C.

2.4.7 DNase digestion of purified RNA

Following extraction of RNA from mammalian endothelial cells (HUVECs & EA.hy 926) with TRIzol reagent (Gibco-BRL Ltd. Paisley, UK); see section 2.4.6, DNase digest of RNA was carried out using the RNeasy kit (Qiagen Ltd. Crawley, West Sussex, UK.) mini protocol for RNA cleanup, according to the manufacturers' instructions.

Initially, the RNA sample, prepared as described in section 2.4.6, was adjusted to a volume of 100 µl with RNase-free water. 350 µl of lysis buffer (RLT buffer), together with β-mercaptoethanol (β-ME) was added to the sample followed by the addition of 250 µl 70% ethanol. The sample was then added to a spin column and centrifuged at 8000g for 15 seconds. The flow-through was discarded, 350 µl of buffer RW1 added to the column and the sample centrifuged at 8000g for 15 seconds. 80 µl DNase I incubation mix (10 µl DNase stock solution + 70 µl RDD buffer) was added directly to the silica gel membrane of the spin column for 15 minutes, followed by a second RW1 wash. The sample was then washed twice with 500 µl of buffer RPE. Finally, the RNA was eluted in 30 µl of RNase-free water added directly to the silica membrane with subsequent centrifugation for one minute at 8000g into a fresh eppendorf tube. The RNA was stored at -80°C.

2.4.8 Reverse transcriptase PCR (RT-PCR)

Reverse transcription was carried out on total RNA extracted from mammalian endothelial cells (HUVECs & EA.hy 926), using SUPERSCRIPT II reverse transcriptase and random hexamer primers (Gibco-BRL Ltd. Paisley, UK), according to the manufacturers' instructions. In brief, 10 µl RNA (1-5 µg), 1 µl random hexamer primers (50-250 ng) and 1 µl dNTP mix (10 mM each) were mixed together in a nuclease-free eppendorf tube. The reaction was incubated at 65°C for 5 minutes and chilled quickly on ice. 4 µl first-strand buffer (5x) and 2 µl DTT (0.1 M) were then added, incubated at 42°C for 2 minutes and 1 µl Superscript II reverse transcriptase (200 units) added. The reaction was subsequently incubated at 42°C for 1 hour and stopped by incubation at 70°C for 15 minutes. To completely remove RNA from the newly synthesised cDNA, 1 µl *E.coli* RNase H was added to the reaction and incubated at 37°C for 20 minutes. The cDNA was subsequently used as a template for amplification in PCR; see section 2.2.3.1.

2.4.8.1 Tm mRNA production by mammalian cells (HUVECs & EA.hy 926)

The RT-PCR method was used to investigate Tm mRNA production by the mammalian endothelial cells (HUVECs & EA.hy 926) used in the *in vitro* reporter gene analysis. For this purpose RT-PCR of total RNA extracted from the mammalian cells (see section 2.4.6) was followed by amplification of Tm and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA. GAPDH, one of the most common 'housekeeping' genes, was used as an internal control, as has been previously reported (Franscini et al., 2004) (Barber et al., 2005). A genomic DNA sample was also used as a positive control. To ensure no contaminating genomic DNA was present, RNA samples were treated

with RNase free DNase; see section 2.4.7. Furthermore, intron flanking primers were used to investigate GAPDH mRNA production by HUVECs and EA.hy 926 cells. For intron flanking primers the forward primer must hybridize to one exon and the reverse primer to another exon. PCR products from cDNA, which contains no introns, will be smaller than PCR products from genomic DNA, which contain introns. The forward and reverse GAPDH primers were 5'-AAGGTCGGACTCAACG-3' and 5'-CAAGCTTCCCGTTCTCA-3', respectively. cDNA amplification (which contains no introns) gave a 281 bp product, whereas genomic DNA amplification (which contains introns) gave a 543 bp product. 100-150 ng cDNA was amplified in a final volume of 20 µl, using polymerase buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 0.01% gelatine, 2 mM dATP, 2 mM dTTP, 2mM dGTP, 2 mM dCTP], 8 pmol of each primer, 0.2 units *Taq* polymerase (Bioline Ltd. London, UK) in a PTC-225 Peltier Thermocycler (MJ Research, UK). Cycling conditions consisted of an initial denaturation step, 95°C for 5 min; followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min; and a final extension of 5 min at 72°C.

As mentioned in section 1.4.4, Tm is an intronless gene (Jackman et al., 1987). As such, intron flanking primers could not be designed to investigate Tm mRNA production by HUVECs and EA.hy 926 cells. In the case of intronless genes, contamination with genomic DNA can only be avoided by DNase treatment of the RNA (Vandesompele et al., 2002). The primers used to study the A455V Tm variant were used to amplify cDNA and investigate Tm mRNA production by HUVECs and EA.hy 926 cells; see Table 2.1. As an added control step, the primers used to study the -1166G>A Tm variant (see Table 2.1), which are complementary to DNA sequences within the promoter region of the Tm gene, were incorporated in the same PCR mix to identify amplification of any contaminating genomic DNA. PCR conditions used for the

amplification of Tm cDNA are shown in Table 2.2. PCR products were run on an agarose gel, visualised under UV light and photographed using a Syngene Trans-illuminator and the Gene-Snap Syngene software (Syngene, Cambridge, UK); see section 2.2.4.1.

CHAPTER THREE

CONTRIBUTION TO RISK OF MI BY TM VARIANTS

3. Contribution to risk of MI by Tm variants

3.1 Gene variants and their role in investigating the pathogenesis of CHD

3.1.1 Methods employed to study complex disease

The genetics of multifactorial disorders, such as CHD and diabetes mellitus, is complex involving many genes, most of which will have small effects (Lander and Schork, 1994) (Cordell and Clayton, 2005) (Farrall and Morris, 2005) (Suh and Vijg, 2005). Susceptibility to such complex disorders does not follow classic Mendelian recessive or dominant inheritance attributed to a single gene locus (Lander and Schork, 1994). Three approaches have been proposed to study complex traits: association studies, linkage analysis and allele-sharing studies.

i) Association studies

Association studies, performed on samples of the population, are designed to investigate whether genetic variation, within or close to a gene of interest, is associated with inter-individual differences in the intermediate phenotype (biochemical or clinical) or with increased risk of disease (Lander and Schork, 1994) (Cordell and Clayton, 2005) (Farrall and Morris, 2005) (Suh and Vijg, 2005). Associations between a genetic polymorphism and a trait might exist in a given population either because the genetic variation is causal, termed direct association, or because the polymorphism is in LD with the actual causal variant, termed indirect association (Cordell and Clayton, 2005) (Suh and Vijg, 2005). Two different experimental designs are typically used in association studies. Prospective (longitudinal) studies are preferred for measuring risk with a specific gene variant, as they measure risk over time in a population. These

studies are also useful in studying gene-environment interactions because of their longitudinal design measuring exposure before disease onset. The population is often carefully selected at baseline to have similar characteristics regarding non modifiable traits such as age and ethnic origin and does not present individuals with disease (Farrall and Morris, 2005). Case-control (reciprocal) studies, however, predominate because they are easier to implement and do not require long-term follow-up, making results available quickly (Farrall and Morris, 2005). In case-control studies, samples of unrelated affected (case) and unaffected (control) individuals are ascertained from the study population (Lander and Schork, 1994) (Farrall and Morris, 2005). A specific gene variant is said to be associated with a particular trait if it occurs at a significantly higher frequency among affected compared to control individuals (Lander and Schork, 1994). Case-control studies allow a more precise characterisation of outcome, as well as greater statistical power to detect association than would be feasible with a prospective design, by ensuring all cases are scanned properly for a particular phenotype (Lander and Schork, 1994). As such, case-control studies are useful to study the association between gene variants and various intermediate phenotypic measures. The main disadvantages of such studies is that errors in the measurements of exposures (e.g. age, ethnic origin) can differ systemically between cases and controls, giving misleading results (Farrall and Morris, 2005). Knowledge of prior events is often limited in cross sectional studies since they are carried out at a single time point.

Association studies (prospective or case-control) are able to detect a small effect of variation in genes and are thus considered to be effective tools for studying complex multifactorial traits (Suh and Vijg, 2005). However, difficulties still exist with these types of studies, namely a lack of reproducibility, which questions the reliability of their findings (Colhoun et al., 2003) (Farrall and Morris, 2005). Typically associations are

detected in a number of modestly sized cohorts, with inadequate numbers of cases to be sufficiently powered to examine disease genetics. As such relative risk arising from genetic variation may be small, which may lead to an over-estimation of the size of genetic effects. When associations are assessed in large samples drawn from the population as a whole or a meta-analysis conducted, the genetic effect is greatly reduced (Farrall and Morris, 2005). One possible explanation for inconsistent results with replication is the phenomenon of publication bias, because only positive associations are published (Colhoun et al., 2003). Alternatively, inconsistent results with replication may be due to the fact that studies vary in terms of phenotype and samples of subjects (e.g. diabetes/non diabetes, male/female) (Farrall and Morris, 2005).

Another important consideration is that variation may exist in the underlying association between genotype and the outcome in different populations studied. This could arise by different disease-causing alleles predominating in different populations or by variation in LD existing between marker and disease (Colhoun et al., 2003). LD depends on population history and on the genetic makeup of the founders of a given population. As such, population admixture raises the possibility of generating false findings (false positive) or obstructing true association (false negative) (Lander and Schork, 1994) (Cordell and Clayton, 2005) (Farrall and Morris, 2005). This difficulty can be addressed by matching samples by geographic region and by any other markers of ethnic origin (Lander and Schork, 1994) (Farrall and Morris, 2005). Another reason for the discrepancies found in some studies is that gene-gene and gene-environment interactions may differ between populations. Variants may only show their effects in populations with a particular genetic or environmental background, indicating that replication studies should take these impacts into account (Talmud and Humphries, 2002). Furthermore, many studies do not stratify by risk factors or look for evidence of

a gene-environment interaction (e.g. smoking), thereby weakening the ability to detect significant associations.

Many of the aforementioned problems can be addressed by using accurately phenotyped, homogeneous large association studies that are powered to detect the small to moderate effects of common functional variants. Reproduction of statistically significant associations in several independent samples is advisable. Meta-analysis of all published data may provide a quantitative estimate of the genetic risk factor and disease (Humphries and Donati, 2002).

ii) Linkage analysis

With linkage analysis the whole genome is scanned in a number of families with a history of disease, to identify chromosomal regions linked to disease and related factors (Lander and Schork, 1994) (Farrall and Morris, 2005). A disease model is constructed, to explain the inheritance pattern of the disease, by comparing the observed segregation of gene markers with trait in affected pedigrees. Linkage analysis is the method of choice for studying simple Mendelian traits in monogenic disorders because the allowable models are few and easily tested (Lander and Schork, 1994). However, the success rate of linkage analysis with complex multifactorial disorders has been considerably lower as the genetic analysis may be confounded by incomplete penetrance, phenocopy, genetic heterogeneity and polygenic inheritance (Lander and Schork, 1994) (Suh and Vijg, 2005). Although linkage studies across the genome may find the broad location of a gene, they do not provide any information about the gene itself (e.g. what it does). Linkage analysis is well powered to detect major gene effects but not modest gene effects.

iii) Allele-sharing studies

The allele sharing approach has also been used for the study of complex diseases. This involves studying affected relatives (or siblings) in a pedigree to see how often they inherit identical copies of a chromosomal region from a common ancestor within the pedigree (Kurtz and Spence, 1993). If affected relatives inherit identical copies of a chromosomal region more often than would be expected by chance, the inheritance pattern of the region is not consistent with random Mendelian segregation. This method applies to a single generation of disease sufferers. It is independent of the pattern of disease inheritance and is therefore frequently used in the analysis of complex disorder with a late onset, such as risk of CHD (Lander and Schork, 1994).

3.1.2 Common gene variants and association analysis

In the past, linkage disequilibrium analysis was used to define regions of the genome where sequence markers (SNPs) were correlated to chronic diseases with a genetic component, limiting the search for causative variants to the region associated with disease (Kruglyak, 1999). In the past this approach could only be carried out for a limited number of sequence variants of one or few genes. The International Haplotype Map (Hapmap) project, a comprehensive view of the structure of LD throughout the genome in multiple populations, will however provide coverage of the human genome with non-redundant tagging SNPs (tag-SNPs) (Thorisson et al., 2005). The use of such tag-SNPs may help capture most of the common genetic variants contributing to complex human disease. This approach will allow a comprehensive genome-wide survey, but is limited by a lack of knowledge concerning LD heterogeneity across different populations (population stratification) and the requirement of large group sizes to account for multiple testing errors (Suh and Vijg, 2005). These problems can be

further exacerbated if the disease gene variant occurs at a low frequency. An alternative approach is that of indirect/direct candidate gene pathway, which is based on knowledge of the disease phenotype, genetic studies in model organisms or location (as defined by linkage analysis) (Tabor et al., 2002). An indirect candidate SNP approach utilizes tagging SNPs across a gene or region of interest to investigate the aetiology of complex diseases such as CHD, whereas a direct candidate SNP approach tests the association between putative functional variants and disease risk (Suh and Vijg, 2005).

SNPs that occur within coding regions and alter the amino acid sequence, or within the promoter region that alter gene expression, or alter the RNA splice sites causing mis-splicing in the intron or exon are characterized as functional. SNP functionality cannot be assessed solely on nucleotide sequence, especially when SNPs do not alter an amino acid or disrupt a known motif that effect protein structure or function. Defining the variants that are functionally implicated in the disease is an important challenge for any study, since only a small subset of variants will confer moderate to small effects on disease related phenotypes. Large, sufficiently powered studies are required to detect any potential association, as many of the variants are low in frequency. Making this approach even more difficult is the fact that elevated risk may be due to the combined effect of several susceptibility alleles (Pritchard and Cox, 2002). As such determining the functional SNPs as precisely as possible is of the utmost importance before proceeding with a direct candidate SNP approach. A final consideration is that gene-gene and gene-environment interactions are likely to affect the statistical significance of any association obtained.

In the current thesis, three studies of differing design were employed to investigate the association of the potentially functional Tm variants with risk for CHD (-1748G>C, -1208/09TT>delTT, -1166G>A, A455V). The HIFMECH case-control study (Hypercoagulability and Impaired Fibrinolytic Functions Mechanisms predisposing to myocardial infarction study, MI), consisting of 598 male MI survivors and 653 controls, was used to assess the association of these polymorphisms with MI risk. Those variants associated with MI risk were subsequently assessed for their contribution to CHD risk in the second Northwick Park Heart study (NPHSII), a large prospective study encompassing over 3000 individuals, to verify and extend the results obtained in the case-control study. The EDSC cross sectional study of type 2 diabetes (Ealing Diabetes Study of Coagulation; n=927) was used subsequently to investigate Tm in individuals within the same pathological environment and to characterise the relationship of Tm genotype with sTm levels. It was hoped that the use of such a range of studies would increase the understanding of the Tm gene and protein in atherosclerosis and MI.

3.2 Contribution to risk of MI by Tm variants

As mentioned in section 1.4.7, numerous variants have been identified in the Tm gene to date, some of which have been associated with CHD risk. Whereas the proximal promoter region (nucleotides -1 to -299, (Le Flem et al., 2001)) of the Tm gene has been extensively studied, and numerous variant sites identified and assessed with regards to CHD (see section 1.4.7), the distal promoter region (nucleotides -300 to -2052,. (Le Flem et al., 2001)), known to contain several important consensus sequences, has received relatively little attention. 47 variants (26 validated) are listed for the Tm distal promoter region on the CHIP Bioinformatics database (http://snpper.chip.org). At the start of this work, eight sequence variants in the Tm distal promoter region had been

studied in a large French case-control study of venous thrombosis (Le Flem et al., 2001). Among them, –1166G>A, -1208/09TT>delTT and –1748G>C appeared to have functional consequences; see section 1.4.7.2. The association of these Tm distal promoter variants with risk of CHD had not been addressed previously. The functional consequences of the coding region variant, A455V were unknown. Data have suggested that A455V is neutral with regards to venous thrombosis (van der Velden et al., 1991) (Aleksic et al., 2002), but assessment of CHD risk has found the rare allele to be associated with both risk and protection of heart disease (Norlund et al., 1997a) (Wu et al., 2001); see section 1.4.7.2.

3.3 Aim

The purpose of the work described in this section was to assess the contribution to risk of MI by four potentially functional variants in the Tm gene (-1748G>C, -1208/09TT>delTT, -1166G>A and A455V), in the North and South of Europe. Tm gene variant interaction with other clinical and life style factors was also studied in relation to MI.

3.4 Methods

3.4.1 DNA extraction from blood using the salting out method

Genomic DNA from participants of the HIFMECH study had been isolated previously using the salting out method (Miller *et al.* 1988); see section 2.2.2.1.

3.4.2 Description of study subjects (HIFMECH)

Hypercoagulability and Impaired Fibrinolytic functions MECHanisms predisposing to myocardial infarction (HIFMECH) is a Pan-European study of male Caucasian patients who survived a first MI event below 60 years of age and population-based individuals of the same age. The study was designed to identify differences in MI risk factors between individuals living in the North or South of Europe. Subjects were recruited from four European centres: Stockholm-Sweden; London-England (Northern Europe), and Marseille-France; San Giovanni Rotondo-Italy (Southern Europe). Patients with familial hypercholesterolemia and insulin-dependent diabetes mellitus were excluded from the study. Consecutive patients along with randomly selected healthy individuals from the same area were invited to participate. A total of 598 MI survivors and 653 healthy control subjects were included in the initial study (Juhan-Vague et al., 2002). In the current study, only those with partial or complete environmental data were considered. Matching of controls to cases was done on the basis of centre and age. While the study design set out to recruit one to one matching, a few more controls to patients were recruited in some centres. Survivors were examined 3-6 months after MI. Examination of both patient and control subjects, was performed in the early morning following an overnight fast. A structured interview concerning socio-economic and lifestyle information was carried out together with a clinical examination involving weight, height, waist and hip circumference measurements, SBP and DBP measurements. Blood samples were taken after 20 minutes rest in the recumbent position, for plasma and DNA analysis. All subjects gave informed consent to their participation, and the local Ethics committees of the four centres approved the study.

3.4.3 Genotyping for polymorphisms in the Tm gene

Tm genotyping for the variant sites under investigation (-1748G>C, -1208/09TT>delTT, -1166G>A and A455V) was performed as described in Chapter 2. PCR conditions and restriction enzyme digestion conditions are summarised in Tables 2.1-2.3. Band patterns for these two Tm polymorphisms studied are shown in Figures 2.4-2.7.

3.4.4 Statistical analysis

Data were entered into an EXCEL spreadsheet (Microsoft). Deviations from Hardy-Weinberg equilibrium were considered via a chi-squared test of observed and expected frequencies. Hardy-Weinberg equilibrium gives the expected genotype distribution based on the observed frequency of the rare allele (q) and common allele (p) as $p^2+2pq+q^2$, where p^2 is the predicted frequency for homozygosity of the common allele, q^2 is the predicted frequency for homozygosity of the rare allele and $2pq$ the heterozygotes. These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection. Deviations from the expected frequencies may suggest selection bias or technical problems with the method of genotyping. Differences in genotype distributions and allele frequencies between groups were analysed using chi-squared tests. LD was considered using the statistic delta (Δ), as described previously (Chakravarti et al., 1984). Δ is essentially a correlation coefficient between different alleles (equivalent to Pearson's correlation coefficient for continuous variables).

In HIFMECH, analysis was carried out with help from Emma Hawe (statistician within CVG). Statistical analysis was conducted using 'Intercooled STATA' version 7.0 (College Station, Texas) and SPSS version 12.0.1. (SPSS Inc., Chicago, USA). HIFMECH was designed to determine differences in CHD risk in the North compared to the South of Europe. The a priori hypothesis was therefore followed and analysis was carried out in the North and South, separately. Analysis was only conducted in individuals with environmental data. Analysis of variance (ANOVA) was performed to compare differences in continuous variables by genotype. Transformed response variables were used as appropriate. Odds ratios were calculated as a measure of relative risk. Differences between cases and controls were analysed by conditional logistic regression. Differences in the risk posed by genotypes was considered either via conditional logistic regression (hence considering the matching of cases and controls by both age and centre) or by logistic regression. The latter was performed when analysis was stratified by a variable on which the cases and controls were not matched. This was done to avoid reducing numbers when stratifying by genotype. Interactions were assessed by standard methods as deviations from multiplicative effects. In addition, logistic models were used, stratified for various lifestyle factors. BMI, the 'metabolic syndrome' factor and the 'inflammation' factor tertiles were set in the controls in the whole of Europe. Haplotype analysis was conducted using the expectation maximisation algorithm, EH. P-values<0.05 were considered as significant.

3.5 Results

3.5.1 HIFMECH risk factor characteristics

Differences in risk factors between cases and controls were examined according to region; see Table 3.1. In the North of Europe, BMI was significantly higher in cases compared to controls (27.2 Kg/m^2 vs 25.8 Kg/m^2, $p<0.0005$), but this was not seen in the South (cases: 26.9 Kg/m^2, control: 26.4 Kg/m^2; $p=0.13$). No case-control difference for SBP was observed either in the North or the South of Europe, but DBP was significantly higher in controls compared to cases in both Northern ($p=0.03$) and Southern ($p<0.0005$) Europe. A significant difference in smoking status existed between cases and controls in both the North and South of Europe, with cases having a higher percentage of current+ex-smokers compared to healthy controls (North: $p=0.0001$; South: $p<0.0005$). Cholesterol levels were lower in cases compared to control subjects in Southern Europe ($p=0.02$). MI survivors had significantly higher levels of plasma triglycerides than the healthy controls in both the North and South of Europe (North: $p<0.0005$; South: $p<0.0005$). The lower cholesterol levels and DBP measurements in cases compared to controls are likely to be caused by the increased use of blood pressure and lipid lowering drugs in cases.

	North			South		
	Controls	**Cases**	**Case-Control difference**	**Controls**	**Cases**	**Case-Control difference**
Age (yrs)	52.7 ± 5.0 n=253	53.51 ± 5.1 n=233	ns	50.5 ± 5.6 n=321	51.0 ± 5.6 n=299	ns
BMI (Kg/m^2)	25.8 ± 11.0 n=253	27.2 ± 3.4 n=229	<0.0005	26.4 ± 3.2 n=322	26.9 ± 3.3 n=229	0.13
SBP (mmHg)	130.2 ± 15.8 n=251	129.5 ± 17.7 n=228	ns	126.2 ± 13.1 n=322	126.4 ± 16.2 n=294	ns
DBP (mmHg)	84.0 ± 8.6 n=251	82.2 ± 9.8 n=228	0.03	84.2 ± 8.6 n=322	81.4 ± 10.7 n=293	<0.0005
Smoking (%)						
Current & Ex	n=194 (76.7%)	n=186 (79.8%)	0.0001	n=232 (72.1%)	n=232 (77.3%)	<0.0005
Never	n=59 (23.3%)	n=47 (20.2%)		n=90 (28.0%)	n=68 (22.7%)	
Triglycerides (mmol/l)	1.52 ± 0.60 n=233	1.99 ± 0.83 n=215	<0.0005	1.39 ± 0.61 n=321	1.79 ± 0.72 n=286	<0.0005
Cholesterol (mmol/l)	5.71 ± 0.99 n=233	5.66 ± 1.22 n=215	ns	5.39 ± 0.94 n=321	5.18 ± 1.11 n=286	0.02

Table 3.1 General characteristics of control and case subjects according to geographical region.
Data are shown as mean ± standard deviation (SD); n, number of subjects in groups; ns, non significant.

3.5.2 Allelic association

Allelic association between the -1748G>C, -1208/09TT>delTT, -1166G>A, and A455V polymorphisms in the Tm gene was determined. Allelic association was considered using the statistic delta (Δ), as described previously (Chakravarti et al., 1984). Strong LD was observed between the –1208/09TT>delTT and A455V variants in both Northern and Southern European controls (Δ=0.64 p<0.0005, North; Δ=0.76 p<0.0005, South), as has also been reported in a French case-control study of venous thrombosis (Δ= 0.89 p<0.01) (Le Flem et al., 2001). No significant allelic association was observed between any of the other polymorphisms studied, either in Northern or Southern European controls. Results are given in Table 3.2.

3.5.3 Tm genotype distribution and allele frequencies

Genotype distribution and allele frequencies for the -1748G>C, -1208/09TT>delTT, -1166G>A, and A455V polymorphisms were determined in the North and South of Europe; see Table 3.3. The frequency of the –1748C and –1208/09delTT alleles in the healthy control men did not differ significantly between the North and South of Europe (-1748C: 0.20 vs 0.22, p=0.28; -1208/09delTT: 0.11 vs 0.09, p=0.37). For the –1166G>A variant, the frequency of the A allele was very low in Northern European controls (frequency: 0.002), as opposed to their Southern European counterparts where the frequency of the A allele was 10-fold higher (frequency: 0.022) (p=0.01). The frequency of the 455V allele amongst Northern European controls (frequency: 0.19) was significantly higher than the South of Europe (frequency: 0.12) (p=0.01).

Polymorphism	-1748G>C	-1208/09TT>delTT	-1166G>A	A455V	
-1748G>C		-0.14 (p=0.14)	0.07 (p=0.53)	-0.13 (p=0.17)	**S**
-1208/09TT>delTT	-0.11 (p=0.54)		0.05 (p=0.96)	**0.76 (p<0.0005)**	**O**
-1166G>A	0.09 (p=0.71)	-0.02 (p=1.00)		-0.05 (p=0.91)	**U** **T**
A455V	-0.14 (p=0.33)	**0.64 (p<0.0005)**	-0.02 (p=1.00)		**H**
		N O R T H			

Table 3.2 Allelic association between polymorphisms studied in the HIFMECH study.
Allelic association determined in Northern (Below the line) and Southern (above the line) European controls. Results shown as Δ. P-value in bracket.

Polymorphism		North		South		P-value for N-S control allele frequency differences
		Controls	Cases	Controls	Cases	
-1748G>C	GG/GC/CC Rare allele freq. (95% CI)	160/83/6 0.20 (0.16-0.23)	157/78/6 0.187 (0.15-0.22)	214/118/22 0.229 (0.20-0.26)	186/114/17 0.233 (0.20-0.27)	ns
-1166G>A	GG/GA/AA Rare allele freq. (95% CI)	247/1/0 0.002 -	241/2/0 0.004 -	341/16/0 0.022 (0.01-0.03)	312/8/0 0.0125 (0.00-0.02)	0.01
-1208/09TT>delTT	Rare allele freq. (95% CI)	197/47/5 0.114 (0.10-0.13)	174/68/1 0.144 (0.12-0.17)	293/62/1 0.09 (0.07-0.11)	277/38/1 0.063 (0.05-0.08)	ns
A455V	AA/AV/VV Rare allele freq. (95% CI)	160/74/8 0.186 (0.16-0.21)	129/100/12 0.257 (0.21-0.29)	275/73/7 0.123 (0.10-0.15)	244/62/3 0.110 (0.09-0.13)	0.01

Table 3.3 Genotype distribution and allele frequency in control and case subjects according to geographical region.
The variable number of samples genotyped for the different polymorphisms was due to repeated failure to amply the target sequence during the PCR reactions. This is a random event and could not be responsible for any association seen. The genotype distribution of all variants studies was consistent with Hardy-Weinberg equilibrium.

3.5.4 Tm genotype and risk of MI

Due to the low number of rare homozygotes identified for each of the four Tm variants studied (see Table 3.3), subjects with one and two rare alleles were combined and compared to those with two common alleles at each variant site.

No analysis was carried out for the –1166G>A variant in relation to risk of MI due to the low frequency; see Table 3.3. It was estimated that a cohort of 6000 individuals would be required to detect a statistically significant effect on risk by the -1166A allele (power 80%, p=0.05, allele frequency 0.0125 in cases and 0.022 in controls as noted for Southern Europeans; see Table 3.3).

There was no evidence to suggest an association with risk for the –1748C allele either in the North or the South of Europe (North OR 0.96 CI 0.66-1.39 p=0.84; South OR 1.08 CI 0.79-1.47 p=0.64); see Table 3.4.

When risk was assessed for the -1208/09TT>delTT variant, carriers of the rare -1208/09delTT allele had an odds ratio (OR) of 1.48 (CI 0.98-2.24; p=0.06) in the North of Europe and 0.65 (CI 0.43-1.01; p=0.06) in the South of Europe; see Table 3.4. Similarly, when the contribution to risk of MI by the A455V variant was assessed, carriers of the rare 455V allele had an OR of 1.40 (CI 0.96-2.05; p=0.06) in the North of Europe and 0.92 (CI 0.63-1.33; p=0.22) in the South of Europe; see Table 3.4. However, this analysis has limited value for understanding the contribution to risk of MI by each variant site, because of the strong LD between the two variant sites; see section 3.5.2.

		North		South	
Variant	**Genotype**	**Odds ratio (95% CI)**	**P-value**	**Odds Ratio (95% CI)**	**P-value**
-1748G>C	**GG**	1.00	p=0.84	1.00	p=0.64
	GC+CC	0.96 (0.66-1.39)		1.08 (0.79-1.47)	
-1208/09TT>delTT	**TT/TT**	1.00	p=0.06	1.00	p=0.06
	TT/delTT+ delTT/delTT	1.48 (0.98-2.24)		0.65 (0.43-1.01)	
-1166G>A	**GG**	NA		NA	
	GA+AA				
A455V	**AA**	1.00	p=0.06	1.00	p=0.22
	AV+VV	1.40 (0.96-2.02)		0.92 (0.63-1.33)	

Table 3.4 Odds ratio to estimate the contribution to risk of MI the contribution to risk of MI of the four Tm variants studied.
Results are adjusted for age and centre. See Table 3.3 for numbers used to calculate OR.

To try to understand contribution to risk by the -1208/09TT>delTT and A455V variant sites, risk was analysed for those heterozygous and homozygous for 455V alone (n=42 cases, n=37 controls in the North; n=27 cases, n=20 controls in the South), compared to those homozygous for the common alleles at both sites (n=126 cases, n=151 controls in the North; n=225 cases, n=240 controls in the South; see Table 3.5). OR for 455V heterozygotes and homozygotes combined were 1.36 (CI 0.82-2.25 p=0.22) in the North and 1.44 (CI 0.79-2.64 p=0.23) in the South. Risk associated with –1208/09TT>delTT alone could not be assessed, because the –1208/09TT>delTT variant occurred on the 455V allele in most individuals. Only 9 individuals were heterozygous for -1208/09delTT but homozygous for A455; see Table 3.5.

Genotype		North		South	
A455V	-1208/09 TT>delTT	Controls (n)	Cases (n)	Controls (n)	Cases (n)
AA	TT/TT	151	126	240	225
AA	TT/delTT	3	1	5	0
AV	TT/TT	35	37	18	27
AA	delTT/delTT	0	0	0	0
VV	TT/TT	2	5	2	0
AV	TT/delTT	36	53	49	30
AV	delTT/delTT	1	0	0	0
VV	TT/delTT	3	6	3	2
VV	delTT/delTT	3	1	1	1

Table 3.5 Number of individuals with rare alleles for A455V and -1208/1209TT>delTT combined in cases and controls in the North and South of Europe.

Risk was also assessed in those individuals heterozygous and homozygous for both 455V and -1208/09delTT (V/delTT) (n=60 cases, n=43 controls in the North; n=33 cases, n=53 controls in the South), compared to those homozygous for the common alleles at both sites (AA/TT) (numbers as above; see Table 3.5). A 1.6-fold elevation in risk was determined for those in the North (OR 1.67 CI 1.06-2.64 p=0.03), but there was no increased risk in the South; see Figure 3.1. In fact, there appeared to be reduced risk in the South, though this failed to reach statistical significance (OR 0.66 CI 0.41-1.06 p=0.09).

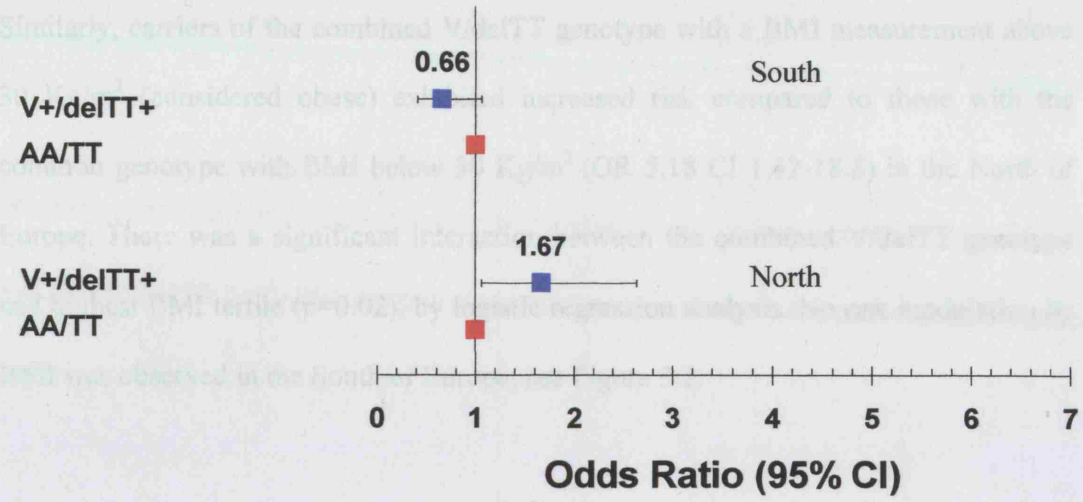


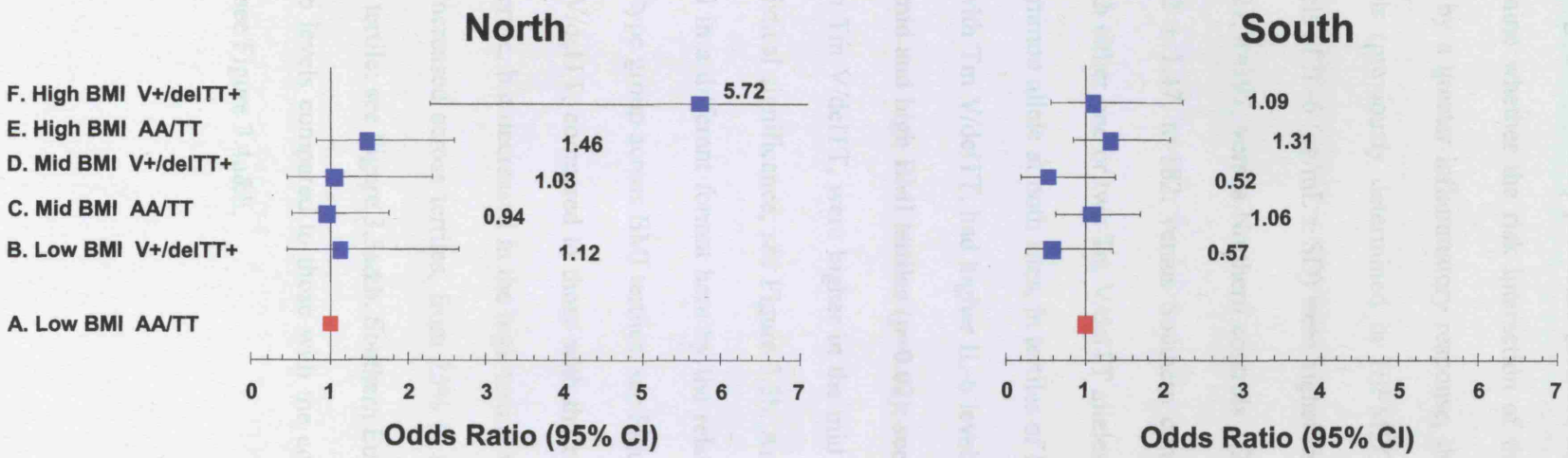
Figure 3.1 Risk for MI in carriers of the 455V/-1208/09delTT genotype combination in the North and South of Europe.
AA/TT = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

3.5.5 Combined Tm 455V/-1208/09delTT genotype (V/delTT) effect in association with other clinical characteristics

The V/delTT genotype effect was analysed together with clinical and life style factors (see Table 3.1) to determine whether there were any interactions with these factors. "Genotype-phenotype interaction" implies that in combination, the risk associated with a particular gene variant in a particular environment is more than the effects of each independently and may help elucidate pathological mechanisms.

No interaction between smoking and V/delTT was identified either in the North or South of Europe, with risk remaining the same in smokers and non-smokers. However, when risk was assessed for the highest BMI tertile in the North of Europe, carriers of V/delTT were associated with a 5.7-fold increased risk of MI (OR 5.72 CI 2.24-14.6

$p<0.0005$), compared to those with the common genotype in the lowest tertile and was increased also above the risk for BMI alone (OR 1.46 CI 0.82-2.60); see Figure 3.2. Similarly, carriers of the combined V/delTT genotype with a BMI measurement above 30 Kg/m^2 (considered obese) exhibited increased risk compared to those with the common genotype with BMI below 30 Kg/m^2 (OR 5.18 CI 1.42-18.8) in the North of Europe. There was a significant interaction between the combined V/delTT genotype and highest BMI tertile ($p=0.02$), by logistic regression analysis. No risk modulation by BMI was observed in the South of Europe; see Figure 3.2.

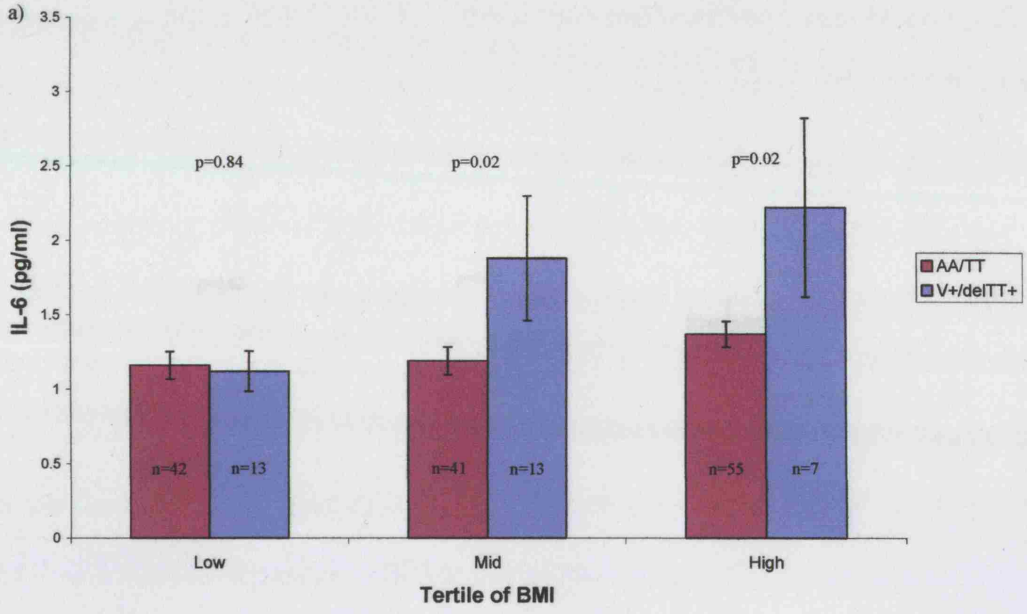


Numbers in each group
A. Controls n=47, cases n=34
B. Controls n=39, cases n=22
C. Controls n=50, cases n=34
D. Controls n=30, cases n=32
E. Controls n=54, cases n=57
F. Controls n=14, cases n=32

Numbers in each group
A. Controls n=69, cases n=57
B. Controls n=26, cases n=15
C. Controls n=83, cases n=73
D. Controls n=24, cases n=13
E. Controls n=88, cases n=95
F. Controls n=28, cases n=31

Figure 3.2 Risk for MI in carriers of the 455V/-1208/09delTT genotype combination for tertiles of BMI.
BMI tertiles: Low → 17.28-24.82 KG/m^2; Middle → 24.85-27.40 kg/m^2; High → 27.41-41.45 kg/m^2. BMI, tertiles were set in the controls in the whole of Europe. **AA/TT** = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

Adipose tissue has been suggested to produce IL-6 (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999) and the PC pathway may participate in regulating inflammatory processes (Esmon, 2001) (Esmon, 2002); see section 1.4.3. To determine whether the risk interaction of the V/delTT allele with BMI could be mediated by a greater inflammatory response, the effect of BMI and Tm genotype on IL-6 levels (previously determined in HIFMECH) was analysed. Overall geometric mean levels of IL-6 (pg/mL ± SD) were higher in cases than in controls: Northern cases 1.77 ± 1.21, n=197, versus Northern controls 1.29 ± 0.74, n=227, $p<0.0001$; Southern cases 2.22 ± 1.47, n=182, versus Southern controls 1.20 ± 0.81, n=202, $p<0.0001$). Those with either one or two Tm V/delTT alleles were compared to those homozygous for the common allele at both sites, in tertiles of BMI. Surprisingly, Northern European controls with Tm V/delTT, had higher IL-6 levels compared to those with the common allele, at mid and high BMI tertiles ($p=0.02$); see Figure 3.3a. Although IL-6 levels, in cases with Tm V/delTT, were higher in the mid BMI tertile in the North, this did not reach statistical significance; see Figure 3.3b. An indication of an effect upon risk can be viewed in a different format here by the relative numbers of cases and controls in each genotype group across BMI tertiles; see Figure 3.3a&b. The proportion of controls with Tm V/delTT, compared to those with the common allele, was similar in the low and mid tertile, but decreased in the high tertile, while the proportion of cases with Tm V/delTT increased across tertiles, from 25% in the low tertile to 50% of cases at the high BMI tertile; see Figure 3.3a&b. Southern European controls with Tm V/delTT had lower IL-6 levels compared to those with the common allele, in the high BMI tertile ($p=0.03$); see Figure 3.4a&b.



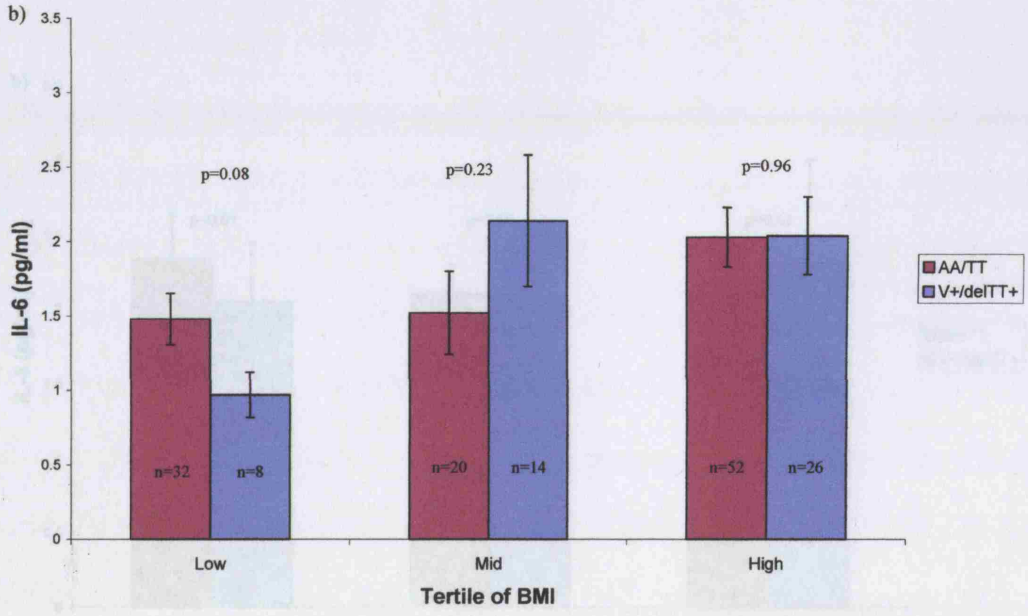


Figure 3.3 Geometric mean levels and approximate standard deviation of IL-6, across BMI tertiles for those with Tm AA/TT (darker shaded bars) compared to those heterozygous or homozygous for Tm V/delTT (lighter shaded bars) in Northern European controls (a) and Northern European cases (b).

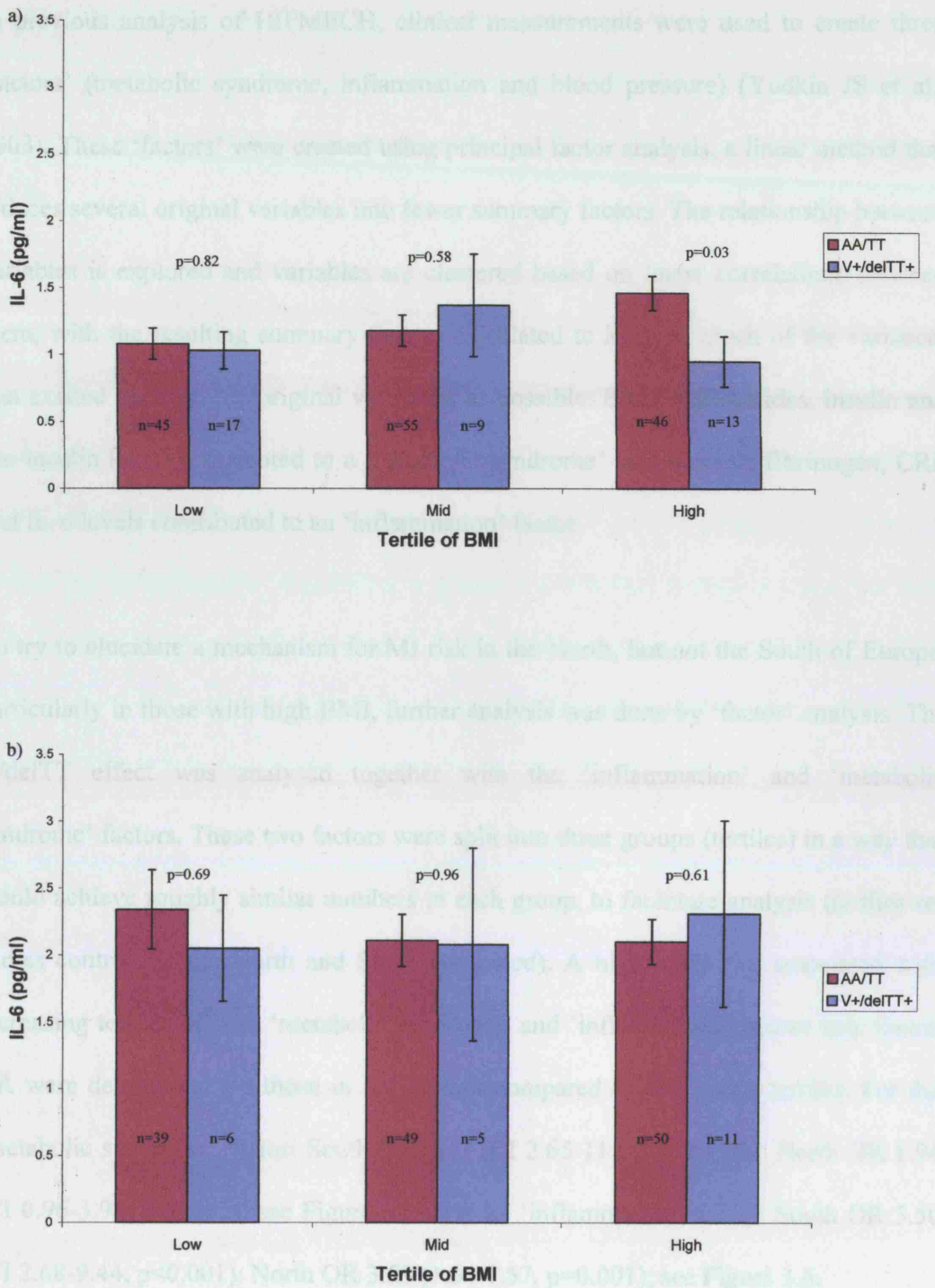


Figure 3.4 Geometric mean levels and approximate standard deviation of IL-6, across BMI tertiles for those with Tm AA/TT (darker shaded bars) compared to those heterozygous or homozygous for Tm V/delTT (lighter shaded bars) in Southern European controls (a) and Southern European cases (b).

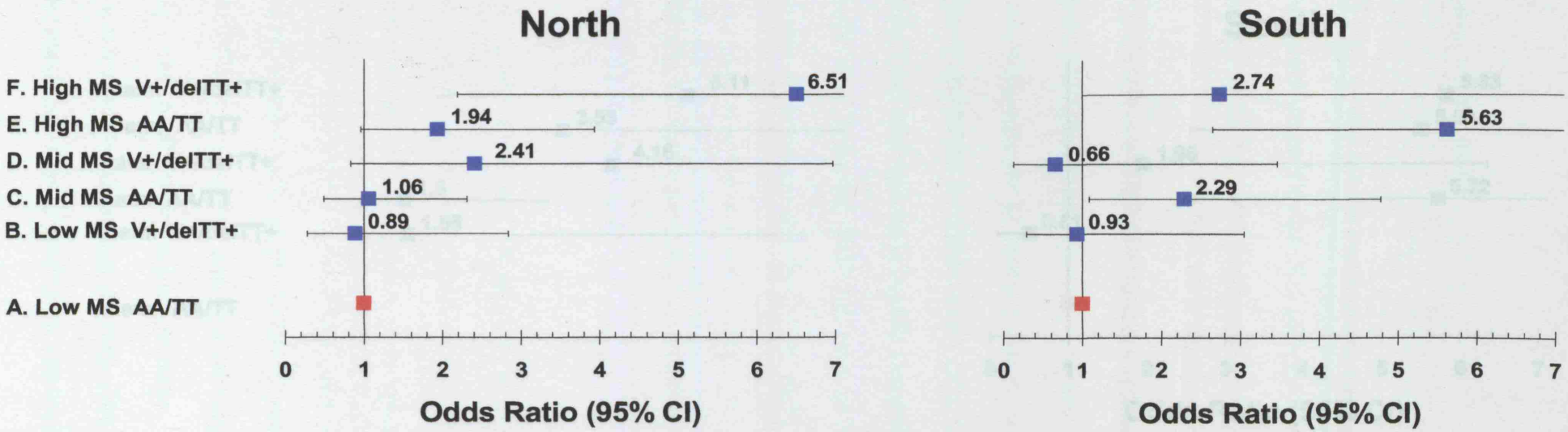
In previous analysis of HIFMECH, clinical measurements were used to create three 'factors' (metabolic syndrome, inflammation and blood pressure) (Yudkin JS et al., 2003). These 'factors' were created using principal factor analysis, a linear method that reduces several original variables into fewer summary factors. The relationship between variables is explored and variables are clustered based on linear correlations between them, with the resulting summary factors calculated to keep as much of the variance, that existed between the original variables, as possible. BMI, triglycerides, insulin and pro-insulin levels contributed to a 'metabolic syndrome' factor, while fibrinogen, CRP and IL-6 levels contributed to an 'inflammation' factor.

To try to elucidate a mechanism for MI risk in the North, but not the South of Europe, particularly in those with high BMI, further analysis was done by 'factor' analysis. The V/delTT effect was analysed together with the 'inflammation' and 'metabolic syndrome' factors. These two factors were split into three groups (tertiles) in a way that would achieve roughly similar numbers in each group, to facilitate analysis (tertiles set across controls in the North and South combined). A higher MI risk associated with increasing tertiles of both 'metabolic syndrome' and 'inflammation' scores was found. OR were determined for those in the highest compared to the lowest tertiles. For the 'metabolic syndrome' factor: South OR 5.63 (CI 2.65-11.97, $p<0.001$); North OR 1.94 (CI 0.96-3.94, $p=0.065$); see Figure 3.5. For the 'inflammation' factor: South OR 5.50 (CI 2.68-9.44, $p<0.001$); North OR 3.53 (1.64-7.57, $p=0.001$); see Figure 3.6.

In the North, the effect of Tm genotype in tertiles of 'metabolic syndrome' and 'inflammation' followed a similar pattern to that shown for BMI. In the highest tertile of 'metabolic syndrome', in the North of Europe, those with the common allele reached an OR 1.94 (CI 0.96-3.94, $p=0.065$), while those with Tm V/delTT reached an OR 6.51 (CI

2.19-19.33, p=0.001); see Figure 3.5. Similarly, in the highest tertile of 'inflammation', in the North, those with the common allele reached an OR 3.53 (1.64-7.57, p=0.001), while those with Tm V/delTT reached an OR 5.11 (CI 1.93-13.5, p=0.001); see Figure 3.6.

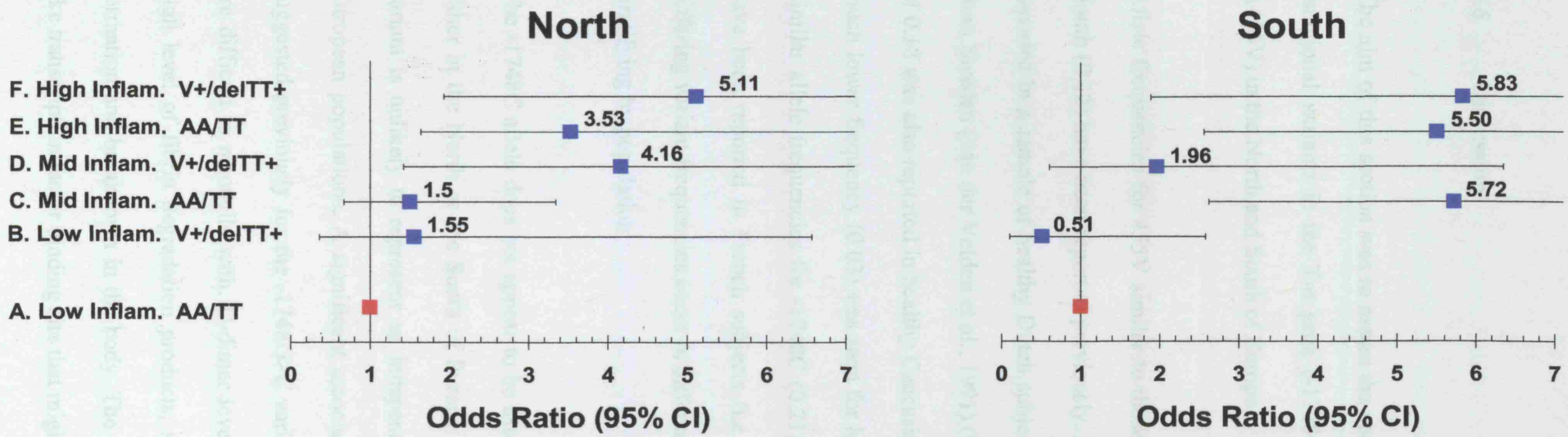
In the mid and high tertiles of 'metabolic syndrome', a higher risk was determined for those with the common Tm allele in the South, compared to the North of Europe (mid: South OR 2.29; CI 1.09-4.78; North OR 1.06; CI 0.49-2.31, high: South OR 5.63; CI 2.65-11.97; North OR 1.94; CI 0.96-3.94); see Figure 3.5. Findings were similar in tertiles of 'inflammation', suggesting a greater contribution generally to MI by both inflammation and metabolic syndrome in the South. An apparent reduction in risk, particularly in the mid and high tertiles of 'metabolic syndrome' was observed in those with Tm V/delTT in the South, compared to those with the common allele in the mid and high tertiles.



Numbers in each group
A. Controls n=32, cases n=20
B. Controls n=12, cases n=6
C. Controls n=34, cases n=23
D. Controls n=8, cases n=12
E. Controls n=43, cases n=52
D. Controls n=6, cases n=23

Numbers in each group
A. Controls n=41, cases n=15
B. Controls n=14, cases n=5
C. Controls n=53, cases n=40
D. Controls n=9, cases n=2
E. Controls n=41, cases n=72
D. Controls n=14, cases n=12

Figure 3.5 Risk for MI in carriers of the 455V/-1208/09delTT genotype combination for tertiles of 'metabolic syndrome' factor.
AA/TT = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.



Numbers in each group
A. Controls n=34, cases n=15
B. Controls n=6, cases n=4
C. Controls n=39, cases n=25
D. Controls n=8, cases n=14
E. Controls n=36, cases n=55
D. Controls n=11, cases n=23

Numbers in each group
A. Controls n=47, cases n=11
B. Controls n=16, cases n=2
C. Controls n=39, cases n=52
D. Controls n=13, cases n=6
E. Controls n=49, cases n=64
D. Controls n=8, cases n=11

Figure 3.6 Risk for MI in carriers of the 455V/-1208/09delTT genotype combination for tertiles of 'inflammation' factor.
AA/TT = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

3.6 Discussion

The aim of this section was to assess the contribution to risk of MI by four potentially functional variants in the Tm gene (-1748G>C, -1208/09TT>delTT, -1166G>A and A455V) in the North and South of Europe.

Allele frequencies for 455V similar to those determined here for the North (0.19) and South (0.12) have been reported previously. A rare allele (455V) frequency of 0.18 was reported in a sample of healthy Dutch subjects and 0.26 in a group of healthy subjects from Sweden (van der Velden et al., 1991) (Norlund et al., 1997a). An allele frequency of 0.18 was also reported in healthy Caucasian Americans, but in the same study a very much lower frequency (0.03) was seen for healthy Black Americans (Wu et al., 2001). Similar allele frequencies for -1748C (0.21), -1208/09delTT (0.10) and-1166A (0.01) have been reported in French subjects (Le Flem et al., 2001). The observation that differing variant frequencies occur in different populations, highlights the importance of stratifying by population.

The -1748C allele does not appear to be associated with increased risk of an MI event either in the North or the South of Europe. This finding suggests that the -1748G>C variant is unlikely to represent an independent risk factor for arterial thrombosis in European populations. A significant association with higher D-dimer levels has been suggested previously for the -1748G>C variant (Le Flem et al., 2001). These findings are difficult to reconcile with. D-dimer levels indicate the presence of an abnormally high level of fibrin degradation products, suggesting there has been significant clot formation and breakdown in the body. The -1748G>C variant creates a putative Sp1-like transcription factor binding site that might be expected to increase expression of the

Tm gene under basal or induced conditions (Le Flem et al., 2001). Upregulation of Tm expression would be expected to decrease thrombin generation, through its anti-thrombotic and anti-iflammatory properties, thus reducing clot formation in the body. As such, the -1748G>C variant might be expected to be associated with reduced D-dimer levels.

The -1208/09TT>delTT variant was found to be in strong LD with A455V in both Northern and Southern European controls, as has also been reported in a recent French case-control study of venous thrombosis (Δ= 0.89 $p<0.01$) (Le Flem et al., 2001). Risk associated with –1208/09delTT alone could not be assessed, since the -1208/09TT>delTT variant occurs on the 455V allele in most individuals. There was, however, a suggestion of elevated risk associated with 455V, alone, in both the North and South of Europe, but this did not reach statistical significance. It was estimated that a total of 550 cases would be required to detect a statistically significant effect on risk by the 455V allele (power 80%, p=0.05, allele frequency 0.19). The number of cases used to assess risk associated with the A455V variant was 229 in the North and 285 in the South of Europe.

The A455V variant is situated in the 6^{th} EGF-like repeat of the Tm molecule, a region necessary for thrombin binding (Kurosawa et al., 1988) (Ye et al., 1992), but has not been identified as a residue critical for either thrombin or Ca^{2+} binding; see section 1.4.3.2. The Ala to Val substitution at position 455 would not be expected to impose an extreme electrostatic or steric effect since both residues are hydrophobic aliphatic amino acids (Cunningham and Wells, 1989). It is possible, however, that this substitution alters folding of the mature protein, since the residue occupies a position within the

minor β-sheet of EGF6 (Fuentes-Prior et al., 2000). No direct *in vitro* functional studies have been carried out to date to address whether this variant is dysfunctional.

Carriers of both 455V and –1208/09delTT had a 1.6-fold increased risk of MI in the North of Europe, but risk appeared to be reduced in Southern Europe, again highlighting the importance of stratifying by population. No evidence could be found for risk modification by smoking. The most interesting finding was an increased risk of MI in carriers of the V/delTT genotype combination in the highest BMI, 'inflammation' and 'metabolic syndrome' factor tertiles in the North of Europe. BMI is a measure of obesity (Stevens et al., 1998) (Tracy, 2001). As aforementioned (see section 1.3.2), obese individuals have a higher risk of developing CHD compared to lean individuals (Lamarche, 1998) (Stevens et al., 1998) and there is increasing evidence that adipose tissue in general and abdominal adiposity in particular, is a key player in the inflammatory process (Yudkin, 1999). Inflammatory mediators contribute to progression of atherosclerosis by several mechanisms; see section 1.3.3.1. In relation to haemostasis, they have been shown to stimulate TF expression (responsible for the initiation of the coagulation cascade) and to cause natural anti-coagulant and fibrinolytic deregulation (Nawroth et al., 1986) (Moore et al., 1987) (Gregory et al., 1989) (Esmon et al., 1991) (Nemerson, 1995) (Orbe et al., 1999) (Speiser et al., 2001); see section 1.3.3.1. In this context, the expression of Tm on the surface of endothelial cells exposed to TNF-α *in vitro* has been shown to decrease (Moore et al., 1989) (Lentz et al., 1991) and the intradermal application of TNF-α was also found to downregulate the expression of Tm on the surface of human endothelial cells (Speiser et al., 2001). *In vitro*, IL-1 was found to cause a decrease in Tm expression and activity on the cell surface, and intravenous infusion of IL-1 was also found to decrease Tm cell surface co-factor activity (Nawroth et al., 1986) (Archipoff et al., 1991). Although Tm is itself

regulated by inflammatory cytokines, it also makes an important contribution to anti-inflammatory processes, through direct and indirect mechanisms; see section 1.4.3.4.

A general process contributing to CHD by inflammatory cytokines and Tm may be increased release of Tm from the surface of vascular endothelial cells and decreased Tm expression. Reduced expression of Tm on the surface of vascular endothelial cells contributes towards a pro-thrombotic and pro-inflammatory state. An associated increased inflammatory response, measured by IL-6 levels, was noted in carriers of the V/delTT genotype with high BMI in Northern Europeans. In addition, when risk was assessed for the highest tertile of the 'inflammation' factor, carriers of V/delTT were associated with increased risk of MI in the North of Europe. A mechanism for interaction between BMI and Tm genotype may be an altered expression of Tm in response to elevated circulating inflammatory cytokines and reduced function of the expressed molecule on the surface of endothelial cells in individuals with the combined genotype V/delTT. Alternatively, the risk effect may be mediated by the promoter variant alone. Analysis of promoter variants has been studied in this thesis; see Chapter 7.

Obesity clusters with established cardiovascular risk factors that include insulin resistance, hyperglycaemia, hypertension, dislipidemia, termed collectively metabolic syndrome (Kaplan, 1989) (Haffner et al., 1992). In the current study, increased risk of MI was observed in carriers of the V/delTT genotype combination in the highest tertile of the 'metabolic syndrome' factor. Whether the interactive risk is directed as an independent mechanism through elevated secretion of inflammatory cytokine in obese individuals or through other aspects of the metabolic syndrome has been assessed further in the studies for this thesis; see Chapters 4, 7 & 8.

The Tm-protein C pathway has both anti-thrombotic and anti-inflammatory characteristics; see sections 1.4.3. Down–regulation of Tm expression is likely to lead to increased thrombin generation and exacerbation of inflammation. Results of the current study have suggested a risk interaction for a Tm haplotype, with obesity or metabolic syndrome, and an associated increased inflammatory response, measured by IL-6 levels. Reasons for the differences in effect of Tm V/delTT in the North and South of Europe are not entirely clear. The higher risk generally, for both 'metabolic syndrome' and 'inflammation' in the South of Europe, and the apparent reduced risk associated with 'metabolic syndrome' and Tm V/delTT in the South, may point towards a survival bias, i.e. those with Tm V/delTT with the greatest 'inflammation' and 'metabolic syndrome' factors might have a higher mortality and may not have survived, rendering them unavailable for analysis. This could lead to an underestimation of risk associated with the Tm genotype combination and elevated inflammation or obesity. Another reason for the discrepancies is that gene-gene and gene-environment interactions may differ between in Northern and Southern Europeans, with variants only showing their effects in populations with a particular genetic or environmental background (Talmud and Humphries, 2002). Further studies are required to address these issues.

In conclusion, the most frequent distal promoter variant (-1748G>C) was not associated with risk for MI in the European population, while an elevation in risk was determined for the combined V/delTT Tm genotype in Northern Europeans. Further, a risk interaction for the V/delTT haplotype with obesity and a 'metabolic syndrome' or 'inflammation' factor was identified in Northern Europeans. An increased inflammatory response was also noted in V/delTT carriers with high BMI.

CHAPTER FOUR

TM POLYMORPHISMS AND CHD

4. Tm polymorphisms and CHD

4.1 Introduction

In Chapter 3, the contribution to risk of MI by four, previously reported (Le Flem et al., 2001), potentially functional variants in the Tm gene was assessed. A haplotype comprising the rare alleles for two of these variants (-1208/09TT>delTT & A455V; V/delTT) contributed to the risk of MI, in the North of Europe, particularly in individuals with obesity, metabolic syndrome or inflammation. An associated increased inflammatory response was also noted. These two variant sites were therefore subsequently assessed for their contribution to CHD risk in a large prospective study, the second Northwick Park Heart study (NPHSII), to verify and extend the results for Northern Europeans in the HIFMECH study. An inherent limitation of case-control studies, and thus HIFMECH, is that only survivors can be studied. This could lead to an underestimation of risk associated with factors having a greater impact on survival, as subjects with these factors will not be available for analysis. A study where individuals, who have not yet had the outcome event in question, are monitored for the number of such events over time (prospective study) will help address this limitation.

4.2 Aim

The purpose of the work described in this section was to assess risk for CHD in those with -1208/09delTT/455V in a large prospective study of heart disease, the second Northwick Park Heart study (NPHSII). Tm gene variant interaction with other clinical and plasma markers of CHD and markers of thrombin generation and inflammation were studied also in relation to CHD.

4.3 Methods

4.3.1 DNA extraction from blood using the salting out method

Genomic DNA from participants of the NPHSII study had been isolated previously using the salting out method (Miller *et al.* 1988); see section 2.2.2.1.

4.3.2 Description of study subjects (NPHSII)

The second Northwick Park Heart Study (NPHSII) is a large prospective study of healthy Caucasian middle aged men, recruited from 9 general medical practices in the UK, and designed to examine risk factors for CHD. From April 1989 to April 1994 (15 years of follow-up), 3012 healthy European, Caucasian men, aged 51-60 years, registered with 9 general medical practices in the UK, were recruited for prospective surveillance (Miller et al., 1995) (Miller et al., 1996). Follow-up is still continuing. All eligible subjects were free of a history of unstable angina, MI or evidence of silent infarction, coronary surgery, aspirin or anticoagulant therapy, cardiovascular disease, malignancy (except skin cancer other than melanoma), or any condition precluding informed consent. Participants were asked to attend a clinic in a non-fasting state, having been instructed to avoid heavy meals before examination and to refrain from smoking and vigorous exercise from midnight of the previous night. DNA was obtained for 2700 people whose baseline characteristics were not significantly different from the whole study group (Humphries et al., 2001).

Participants answered a questionnaire for previous medical history and smoking habit and were classified as never smokers, ex-smokers (cessation for minimum 1 year) and

current smokers. A standard electrocardiogram (ECG) was recorded and coded according to Minnesota criteria. This resulted in the exclusion of 42 men with changes indicative of MI. Weight, height and SBP measurements were recorded and venous blood samples were collected for plasma and DNA analysis. Participants were recalled annually for interview and repeat plasma analysis. A routine ECG was repeated at the sixth examination. Endpoints for CHD were fatal and non-fatal MI, silent MI or coronary revascularization procedures (stroke, n=54, was not included in the current study). The numbers for these end points as of April 2003 were: fatal and non-fatal MI (non-fatal n=113, fatal n=49), silent MI (n=20), coronary revascularization (n=45), total 227. Forty-nine had a fatal MI. These events occurred from 64 days to 13 years of follow up.

Biochemical and clinical measurements

A 5 ml sample of venous blood was taken using a Vacutainer (Becton Dickinson, Cowley, Oxford, UK) from each patient and transferred to plastic screw-cap vials, which were stored at -4^{0}C pending analysis. Cholesterol and triglyceride concentrations were measured using automated enzyme procedures (Humphries et al., 2001). Fibrinogen concentration was measured by a thrombin-clotting method, calibrated against a standard plasma (Immuno, Vienna). F1+2 and fibrinopeptide A (FPA) levels had been measured previously (Cooper et al., 2000). Blood pressure was recorded twice with a random zero sphygmomanometer after the subjects had been seated for 5 min, and the results were averaged for statistical analysis. Height (m) was measured on a stadiometer and weight (kg) on a balance scale to calculate BMI (kg/m^2). CRP levels were measured in 587 randomly selected baseline samples (509 controls, 78 cases) using a commercial assay (Cordia High Sensitivity CRP). Baseline measurements were used for analysis in the current study.

4.3.3 Genotyping for polymorphisms in the Tm gene

Tm genotyping for the variant sites under investigation (-1208/09TT>delTT & A455V) was performed as described in Chapter 2. PCR conditions and restriction enzyme digestion conditions are summarised in Tables 2.1-2.3. Band patterns for these two Tm polymorphisms studied are shown in Figures 2.5 and 2.7. A total of 2367 controls and 201 cases with CHD were successfully genotyped for both Tm variants in the current study.

4.3.4 Statistical analysis

Data were entered into an EXCEL spreadsheet (Microsoft). Deviations from Hardy-Weinberg equilibrium were considered via a chi-squared test of observed and expected frequencies. Differences in genotype distributions and allele frequencies between groups were analysed using chi-squared tests. LD was considered using the statistic delta (Δ) (Chakravarti et al., 1984).

In NPHSII statistical analysis was carried out with help from Jackie Cooper (statistician in CVG who solely analyses NPHSII data), using 'Intercooled STATA' version 7.0 (College Station, Texas). Continuous variables are expressed as mean $\pm$ SD for normally distributed variables or geometric means and approximate SD for variables where normality was reached after ln-transformation. Triglyceride, FPA, F1+2 and CRP levels were ln-transformed before analysis. Normality was considered graphically with QQ-norm plots and formally via a Kolmogorov-Smirnov test. Continuous variables were assessed by analysis of variance (ANOVA) on normally distributed data, or after appropriate transformation. Chi-squared tests were used to compare differences in

categorical variables. Association of Tm genotype with risk of CHD was assessed using Cox's proportional hazards model, with significance assessed by the likelihood ratio test. Results are presented as Relative Risk (RR) with 95% CI. Results were adjusted for age and practice unless otherwise stated. This was carried out to allow for differences in baseline data according to age and practice. Age was included as a covariate in the model and data stratified by practice (using the strata option in STATA). Interaction between Tm genotype and other clinical characteristics was tested by a model including an interaction term. P-values <0.05 were considered as significant.

4.4 Results

4.4.1 Characteristics of NPHSII samples

Baseline clinical and biochemical measurements in those who had an event were compared to those without an event, for those genotyped in the current study; see Table 4.1. Age, BMI, prevalence of smoking, SBP, cholesterol, triglyceride and CRP levels were significantly higher in those with CHD, compared to those without CHD ($p<0.05$).

	No CHD event (n=2367)	CHD event (n=201)	P-values
Age (years)	56.0±3.4	56.6±3.5	0.03
BMI (Kg/m2)	26.4±3.5	27.1±3.4	0.009
Smokers (%)	27.6	39.8	<0.0001
SBP (mmHg)	137.9±19.1	144.0±20.1	<0.0001
Cholesterol (mmol/l)	5.71±1.00	6.07±1.01	<0.0001
Triglyceride (mmol/l)	1.79±0.94	2.09±1.11	<0.0001
FPA (μmol/l)	1.19±0.60	1.22±0.60	0.55
F1+2 (μmol/l)	0.70±0.27	0.71±0.25	0.82
CRP (pg/ml)	1.18±1.30	1.98±2.18	<0.0001

Table 4.1 Base line characteristics of subjects in NPHSII.
Data are mean±SD unless otherwise stated. Triglyceride, F1+2, FPA and CRP levels ln-transformed. **CHD event**: fatal and non-fatal MI, coronary artery surgery or ECG evidence of MI.

4.4.2 Allelic association

Allelic association between the -1208/09TT>delTT and A455V polymorphisms was considered using the statistic delta (Δ), as described previously (Chakravarti et al., 1984). As was the case for HIFMECH (see section 3.5.2), strong LD was observed between the –1208/09TT>delTT and A455V variants (Δ=0.67 p<0.0005), with only 26 individuals heterozygous for -1208/09delTT but homozygous for A455 (cases n=5, controls n=21).

4.4.3 Tm genotype distribution and allele frequencies

Genotype distribution and allele frequencies for the -1208/09TT>delTT and A455V polymorphisms were determined in NPHSII; see Table 4.2. No significant difference in genotype distribution for either variant was observed between those with and without a CHD event; see Table 4.2. The variable number of samples genotyped for the different polymorphisms was due to repeated failure to amply the target sequence during the PCR reactions. This is a random event and could not be responsible for any association seen. Genotype distribution was in Hardy-Weinberg equilibrium for both variant sites.

Polymorphism		N	Genotypes			Rare Allele Freq. (95% CI)	P-value for CHD-no CHD allele frequency
			11	12	22		
-1208/09	Total	2663	2138	492	33	0.104 (0.09-0.12)	
TT>delTT	No CHD	2450	1978	441	31	0.104 (0.08-0.13)	0.10
	CHD	213	160	51	2	0.129 (0.11-0.15)	
A455V	Total	2635	1751	784	100	0.184 (0.15-0.21)	
	No CHD	2424	1617	716	91	0.185 (0.16-0.21)	0.64
	CHD	211	134	68	9	0.204 (0.18-0.21)	

Table 4.2 Genotype distribution and allele frequencies of the -1208/09TT>delTT and A455V polymorphisms in the whole NPHSII study and in those with and without CHD.
Tm -1208/09TT>delTT: TT/TT (11), **TT/delTT** (12), **delTT/delTT** (22). **Tm A455V: AA** (11), **AV** (12), **VV** (22).

4.4.4 Tm genotype and risk of CHD

Due to the low number of rare homozygotes identified for each of the Tm variants studied (see Table 4.2), subjects with one and two rare alleles were combined and compared to those with two common alleles at each variant site.

There was no statistically significant evidence to suggest a contribution to CHD, when risk was assessed for each variant site individually, although risk approached significance for -1208/09TT>delTT. Overall risk for each variant site was: -1208/09TT>delTT: RR 1.36 (CI 0.99-1.87, p=0.06) and 455V RR 1.12 (CI 0.84-1.49, p=0.89), after adjustment for age and practise.

Risk was analysed further in carriers of the 455V allele who were homozygous for the common allele at –1208/09 (CHD events n=26; no event n=358), as compared to those homozygous for the common allele at both sites (CHD events n=123; no event n=1558): RR 0.90 (CI 0.59-1.37, p=0.71). Risk for –1208/09delTT alone could not be assessed because -1208/09delTT occurs on the 455V allele in most individuals. When risk was assessed in men heterozygous and homozygous for both -1208/09delTT and 455V (CHD events n=47; no event n=430), compared to those homozygous for the common allele at both sites (CHD events n=123; no event n=1558); see Table 4.3, risk was 1.35 (CI 0.96-1.89, p=0.08), after adjustment for age, practise, SBP, cholesterol, triglycerides, fibrinogen and smoking. These parameters used for adjustment had been identified previously as statistically significant independent predictors of CHD risk in NPHSII (Miller et al. 1995).

	-1208/09TT>delTT		
A455V	**TT/TT**	**TT/delTT**	**delTT/delTT**
AA	123/1681 (7.3%)	5/26 (19.2%)	0/0
AV	24/360 (6.7%)	40/402 (9.9%)	0/1
VV	2/24 (8.3%)	5/43 (11.6%)	2/31 (6.4%)

Table 4.3 Combined Tm genotype distribution.
Numbers of CHD events/total number in each group (%).

4.4.5 Combined Tm genotype (V/delTT) effect in association with other baseline clinical characteristics

As there was a suggestion for increased risk (although not significant) for both the -1208/09TT>delTT (RR 1.36 CI 0.99-1.87, p=0.06) and the combined V/delTT genotype (RR 1.35 CI 0.96-1.89, p=0.08), the effect of the V/delTT Tm genotype on CHD risk was analysed together with clinical and life style factors; see Table 4.1. In HIFMECH, CHD risk interaction was identified with a 'metabolic syndrome' and 'inflammation' factor; see Chapter 3. Interaction with factors present in metabolic syndrome, and other factors (smoking), was therefore further assessed. No interaction between smoking and V/delTT genotypes was identified, as risk for carriers of the V/delTT haplotype, compared to those with the common allele at both sites, was consistent when data were analysed for smokers and never+ex-smokers (RR 1.42, CI 0.93-2.16, p=0.1 in non-smokers; RR 1.30, CI 0.74-2.29, p=0.36 in smokers).

NPHSII men were analysed for CHD risk in those with the V/delTT haplotype after stratifying by BMI. A gradual increase in CHD risk was observed when the rare allele combination was assessed by tertile of BMI (tertiles: low <24.9 Kg/m^2; mid 24.9-27.5 Kg/m^2; high >27.5 Kg/m^2), as compared to those with the common allele in the lowest third (mid-third RR 1.86, CI 1.02-3; high-third RR 2.48, CI 1.41-4.34, adjusted for age and practice). Although a significant risk-trend from the lowest to the highest BMI tertile was observed for V/delTT carriers (p=0.05), there was no significant interaction between Tm genotype and BMI; see Table 4.4. When results were adjusted for smoking, SBP and triglyceride, in addition to age and practice, the significant risk-trend observed for V/delTT carriers was removed; see Table 4.4.

Tertile of BMI	A455V	-1208/09 TT>delTT	No of events	Total N	% with events	RR (95% CI)	RR (95% CI)
1	AA	TT	30	563	5.3%	1.00	1.00
(<24.9)	V+	delTT+	9	147	6.1%	1.05 (0.50-2.22)	1.09 (0.51 – 2.30)
2	AA	TT	45	553	8.1%	1.52 (0.95-2.41)	1.48 (0.92 – 2.38)
(24.9 – 27.5)	V+	delTT+	17	171	9.9%	1.86 (1.02-3.38)	1.96 (1.07 – 3.60)
3	AA	TT	48	563	8.5%	1.57 (0.99-2.49)	1.40 (0.86 – 2.28)
(>27.5)	V+	delTT+	21	158	13.3%	2.48 (1.41-4.34)	2.29 (1.28 – 4.10)
						Adjusted for age and practice	Adjusted for age, practice, smoking, SBP & triglycerides
						Test for interaction p=0.65	Test for interaction p=0.67
						Trend p=0.05	Trend p=0.20

Table 4.4 CHD risk for BMI tertile by Tm genotype.
BMI units: Kg/m^2. **AA/TT** = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

As aforementioned (see section 1.3.2), obesity clusters with established cardiovascular risk factors that include insulin resistance, hyperglycemia, hypertension, elevated triglyceride levels and reduced HDL-cholesterol levels, termed collectively metabolic syndrome (Kaplan, 1989) (Haffner et al., 1992). In the current study, BMI correlated well with SBP ($r=0.2$, $n=3006$, $p<0.0001$) and triglyceride levels ($r=0.29$, $n=2910$, $p<0.0001$), as assessed by Pearson's correlation analysis. The data were therefore analysed further to determine any interaction between Tm genotype and tertiles of SBP or plasma triglyceride that could have been underlying the risk with BMI in the case-control study. Although carriers of V/delTT in the highest SBP tertile were associated with increased risk of CHD (RR 2.63 CI 1.52-4.55), compared to those with the common genotype in the lowest tertile, no significant risk-trend was observed from the lowest to the highest SBP tertile (low <128.5mmHg, mid: 128.5-145mmHg, high: >145mmHg) for V/delTT carriers ($p=0.09$), and there was no significant interaction between Tm genotype and SBP ($p=0.50$). An increase in risk was, however, observed when V/delTT was assessed by tertile of triglyceride (low: <1.4mmol/l, mid: 1.4-2.19mmol/l, high: >2.2mmol/l) and reached a statistical interaction ($p=0.016$). V/delTT carriers had a 2-fold increased risk of CHD (RR1.95 CI 1.12-3.40) amongst subjects in the mid triglyceride third, and 1.8-fold increased risk in subjects within the high third (RR 1.77 CI 1.02-3.09), compared to those with the common genotype in the lowest tertile, after adjustment for age, practice, smoking, SBP and BMI; see Table 4.5. When results were adjusted for the classical CHD risk factors (age, cholesterol, smoking, SBP, BMI in addition to practice) the interaction between Tm haplotype and tertiles of triglycerides remained statistically significant ($p=0.02$). For V/delTT carriers, a significant risk-trend was observed from the low to high triglyceride tertile (Mid-tertile RR 3.54 CI 1.42-8.85; High-tertile RR 3.21 CI 1.28-8.05, $p=0.01$) while no significant risk was associated with higher triglyceride levels in non-carriers; see Figure 4.1.

Triglyceride levels did not differ significantly between those subjects with the common genotype (n=1666; geometric mean 1.80; SD 0.96) and those with the rare genotype (n=475; geometric mean 1.79; SD 0.90) (p=0.92), suggesting that the rare Tm allele has no direct causal effect on triglyceride levels.

Tertile of triglyceride	A455V	DelTT	No of events	Total N	% with events	RR (95% CI)
1	AA	TT	35	559	6.3	1.00
(<1.4)	V+	delTT+	6	166	3.6	0.55 (0.23 – 1.32)
2	AA	TT	30	542	5.5	0.85 (0.52 – 1.39)
(1.4 – 2.19)	V+	delTT+	20	146	13.7	1.95 (1.12 – 3.40)
3	AA	TT	56	565	9.9	1.22 (0.79 – 1.90)
(>=2.2)	V+	delTT+	21	163	12.9	1.77 (1.02 – 3.09)
						Adjusted for age, practice, smoking, SBP & BMI
						Test for interaction=0.016

Table 4.5 Risk for triglyceride tertile by Tm genotype.
Triglyceride units (mmol/l). **AA/TT** = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

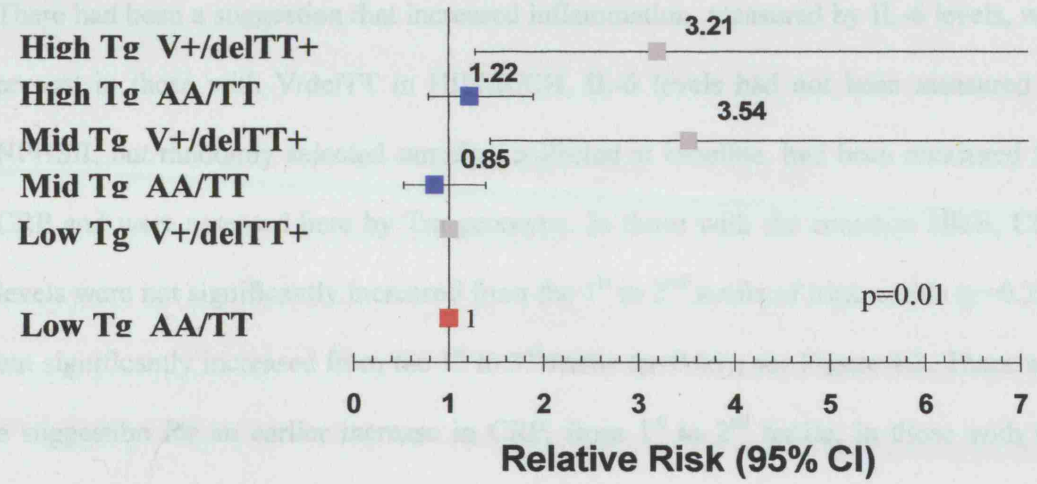


Figure 4.1 CHD risk-trend in carriers of Tm V/delTT haplotype, compared to Tm AA/TT, in lowest to highest triglyceride (Tg) tertile.
In each genotype group low Tg tertile set at RR 1.00. Tg tertiles: Low<1.4mmol/l; Mid=1.4-2.19mmol/l; High>2.2mmol/l. **AA/TT** = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles. Results adjusted for age, practice, smoking, SBP, BMI.

4.4.6 Effects of genotype (V/delTT) on measures of thrombin generation and a marker of inflammation

Baseline F1+2 and FPA levels were analysed to determine any effect upon thrombin generation or activity. There was no evidence to suggest differences in these markers by Tm genotype (for F1+2: AA/TT 0.68 $\pm$ 0.25, n=1511 v V/delTT 0.67 $\pm$ 0.24, n=435; p>0.29 & for FPA: AA/TT 1.21 $\pm$ 0.6; n=1484 v V/delTT 1.17 $\pm$ 0.6; n=426 for F1+2 p>0.29, n=1910; p>0.18). Furthermore, Tm genotype had no effect on these markers when assessed in tertiles of triglyceride (genotype-triglyceride interaction p=0.12 for F1+2 & p=0.9 for FPA).

There had been a suggestion that increased inflammation, measured by IL-6 levels, was present in those with V/delTT in HIFMECH. IL-6 levels had not been measured in NPHSII, but randomly selected samples, collected at baseline, had been measured for CRP and were assessed here by Tm genotype. In those with the common allele, CRP levels were not significantly increased from the 1st to 2nd tertile of triglyceride (p=0.27), but significantly increased from the 1st to 3rd tertile (p=0.03); see Figure 4.2. There was a suggestion for an earlier increase in CRP, from 1st to 2nd tertile, in those with the V/delTT haplotype, but this finding was of borderline significance (p=0.05). The difference in CRP levels from the 1st to the 3rd tertile did not reach statistical significance, in V/delTT carriers (p=0.07). Numbers were too small to analyse similarly for cases although the trend was similar when controls only were analysed.

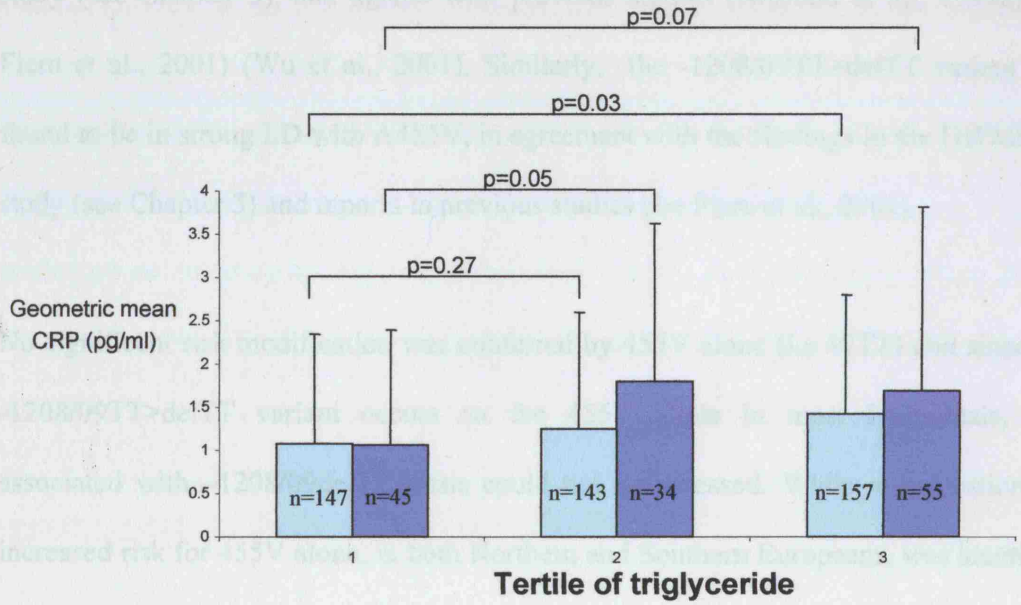


Figure 4.2 Geometric mean and approximate SD for CRP levels by tertile of triglyceride, for those with the common Tm allele (light shaded bar) and those with Tm V/delTT (dark shaded bar).
Tg tertiles: Low<1.4mmol/l; Middle=1.4-2.19mmol/l; High>2.2mmol/l.

4.5 Discussion

A haplotype comprising the rare alleles for two common Tm variants (-1208/09TT>delTT & A455V) contributed to the risk of MI, in the North of Europe, particularly in individuals with obesity, metabolic syndrome or inflammation; see Chapter 3. An associated increased inflammatory response was also noted. The purpose of this section was to investigate the contribution of these two sites to risk of CHD in a large prospective study (NPHSII), to verify and extend the results in the HIFMECH study.

Rare allele frequencies for the -1208/09TT>delTT and A455V variant were similar to those noted in the Northern European control group of the HIFMECH case-control study (see Chapter 3), and agreed with previous studies (Norlund et al., 1997a) (Le Flem et al., 2001) (Wu et al., 2001). Similarly, the -1208/09TT>delTT variant was found to be in strong LD with A455V, in agreement with the findings in the HIFMECH study (see Chapter 3) and reports in previous studies (Le Flem et al., 2001).

No significant risk modification was conferred by 455V alone (i.e V/TT) and since the -1208/09TT>delTT variant occurs on the 455V allele in most individuals, risk associated with –1208/09delTT alone could not be assessed. While a suggestion for increased risk for 455V alone, in both Northern and Southern Europeans, was identified in the HIFMECH case-control study (OR≥1.36), no suggestion for increased risk was observed in the prospective study (RR 0.9). It is possible that both studies were underpowered to determine any significant effect; see Chapter 3. However, the results from the current study and previous case-control study may suggest that risk is acting through either the combination of 455V and -1208/09delTT or through the distal

promoter variant alone. Past association studies of the effect of A455V alone have shown both risk and protection associated with the 455V allele in different populations (Norlund et al., 1997a) (Wu et al., 2001). It is possible that this discrepancy is due to differences in allele frequency of the –1208/09delTT in different populations. The mechanism of action of this promoter variant was examined by *in vitro* reporter gene analysis under basal and suppressed conditions; see Chapter 7. Tm -1208/09TT>delTT lies between a DR4 sequence (-1531 to –1516) and SSRE (-1163 to -1168) (Ishii et al., 2003); see sections 1.4.4 & 1.4.7. It is possible that the deletion variant disrupts nuclear protein binding, but it cannot be ruled out that this haplotype is marking an additional functional variant either within or outside the Tm gene.

Carriers of both 455V and –1208/09delTT had an overall small but non-significant elevation in risk for CHD (1.35 CI 0.96-1.89, p=0.08). In Chapter 3, in the Northern Europe subgroup, risk for the rare combined allele reached statistical significance (OR 1.67 CI 1.06-2.64, p=0.03), compared to those with the common allele at both sites. Failure of the current study to detect a significant overall effect of the V/delTT haplotype on risk may be due to a lower power to pick up a significant effect. It was estimated that the NPHSII study has a power of only 54% to detect a 1.35-fold elevation in CHD risk (proportion of individuals with an event, carrying the V/delTT haplotype =0.099; proportion of individuals with an event, homozygous for common allele at both sites =0.073).

A significant interaction (p=0.02) between the combined V/delTT genotype and increasing BMI was previously observed in a case-control study; see Chapter 3. In the current prospective study, however, no significant interaction was observed, despite a trend occurring in the same direction. In NPHSII men, Tm genotype was found to

significantly alter risk associated with triglyceride levels, with V/delTT carriers having higher risk in the mid and top tertiles compared to non-carriers (interaction p=0.016). This was maintained when results were adjusted for classical CHD risk factors (age, smoking, cholesterol, SBP, BMI) along with practice. It should be noted that the interaction of genotype with triglyceride levels in the current study was identified in baseline measurements, compared to the case-control study, where interaction with BMI was post-event. In the HIFMECH case-control study, increased risk for MI was noted for carriers of the V/delTT haplotype in the highest tertile of a 'metabolic syndrome' and 'inflammation' factor; see Chapter 3. This observation together with the significant correlation between BMI and triglyceride levels in NPHSII men, suggests that the true underlying mechanism may be between genotype and metabolic syndrome (see below).

Elevated triglyceride levels often occur in the presence of low HDL-cholesterol and hyperglycaemia (Kaplan, 1989). Low HDL-cholesterol is the most common form of dyslipidemia in type 2 diabetes (Taskinen, 2002). It is unclear, therefore, whether hypertriglyceridemia is an independent predictor of coronary events or an indicator of the presence of a cluster of CHD risk factors that includes also hyperglycaemia, hypertension, and abdominal obesity or elevated BMI, termed collectively metabolic syndrome (Kaplan, 1989) (Haffner et al., 1992). The significant correlation between BMI and triglyceride levels in NPHSII men may thus be an indicator for metabolic syndrome. Whether the interactive risk is directed as an independent mechanism through triglyceride-containing lipoproteins or indirectly through other aspects of the metabolic syndrome will need further assessment. However, it was particularly interesting that the risk associated with increasing triglyceride levels was absent in those carrying the common Tm allele.

Increased triglyceride levels have been shown to be consistently associated with smaller LDL size (Austin, 2000) (Packard et al., 2000). Small dense LDL is associated with formation of ox-LDL (Anber et al., 1996) (Packard et al., 2000). LDL oxidation is thought to be the most important pathological hallmark of atherosclerosis; see section 1.3.1. Ox-LDL also contributes to atherothrombosis by inducing endothelial cell death (leading to plaque erosion), stimulating TF production by SMCs and impairing the anti-coagulant nature of the endothelium (Mertens and Holvoet, 2001). In the context of the current study, it has been suggested that ox-LDL, following endocytosis, releases oxidised phospholipids through a lysosomal pathway and that the released oxidised phospholipids downregulate Tm expression (Ishii et al., 2003); see section 1.4.5. The triglyceride moiety, it seems, does not alter Tm expression (Ishii et al., 1996) (Ishii et al., 2003). A general process underlying contribution to CHD by triglyceride containing lipoprotein and Tm may be increased release of Tm from the surface of the endothelium and downregulation of Tm expression by ox-LDL. A consequence of this would be reduced Tm on the surface of arterial endothelium, with reduced anti-thrombotic and anti-inflammatory potential. A mechanism for interaction between the metabolic syndrome or triglyceride containing lipoprotein and Tm genotype may be an altered expression of Tm in response to ox-LDL in individuals with the combined genotype V/delTT. An alternative explanation is that interactive risk is directed through other aspects of the metabolic syndrome. Pro-inflammatory cytokines, elevated levels of which have been reported in metabolic syndrome (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999), would be a prime candidate, as described in Chapter 3.

The lack of any effect of Tm genotype upon thrombin generation (F1+2 levels) or thrombin activity (FPA levels) may perhaps not seem surprising in clinically healthy

individuals. However, this group has shown that a variant identified in another endothelial cell surface protein (EPCR; Ser219Gly), which participates in the PC pathway, has a significant effect upon F1+2 levels, in baseline samples from NPHSII (Ireland et al., 2005). These results, taken together with the current study of Tm, may suggest that CHD risk is not manifest through increased thrombin generation, but rather perhaps through inflammatory processes and that the V/delTT allele is not rate limiting for thrombin inhibition. By altering the level of Tm expression, function or both, Tm genotype might operate on CHD risk by compromising the direct anti-inflammatory potential of Tm, as well as through long-term affects on APC and its roles in vascular inflammation (Esmon, 2001) (Esmon, 2002); see section 1.4.3. There was evidence to suggest an increased inflammatory response in V/delTT carriers with high BMI in the case-control study. The suggestion of increased inflammatory response, measured by CRP levels for the V/delTT allele, in NPHSII, would support the importance of Tm itself, upon regulation of inflammation, but needs to be confirmed.

In summary, risk for the combined V/delTT Tm genotype and increasing BMI failed to reach statistical significance. Upon further analysis, however, Tm genotype altered risk associated with triglyceride levels, with carriers of the V/delTT haplotype having higher risk at each tertile compared to non-carriers. Risk associated with increasing triglyceride levels was absent in those with the common Tm haplotype. There was a suggestion for greater inflammatory response (CRP levels) in those with V/delTT compared to those with the common allele, as triglyceride levels increased. Overall, these findings suggest that Tm genotype (V/delTT) contributes to risk for CHD through an interaction with either plasma triglyceride-containing lipoproteins or other aspects of the metabolic syndrome.

CHAPTER FIVE

MEASUREMENTS OF STM: FACTORS CONTRIBUTING TO VARIANCE, AND ASSOCIATION WITH RISK FOR CHD IN TYPE II DIABETES

5. Measurements of sTm: factors contributing to variance, and association with risk for CHD in type II diabetes

5.1 Introduction

As described in section 1.3.3.1, investigation of Tm in relation to cardiovascular disease has been hindered due to the proteins relative inaccessibility on the endothelial cell surface, the prime physiological site for Tm function. Smaller heterogeneous soluble Tm fragments (sTm) have, however, been detected in plasma and urine of healthy individuals (Ishii and Majerus, 1985); see section 1.4.2. sTm is traditionally thought to serve as a marker of endothelial cell damage (Ishii and Majerus, 1985) (Takano et al., 1990) (Ishii et al., 1991) (Borawski et al., 2001), with elevated levels detected in various clinical conditions including cardiovascular disease and diabetes (Inukai et al., 1996) (Ohlin et al., 1996). Several studies have demonstrated that the variously sized Tm molecular subspecies, retain Tm functional activity (Hosaka et al., 1998) (Takahashi et al., 1998), though the range of cofactor activity may vary (Ishii et al., 1990) (Uehara et al., 2001). sTm also possesses anti-inflammatory and anti-coagulant properties (Uchiba et al., 1996) (Mohri et al., 1998) (Conway et al., 2002) (Abeyama et al., 2005), thereby conferring protection against atherosclerosis and its complications. A large prospective study (ARIC) demonstrated a reduced risk of CHD in healthy individuals with elevated plasma sTm levels (Salomaa et al., 1999). It has been suggested that plasma sTm levels, in healthy individuals, may result from the constitutive cleavage of membrane bound Tm, and may thus reflect the levels of Tm synthesized and expressed in endothelial cells (Salomaa et al., 1999).

5.2 Aim

The purpose of the work described in this section, was to determine firstly, the relationship between sTm act and ag levels in type 2 diabetes, secondly the relationship between these phenotypic measurements and other variables associated with CHD, and thirdly to determine whether sTm levels are related to personal or family history of CHD. The effect of Tm genotype (-1208/09TT>delTT & A455V) in relation to plasma sTm measurements was also assessed.

5.3 Methods

5.3.1 Measurement of plasma sTm levels

Stored plasma was used to measure circulating levels of sTm, using activity (act) and antigen (ag) assays, as described in detail in section 2.2.1. These measures were performed on plasma samples collected in the Ealing Diabetes Study of Coagulation (EDSC).

5.3.2 Description of study subjects (EDSC)

The Ealing Diabetes Study of Coagulation (EDSC) was designed to address genotype/phenotype associations (particularly in haemostatic and anti-inflammatory pathways) in type 2 diabetes and to identify risk factors for CHD. Patients were recruited consecutively from a diabetes clinic at Ealing Hospital over a 2 year period. Approval was given for the study from Ealing Hospital Ethics Committee and all individuals gave written informed consent (Ireland et al., 2005). A questionnaire was

completed by each patient. Details of age, country of origin, ethnicity, smoking habit, whether they had fasted, heart attack, stroke, family history of CHD and stroke were collected. For the current analysis, all those with >=1 family member with heart disease were grouped together. After exclusion of those who had probable type 1 diabetes, and those of uncertain ethnic origin, 927 patients remained in the study. Reduced numbers may appear in the text and tables, following adjustment for statistical analysis, as data were not available for every variable in all patients. Subjects were classified into three ethnic groups: European Whites (EW), n=331; Indian Asians (IA), n=503; Black African or Afro-Caribbean (AC, n=93). Blood samples were collected for routine biochemical analysis and samples specific to this study. All samples were collected in the morning to eliminate possible effects of diurnal variation. Standard automated techniques were used to measure all biochemical plasma variables [glucose, HbA1c, cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, urea, creatinine, potassium]. F1+2 and CRP levels were measured using ELISA assays (Dade Behring, Marburg, Germany & DiaMed, Switzerland, respectively). Proteinuria was determined by standard dipstick score. Details of duration of diabetes, family history of diabetes, BMI, SBP, DBP, details of CHD, stroke, peripheral vascular disease, retinopathy, medication currently taken, were collected from clinic data. Patients were classified as type 2 diabetic if they presented with diabetes at 40 years or over, or had been taking oral hypoglycaemics for >1 year following diagnosis. Patients were investigated for CHD if they had symptoms (chest pain). If neither the patient nor the clinic records suggested clinical symptoms of CHD, these patients were recorded as negative for CHD. If either the patient or clinic records suggested they had had an event or had angina, the full medical records were searched to identify the criteria used to determine the event. Patients with chest pain but with normal ECG, Holter exercise test, thallium scan or angiogram, were classified negative for CHD. Patients positive for CHD

included those with severe atherosclerosis, shown by angiogram, and/or requiring intervention (angioplasty, percutaneous transluminal coronary angioplasty; PTCA, coronary artery bypass graft; CABG), those who had been admitted with an acute MI determined by ECG/increase in cardiac enzymes, patients who had a silent MI but showed unequivocal ECG changes suggesting previous MI, and those with a positive exercise test or thallium scan for ischemia.

5.3.3 Genotyping for polymorphisms in Tm

Tm genotyping for the variant sites under investigation (-1208/09TT>delTT and A455V) was performed as described in Chapter 2. PCR conditions and restriction enzyme digestion conditions are summarised in Tables 2.1-2.3. Band patterns for these two Tm polymorphisms studied are shown in Figures 2.5 and 2.7.

5.3.4 Statistical analysis

Data were entered into an EXCEL spreadsheet (Microsoft). Deviations from Hardy-Weinberg equilibrium were considered via a chi-squared test of observed and expected frequencies. Differences in genotype distributions and allele frequencies between groups were analysed using chi-squared tests. LD was considered using the statistic delta (Δ), described previously (Chakravarti et al., 1984).

Statistical analysis was carried out primarily by myself with advice from Jackie Cooper. Statistical analysis was conducted using SPSS version 12.0.1. (SPSS Inc., Chicago, USA). Assessment of a normal distribution was considered graphically with QQ-norm plots, and formally via a Kolmogorov-Smirnov test. Ln-transformation was required for

BMI, urea, glucose, HbA_{1C}, SBP, triglyceride, F1+2, CRP, in-house (IH) sTm act, IH sTm ag and Diagnostica Stago (DS) sTm ag levels, whereas square root-transformation was required to normalise creatinine, DBP, HDL-cholesterol and LDL-cholesterol levels. Results are presented as mean ± standard deviation where appropriate. For data that were not normally distributed results are presented as the geometric mean ± approximate standard deviation or median (interquartile range), as indicated in the results. Continuous variables were assessed by analysis of variance (ANOVA) on normally distributed data, or after appropriate transformation.

The relationship between the two sTm ag measurements and sTm act, and between sTm measurements and other variables were assessed using Pearson's correlation coefficient, following appropriate transformation. A univariate step-wise regression model was subsequently applied to assess contributors to variance in sTm assays. The percentage of variation in sTm measurements explained by variables in the model was considered by partial R^2. Pearson's Chi-squared test was used to assess association between sTm measurements and personal- or family-history of CHD, and between sTm measurements and Tm genotype. P-values <0.05 were considered significant.

5.4 Results

5.4.1 Characteristics of EDSC type 2 diabetic subjects

The clinical characteristics of the type 2 diabetic subjects in the three ethnic groups of the EDSC study are shown in Table 5.1. Duration of diabetes was similar in the three ethnic groups, but IA patients were younger when recruited and when they first presented with diabetes ($p<0.001$). The prevalence of personal history of CHD (0.007) and proteinuria ($p=0.03$) were higher in IA, whereas BMI ($p<0.001$) was lower. Prevalence of smoking was lowest in IA and highest in EW ($p<0.001$). DBP was lower in EW ($p=0.02$), whereas urea levels ($p<0.001$) were higher. The prevalence of family history of CHD ($p<0.001$), CRP ($p=0.02$) and triglyceride levels ($p<0.001$) were lower in AC, whereas SBP ($p<0.001$) and HDL-cholesterol levels ($p<0.001$) were higher. Only samples ($n=316$; randomly selected) from IA and EW patients who were not taking lipid-lowering drugs had been analysed for prothrombin F1+2 levels, with IA having lower levels ($p<0.001$).

sTm levels did not differ by gender, in any of the assays. A significantly lower level of sTm was noted for AC, but only in the DS sTm ag assay ($p=0.008$). As lower levels occurred in only one of the three assays and because the numbers of AC were low compared to the other groups, subsequent analysis of sTm was performed in all patients together.

	EW	IA	AC	P-values
Male (%)#	60.8 200/329	56.3 281/499	62.0 57/92	p=0.317
Age at recruitment (years)	63.5 ± 13.8 n=331	58.3 ± 11.6 n=502	62.6 ± 10.3 n=93	**p<0.001**
Age at diagnosis (years)	53.5 ± 13.4 n=327	47.9 ± 11.7 n=495	51.8 ± 10.4 n=91	**p<0.001**
Duration of diabetes (years)#	9.0 (4-15) n=331	10.0 (5-15) n=503	10.0 (6-16) n=93	p=0.700
Family History CHD (%)#	33.4 110/329	27.5 138/501	10.8 10/93	**p<0.001**
CHD (%)#	16.8 53/315	25.8 127/492	16.3 15/92	**p=0.007**
Proteinuria (%) (0,1+,2+,3+)#	79/15.2/4.5/1.3 245;47;14;4/310	70/17.6/7.3/5.1 327;82;34;24/467	68/11.8/9.1/2.3 60;18;8;2/88	**p=0.028**
Smoking status (%) (never/former/current)#	37.9/43.6/18.5 125;144;61/330	79.4/14.4/6.2 397;72;31/500	60.9/27.2/12.0 56;25;11/92	**p<0.001**
BMI (kg/m^2)*	29.3 ± 5.6 n=307	28.0 ± 4.6 n=457	28.8 ± 4.6 n=89	**p<0.001**
Creatinine (μmol/l)†	87.4 ± 34.5 n=314	85.0 ± 31.3 n=490	91.5 ± 20.7 n=92	p=0.473
Urea (mmol/l)*	6.4 ± 2.5 n=314	5.4 ± 2.3 n=490	5.7 ± 1.9 n=92	**p<0.001**
Glucose (mmol/l)*	12.2 ± 4.9 n=321	11.7 ± 4.6 n=489	10.8 ± 4.5 n=92	p=0.068
HbA_{1c} (%)*	8.2 ± 1.85 n=308	8.5 ± 2.45 n=483	8.0 ± 1.9 n=89	p=0.253
SBP (mmHg)*	136.5 ± 21.2 n=326	134.7 ± 20.9 n=493	142.8 ± 19.7 n=92	**p=0.001**
DBP (mmHg)†	72.6 ± 11.5 n=325	74.6 ± 11.4 n=493	76.3 ± 11.7 n=92	**p=0.02**
Triglycerides (mmol/l)*	1.9 ± 1.1 n=295	2.0 ± 1.0 n=444	1.4 ± 0.7 n=76	**p<0.001**
Total cholesterol (mmol/l)	4.9 ± 1.1 n=317	4.9 ± 1.2 n=490	4.9 ± 1.1 n=92	p=0.727
LDL-cholesterol (mmol/l)†	2.8 ± 0.9 n=273	2.8 ± 0.9 n=414	2.8 ± 0.9 n=76	p=0.988
HDL-cholesterol (mmol/l)†	1.2 ± 0.4 n=295	1.1 ± 0.4 n=443	1.4 ± 0.5 n=76	**p=0.001**
F1+2 (μmol/l)*	1.1 ± 0.3 n=133	0.9 ± 0.2 n=183	–	**p<0.001**
CRP (pg/ml)*	2.4 ± 2.6 n=82	2.1 ± 2.4 n=130	1.0 ± 1.8 n=19	**p=0.018** p=0.491 (EW v IA)
IH sTM ag (SEq/ml)*	5.3 ± 1.9 n=188	5.2 ± 2.0 n=302	5.2 ± 2.4 n=50	p=0.639
IH sTM act (SEq/ml)*	3.9 ± 1.2 n=211	3.9 ± 1.2 n=337	3.8 ± 1.1 n=54	p=0.276
DS sTM ag (ng/ml)*	67.8 ± 25.5 n=215	67.8 ± 29.7 n=309	56.0 ± 19.1 n=54	**p=0.008** p=0.979 (EW v IA)

Table 5.1 Biochemical and clinical characteristics for each ethnic group within EDSC.

Mean ± SD or median and interquartile range shown. Continuous variables analysed by ANOVA after appropriate transformation. *Ln-transformed, †Square root-transformed. #Chi-squared test used to compare frequency data. Family history of CHD indicates the % of individuals with 1-4 family members with CHD.

5.4.2 Allelic association between the Tm variants

Genotypes for -1208/09TT>delTT and A455V were determined for 904 (EW n=327; IA n=488; AC n=89) and 900 (EW n=327; IA n=485; AC n=88) participants, respectively, in the EDSC study. The variable number of samples genotyped for the different polymorphisms was due to repeated failure to amply the target sequence during the PCR reactions. This is a random event and could not be responsible for any association seen. The genotype distribution of all variants studies was consistent with Hardy-Weinberg equilibrium.

Allelic association between the -1208/09TT>delTT and A455V polymorphisms in the Tm gene, was determined in EW, IA and AC subjects within the EDSC study. Allelic association was considered using the statistic delta (Δ), as described previously (Chakravarti et al., 1984). As was the case for NPHSII (Δ=0.67 $p<0.0005$; section 4.4.2), strong LD was observed between the –1208/09TT>delTT and A455V variants in all ethnic groups (Δ=0.64 $p<0.0001$, EW; Δ=0.85 $p<0.0001$, IA; Δ=0.82 $p<0.0001$, AC), with only 4 individuals (one in each of EW & IA and 2 in AC) heterozygous for -1208/09delTT but homozygous for A455; see Table 5.3.

5.4.3 Tm genotype distribution and allele frequencies

Genotype distribution and allele frequencies for the -1208/09TT>delTT and A455V polymorphisms were determined in all ethnic groups within the EDSC study; see Table 5.2. The -1208/09delTT allele frequency was similar in the White individuals NPHSII (0.104 CI0.09-0.12; see section 4.4.3) and EDSC (0.09 CI 0.07-0.11). In IA within EDSC, the -1208/09delTT allele frequency was significantly higher (0.15 CI 0.12-0.17)

compared to EW (chi-squared, p=0.006), whereas AC (0.08 CI 0.06-0.10) had a similar rare allele frequency to EW.

Rare allele frequency for the A455V variant was similar in EW (0.19 CI 0.16-0.22) and IA (0.19 CI 0.17-0.22) and analogous to that in NPHSII (0.184 CI 0.15-0.21; see section 4.4.3). The frequency of the 455V allele was, however, significantly lower in AC (0.09 in AC compared to 0.19 in EW & IA; chi-squared, p=0.02). This result is in agreement with previously published data, where a lower 455V allele frequency was reported in healthy Black Americans compared to healthy White Americans (Wu et al., 2001). No significant difference in genotype distribution for either -1208/09TT>delTT or A455V was observed in those with or without CHD; see Table 5.4.

Based on these two variant sites, differences in haplotype structure are indicated in the three ethnic groups. The number of patients heterozygous for both 455V and delTT were similar in EW (15%) and AC (12.5%), but higher in IA (23%). A bigger difference between the ethnic groups was observed for those having only the common allele at the promoter site but being heterozygous for A455V: 18% of EW compared to 8% of IA and 3.4% of AC; see Table 5.3.

Ethnicity	Polymorphism	(n)	Genotypes			Rare allele Freq.
			11	12	22	(95% CI)
EW	-1208/09TT>delTT	327	269	55	3	0.09 (0.07-0.11)
	A455V	327	211	107	9	0.19 (0.16-0.22)
IA	-1208/09TT>delTT	488	358	116	14	0.15 (0.12-0.17)
	A455V	485	316	151	18	0.19 (0.17-0.22)
AC	-1208/09TT>delTT	89	75	13	1	0.084 (0.06-0.10)
	A455V	88	73	14	1	0.09 (0.07-0.11)

Table 5.2 Genotype distribution and allele frequencies for the -1208/09TT>delTT and A455V polymorphisms in the EW and IA subjects within the EDSC study.

		-1208/09TT>delTT								
		TT/TT (11)			TT/delTT (12)			delTT/delTT (22)		
		EW	IA	AC	EW	IA	AC	EW	IA	AC
A455V	AA	210	315	71	1	1	2	0	0	0
	(11)	64%	65%	80.6%	0.3%	0.2%	2.3%	-	-	-
	AV	58	39	3	49	112	11	0	0	0
	(12)	18%	8%	3.4%	15%	23%	12.5%	-	-	-
	VV	1	1	0	5	3	0	3	14	1
	(22)	0.3%	0.2%	-	1.5%	0.6%	-	0.9%	3%	1.2%

Table 5.3 Combined Tm genotype distribution for EW and IA subjects within the EDSC study.
Percentages shown are expressed as a proportion of total number within each ethnic group.

Ethnicity	Polymorphism		(n)	Genotypes			Rare allele Freq. (95% CI)	P-value
				11	12	22		
EW	-1208/09TT>delTT	No CHD	259	214	43	2	0.09 (0.07-0.11)	0.794
		CHD	52	44	8	0	0.07 (0.06-0.10)	
	A455V	No CHD	259	167	84	8	0.19 (0.17-0.22)	0.364
		CHD	52	37	15	0	0.14 (0.12-0.17)	
IA	-1208/09TT>delTT	No CHD	354	262	82	10	0.14 (0.12-0.17)	0.778
		CHD	123	87	32	4	0.16 (0.14-0.19)	
	A455V	No CHD	354	229	112	13	0.19 (0.17-0.22)	0.926
		CHD	120	79	36	5	0.19 (0.16-0.22)	
AC	-1208/09TT>delTT	No CHD	73	61	11	1	0.08 (0.06-0.10)	0.265
		CHD	14	11	3	0	0.14 (0.12-0.16)	
	A455V	No CHD	74	64	9	1	0.09 (0.07-0.11)	0.770
		CHD	14	10	4	0	0.10 (0.08-0.12)	

Table 5.4 Genotype distribution and allele frequencies for the -1208/09TT>delTT and A455V polymorphisms in the EW and IA subjects within the EDSC study, in those with and without CHD.

5.4.4 Correlations between sTm assays

A strong positive correlation (Pearson's) was observed between sTm ag level determined IH and using the commercially available assay ($r=0.734$, $p<0.001$, $n=492$); see Table 5.5. Strong correlations were also observed between sTm cofactor act and both DS sTm ag ($r=0.816$, $p<0.001$, $n=553$) and IH sTm ag levels ($r=0.702$, $p<0.001$, $n=550$).

	IH sTm act* (SEq/ml)	IH sTm ag* (SEq/ml)	DS sTm ag* (ng/ml)
IH sTm act* (SEq/ml)	-	r=0.702 p<0.001 n=550	r=0.816 p<0.001 n=553
IH sTm ag* (SEq/ml)	r=0.702 p<0.001 n=550	-	r=0.734 p<0.001 n=492
DS sTm ag* (ng/ml)	r=0.816 p<0.001 n=553	r=0.734 p<0.001 n=492	-

Table 5.5 Correlations between sTm measurements in the three separate assays (Pearson's test).
*Ln-transformed, †Square root-transformed.

Scattergrams are shown for the two sTm ag measurements plotted against sTm act; see Figure 5.1A&B. Twenty three subjects were identified within the lowest 5th percentile of the IH sTm act distribution. Of these, 52% (n=12/23) also fell within the lowest 5th percentile of the IH sTm ag distribution, with 35% (n=8/23) falling within the lowest 20th percentile of the IH sTm ag distribution, and the remaining 13% (n=3/23) having higher levels (>30th percentile). Results were similar when those in the lowest 5% of the IH sTm act distribution were assessed for their levels in the Ds sTm ag assay; see Figure 5.1C.

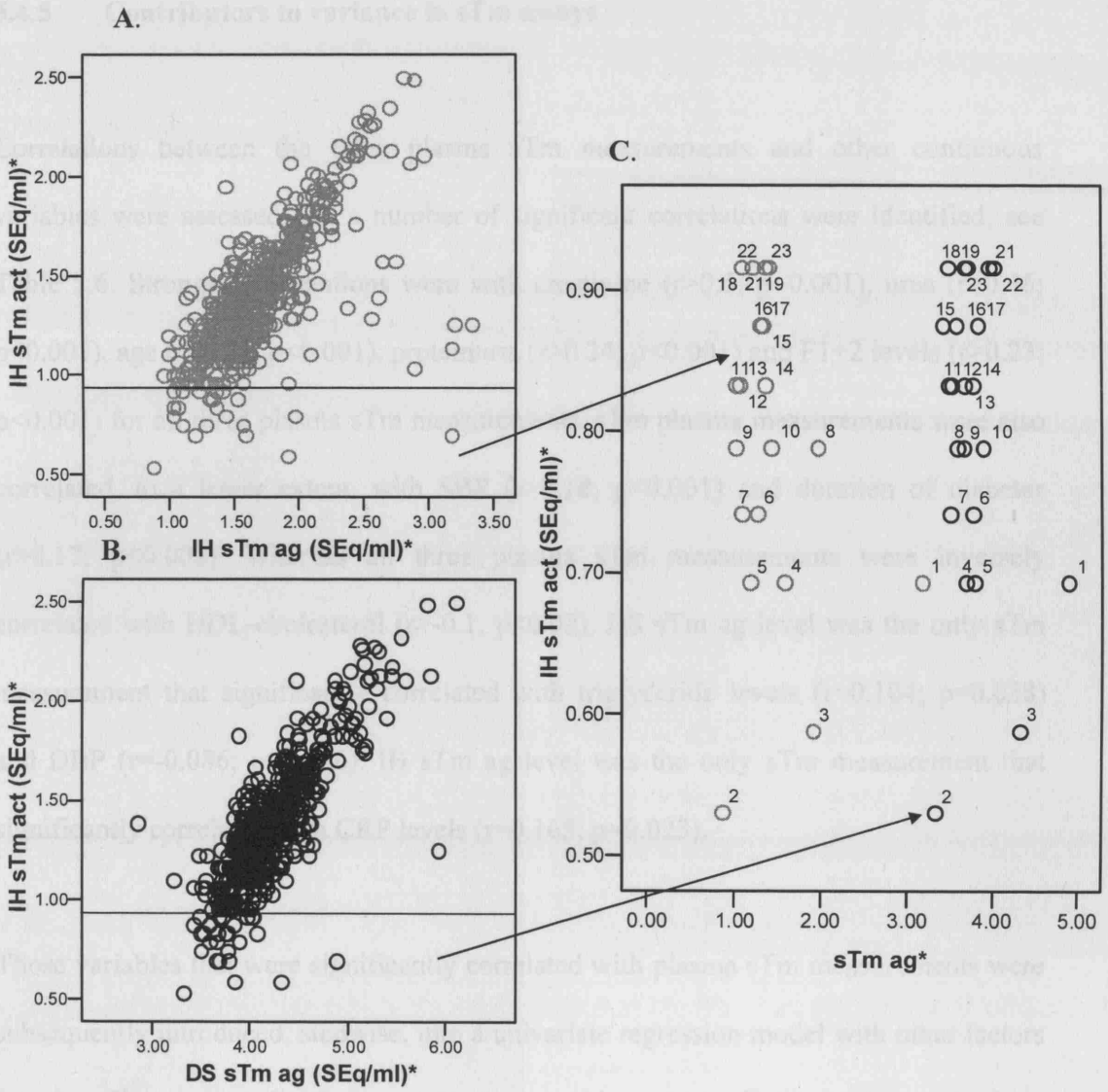


Figure 5.1 The relationship between sTm levels measured in the three separate assays.

A. IH sTm ag levels plotted against IH sTm act levels. **B**. DS sTm ag levels plotted against IH sTm act levels. **C**. The lowest 5th percentile of IH sTm act distribution plotted against IH and DS sTm ag levels. *Ln-Transformed.

5.4.5 Contributors to variance in sTm assays

Correlations between the three plasma sTm measurements and other continuous variables were assessed and a number of significant correlations were identified; see Table 5.6. Strongest correlations were with creatinine ($r>0.5$; $p<0.001$), urea ($r>0.46$; $p<0.001$), age ($r>0.25$; $p<0.001$), proteinuria ($r>0.24$; $p<0.001$) and F1+2 levels ($r>0.23$; $p<0.001$) for all three plasma sTm measurements. sTm plasma measurements were also correlated, to a lesser extent, with SBP ($r>0.18$; $p<0.001$) and duration of diabetes ($r>0.17$; $p<0.002$). Whereas all three plasma sTm measurements were inversely correlated with HDL-cholesterol ($r>-0.1$, $p<0.02$), DS sTm ag level was the only sTm measurement that significantly correlated with triglyceride levels ($r=0.104$; $p=0.038$) and DBP ($r=-0.086$; $p=0.036$). IH sTm ag level was the only sTm measurement that significantly correlated with CRP levels ($r=0.165$; $p=0.023$).

Those variables that were significantly correlated with plasma sTm measurements were subsequently introduced, stepwise, into a univariate regression model with other factors (gender, disease complications, treatments, smoking status, fasting status and ethnic group) to identify the most significant contributors to variance. Creatinine ($p<0.0001$), urea ($p<0.0001$), proteinuria ($p<0.02$) and SBP ($p<0.05$) contributed to variance in all three plasma sTm assays, Table 5.7A, B&C; HDL-cholesterol contributed a significant proportion of the variance in DS sTm ag levels ($p=0.001$), Table 5.7C; Age only contributed to variance in the IH act assay ($p=0.041$), Table 5.7A. No significant contribution to variance in the sTm assays was made by F1+2 levels or duration of diabetes, each identified as significant correlates in the initial correlation analysis.

	IH sTm act*	IH sTm ag*	DS sTm ag*
Age at recruitment (years)	**r=0.292**	**r=0.258**	**r=0.261**
	p<0.001	**p<0.001**	**p<0.001**
	n=614	**n=550**	**n=590**
Duration diabetes (years)	**r=0.177**	**r=0.181**	**r=0.229**
	p=0.001	**p<0.001**	**p<0.001**
	n=608	**n=544**	**n=584**
Proteinuria (%)	**r=0.303**	**r=0.249**	**r=0.297**
(0,1+,2+,3+)	**p<0.001**	**p<0.001**	**p<0.001**
	n=580	**n=518**	**n=556**
BMI (kg/m^2)*	r=-0.014	r=-0.002	r=0.023
	p=0.737	p=0.968	p=0.591
	n=570	n=510	n=546
Creatinine (µmol/l)†	**r=0.602**	**r=0.500**	**r=0.663**
	p<0.001	**p<0.001**	**p<0.001**
	n=598	**n=535**	**n=575**
Urea (mmol/l)*	**r=0.564**	**r=0.468**	**r=0.585**
	p<0.001	**p<0.001**	**p<0.001**
	n=598	**n=535**	**n=575**
Glucose (mmol/l)*	r=0.029	r=0.052	r=0.015
	p=0.476	p=0.232	p=0.719
	n=600	n=536	n=575
HbA1C (%)*	r=0.013	r=-0.023	r=-0.010
	p=0.747	p=0.605	p=0.807
	n=588	n=525	n=564
SBP (mmHg)*	**r=0.204**	**r=0.203**	**r=0.188**
	p<0.001	**p<0.001**	**p<0.001**
	n=606	**n=542**	**n=581**
DBP (mmHg)†	r=-0.073	r=-0.021	**r=-0.087**
	p=0.073	p=0.620	**p=0.036**
	n=606	n=542	**n=581**
Triglycerides (mmol/l)*	r=0.052	r=-0.030	**r=0.090**
	p=0.221	p=0.500	**p=0.038**
	n=560	n=501	**n=538**
Total cholesterol (mmol/l)	r=0.012	r=-0.003	r=-0.023
	p=0.761	p=0.945	p=0.583
	n=601	n=516	n=576
LDL-cholesterol (mmol/l)†	r=-0.001	r=0.011	r=-0.060
	p=0.983	p=0.808	p=0.179
	n=524	n=472	n=502
HDL-cholesterol (mmol/l)†	**r=-0.106**	**r=-0.113**	**r=-0.193**
	p=0.012	**p=0.014**	**p<0.001**
	n=560	**n=501**	**n=538**
F1+2 (µmol/l)*	**r=0.285**	**r=0.238**	**r=0.239**
	p<0.001	**p<0.001**	**p<0.001**
	n=291	**n=262**	**n=271**
CRP (pg/ml)*	r=0.040	**r=0.165**	r=0.135
	p=0.566	**p=0.023**	p=0.063
	n=205	**n=189**	n=190

Table 5.6 Correlations between sTm measurements and variables classically associated with CHD (Pearson's test).
*Ln-transformed, †Square root-transformed.

A – IH sTm act

Source	Change in Tm levels ±SE	% total variance explained	P-value
Creatinine†	0.063 ± 0.008	9.7%	<0.0001
Proteinuria	0.074 ± 0.031	3.9%	<0.0001
SBP*	0.001 ± 0.001	0.8%	0.036
HDL-cholesterol†	-0.048 ± 0.056	0.1%	0.389
Urea*	0.185 ± 0.036	4.8%	<0.0001
Age	0.002 ± 0.001	0.8%	0.041

R^2=0.464 (adjusted R^2=0.435)

Factors tested but not significant in this model: Stroke, Coronary Artery Disease, Family History of CHD, Peripheral Neuropathy, Peripheral Vascular Disease, Retinopathy, Aspirin, Blood Pressure lowering drugs, Lipid lowering drugs, Fasting, Smoking, Prothrombin F1+2*, Triglycerides*, CRP*, Duration Diabetes, Gender, Ethnic group.

B – IH sTm ag

Source	Change in Tm levels ±SE	% total variance explained	P-value
Creatinine†	0.062 ± 0.012	5.2%	<0.0001
Proteinuria	0.113 ± 0.042	2.2%	0.009
SBP*	0.001 ± 0.001	0.8%	0.048
HDL-cholesterol†	0.019 ± 0.083	0%	0.822
Urea*	0.191 ± 0.052	2.8%	<0.0001
Age	0.002 ± 0.001	0.6%	0.096

R^2=0.294 (adjusted R^2=0.253)

Factors tested but not significant in this model: Stroke, Coronary Artery Disease, Family History of CHD, Peripheral Neuropathy, Peripheral Vascular Disease, Retinopathy, Aspirin, Blood Pressure lowering drugs, Lipid lowering drugs, Fasting, Smoking, Prothrombin F1+2*, Triglycerides*, CRP*, Duration Diabetes, Gender, Ethnic group.

C – DS sTm ag

Source	Change in Tm levels ±SE	% total variance explained	P-value
Creatinine†	0.102 ± 0.011	14.5%	<0.0001
Proteinuria	0.113 ± 0.042	4.7%	<0.0001
SBP*	0.001 ± 0.001	0.8%	0.048
HDL-cholesterol†	-0.237 ± 0.072	2.1%	0.001
Urea*	0.242 ± 0.046	5.3%	<0.0001
Age	0.002± 0.001	0.3%	0.185

R^2=0.515 (adjusted R^2=0.486)

Factors tested but not significant in this model: Stroke, Coronary Artery Disease, Family History of CHD, Peripheral Neuropathy, Peripheral Vascular Disease, Retinopathy, Aspirin, Blood Pressure lowering drugs, Lipid lowering drugs, Fasting, Smoking, Prothrombin F1+2*, Triglycerides*, CRP*, Duration Diabetes, Gender, Ethnic group.

Table 5.7 Univariate regression model to identify the most significant contributors to variance in the sTm assays

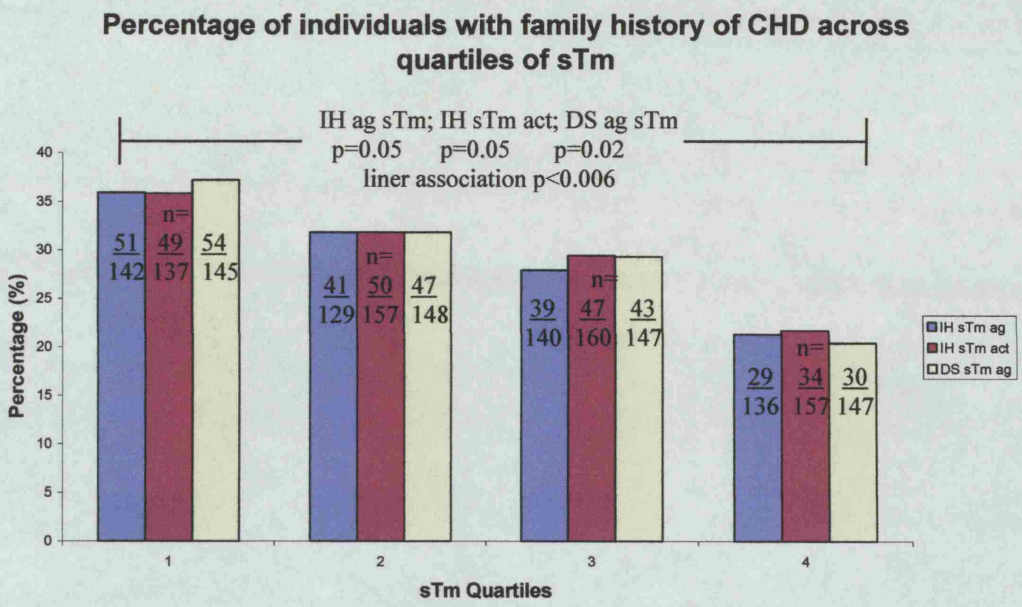
A. Dependent variable: IH sTm act levels; **B.** Dependent variable: IH sTm ag levels; **C.** Dependent variable: DS sTm ag levels. *Ln-transformed, †Square root-transformed.

5.4.6 sTm levels and CHD

Although CHD did not have a significant effect upon sTm levels overall, further analysis was performed to determine whether the frequency of individuals with either a personal or a family history of CHD differed across the sTm distribution. The number of individuals who either had CHD themselves or stated that they had members of their family with an MI or coronary surgery intervention (1-4 family members with CHD grouped together), were analysed across quartiles of the sTm distribution for each assay. Quartiles of sTm were calculated across ethnic group and gender. sTm quartiles were: *DS sTm ag quartiles* **I.** < 50.8 ng/ml, **II.** 50.9 - 61.6 ng/ml, **III.** 61.7 - 80.3 ng/ml, **IV.** >= 80.4 ng/ml; *IH sTm ag quartiles* **I.** < 4.1 SEq/ml, **II.** 4.2 – 4.9 SEq/ml, **III.** 5.0 – 6.2 SEq/ml, **IV.** >= 6.3 SEq/ml; *IH sTm act quartiles* **I.** < 3.2 SEq/ml, **II.** 3.3 – 3.7 SEq/ml, **III.** 3.8 – 4.6 SEq/ml, **IV.** >= 4.7 SEq/ml.

The highest percentage of individuals with a family history of CHD was identified in the lowest quartile for each sTm measurement (IH sTm ag 35.9%, IH sTm act 36.7%, DS sTm 37.2%); see Figure 5.2A. This percentage decreased across the quartiles for both act and ag levels, being lowest in the 4th quartile (IH sTm ag 21.6%, IH sTm act 22.1%, DS sTm ag 20.4%) and was significant by chi-squared analysis ($p<0.05$) and linear association ($p=0.006$). By contrast, the percentage of individuals who had a personal history of CHD was not significantly different in the 1st compared to the 2nd quartile in all three assays (chi squared: $p>0.44$). Whilst the percentage of individuals within each quartile who had a personal history of CHD increased from the 2nd to 4th quartile for the DS sTm ag levels (chi squared: $p=0.02$; Figure 5.2B), this did not reach statistical significance for the other two assays.

A



B

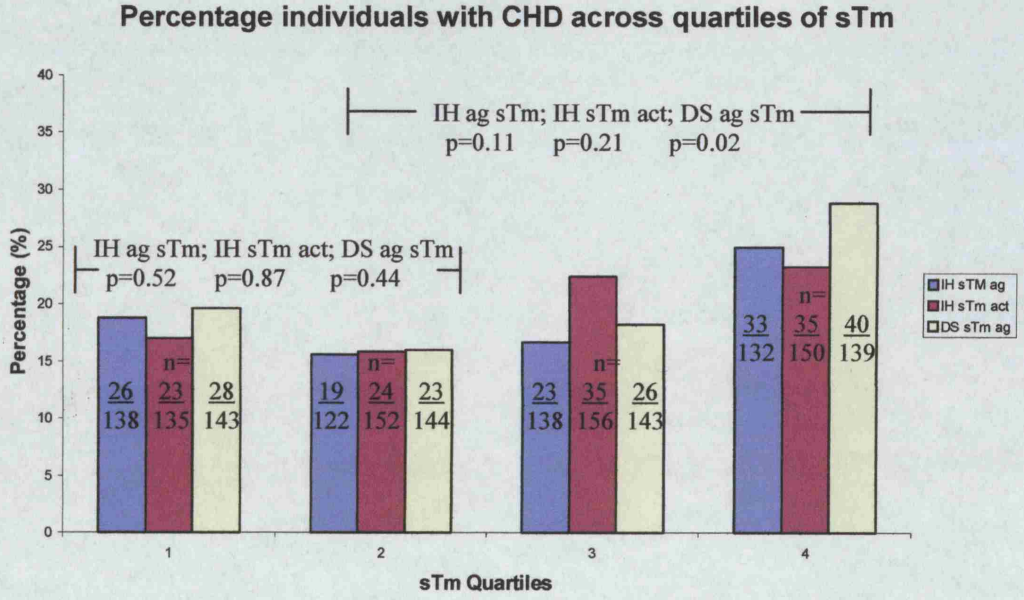


Figure 5.2 sTm levels and CHD.
A. Percentage of individuals with family history of CHD across quartiles of plasma sTm measurements; **B.** Percentage individuals with CHD across quartiles of plasma sTm measurements.

5.4.7 sTm levels and Tm genotype

Analysis of variance (ANOVA) was used to assess the association between Tm genotype (-1208/09TT>delTT & A455V) and sTm measurements in the three ethnic groups within EDSC. The effect of Tm genotype on sTm levels was assessed separately for each ethnic group due to the differences in rare allele frequency, for both -1208/09TT>delTT and A455V, observed between the three ethnic groups. Due to the low numbers of rare homozygotes, these were grouped with heterozygotes for analysis. Reduced numbers may appear following adjustment for statistical analysis, as data were not available for every variable in all patients.

There was no evidence to suggest a reduction in any of the sTm plasma measurements by either -1208/09TT>delTT or A455V genotype in any of the ethnic groups; see Tables 5.8 & 5.9. Similarly, when the association between the combined Tm genotype (V/delTT) and sTm levels was assessed in the three ethnic groups within EDSC, geometric mean levels did not differ significantly in patients heterozygous and homozygous for the rare allele at both sites, compared to those homozygous for the common allele at both sites; see Table 5.10. Numbers were too small to analyse in cases and controls separately.

Ethnicity		-1208/09TT>delTT		P-value
		11	12+22	
EW	IH sTm act (SEq/ml)*	4.0 ± 1.2 n=165	3.7 ± 1.2 n=44	0.236
	IH sTm ag (SEq/ml)*	5.2 ± 1.8 n=146	5.4 ± 2.3 n=40	0.586
	DS sTm ag (ng/ml)*	68.2 ± 25.2 n=169	67.2 ± 27.6 n=44	0.833
IA	IH sTm act (SEq/ml)*	3.9 ± 1.1 n=232	3.9 ± 1.4 n=94	0.826
	IH sTm ag (SEq/ml)*	5.1 ± 1.7 n=205	5.5 ± 2.4 n=87	0.157
	DS sTm ag (ng/ml)*	66.5 ± 26.7 n=217	71.8 ± 37.7 n=81	0.158
AC	IH sTm act (SEq/ml)*	3.7 ± 1.1 n=45	3.7 ± 0.8 n=7	0.856
	IH sTm ag (SEq/ml)*	5.3 ± 2.5 n=41	5.2 ± 1.7 n=7	0.999
	DS sTm ag (ng/ml)*	55.2 ± 19.6 n=45	58.7 ± 17.2 n=7	0.648

Table 5.8 sTm measurements by -1208/09TT>delTT genotype in EW, IA and AC subjects within the EDSC study.
Geometric mean ± approximate SD shown. *Ln-transformed. **-1208/09TT>delTT**: **TT/TT** (11); **TT/delTT** (12); **delTT/delTT** (22).

Ethnicity		A455V		P-value
		11	12+22	
EW	IH sTm act (SEq/ml)*	4.0 ± 1.2 n=132	3.7 ± 1.2 n=77	0.08
	IH sTm ag (SEq/ml)*	5.3 ± 1.9 n=116	5.2 ± 2.0 n=70	0.601
	DS sTm ag (ng/ml)*	70.1 ± 26.1 n=132	64.7 ± 24.6 n=81	0.131
IA	IH sTm act (SEq/ml)*	3.9 ± 1.1 n=205	4.0 ± 1.4 n=120	0.545
	IH sTm ag (SEq/ml)*	5.1 ± 1.7 n=180	5.4 ± 2.3 n=111	0.280
	DS sTm ag (ng/ml)*	66.5 ± 26.0 n=192	60.1 ± 36.2 n=106	0.324
AC	IH sTm act (SEq/ml)*	3.7 ± 1.1 n=42	3.9 ± 0.8 n=7	0.678
	IH sTm ag (SEq/ml)*	5.5 ± 2.6 n=38	5.2 ± 1.7 n=7	0.809
	DS sTm ag (ng/ml)*	56.1 ± 20.3 n=42	60.7 ± 17.2 n=7	0.582

Table 5.9 sTm measurements by A455V genotype in EW, IA and AC subjects within the EDSC study.
Geometric mean ± approximate SD shown. *Ln-transformed. **A455V**: **AA** (11); **AV** (12); **VV** (22).

Ethnicity		Tm Genotype		P-value
		AA/TT	V+/delTT+	
EW	IH sTm act (SEq/ml)*	4.0 ± 1.2 n=132	3.7 ± 1.2 n=44	0.132
	IH sTm ag (SEq/ml)*	5.3 ± 1.9 n=116	5.4 ± 2.3 n=40	0.805
	DS sTm ag (ng/ml)*	70.1 ± 26.1 n=132	67.2 ± 27.6 n=44	0.536
IA	IH sTm act (SEq/ml)*	3.9 ± 1.1 n=205	3.9 ± 1.4 n=94	0.747
	IH sTm ag (SEq/ml)*	5.1 ± 1.7 n=180	5.5 ± 2.4 n=87	0.180
	DS sTm ag (ng/ml)*	66.0 ± 25.1 n=191	70.7 ± 36.1 n=80	0.221
AC	IH sTm act (SEq/ml)*	3.7 ± 1.1 n=42	3.7 ± 0.8 n=9	0.994
	IH sTm ag (SEq/ml)*	5.5 ± 2.6 n=38	4.9 ± 1.6 n=9	0.785
	DS sTm ag (ng/ml)*	56.1 ± 20.3 n=42	54.4 ± 15.5 n=8	0.694

Table 5.10 sTm measurements by Tm genotype in EW, IA and AC subjects within the EDSC study.
Geometric mean ± approximate SD shown. *Ln-transformed. **AA/TT** = homozygous for both common alleles. **V+/delTT+** = heterozygous or homozygous for both rare alleles.

Although the combined Tm genotype (V/delTT) did not have a significant effect upon sTm levels overall, further analysis was performed to determine whether the frequency of individuals who were heterozygous and homozygous for the rare allele at both sites differed in those subjects within the lowest 5th percentile of the sTm distribution for each assay, compared to those in the the remaining 95 percentiles. The percentage of individuals who were heterozygous and homozygous for both rare alleles was higher in those within the lowest 5th percentile of the sTm sTm IH act distribution (47.8%), compared to those in the the remaining 95 percentiles (26.7%) (chi-squared p=0.03). By contrast, in the ag assays, the percentage of individuals who were heterozygous and homozygous for the rare allele at both sites did not differ significantly in the lowest 5th percentile of the distribution (sTm IH ag: 24%; sTm DS ag: 22%) compared to the remaining 95 percentiles (sTm IH ag:28.4%; sTm DS ag: 26.6%) (chi squared: p>0.14).

5.5 Discussion

In this section, circulating levels of sTm ag and act were measured in a large cohort of patients with type 2 diabetes, to determine contributors to sTm variance, and the impact of these three assays by a personal or family history of CHD and by Tm genotype. Although numerous studies have examined plasma sTm concentrations in type 2 diabetic patients, numbers have been relatively small (Oida et al., 1990) (Shimano et al., 1994) (Inukai et al., 1996) (Aso et al., 1998) (Aso et al., 2000) (Aso et al., 2001) (Uehara et al., 2001). Furthermore, the association between act and ag has not, to date, been assessed in a large cohort of patients with type 2 diabetes.

Although the two assays employed to measure circulating levels of sTm ag use different immobilizing and tagging antibodies, there was a strong correlations between them. Similarly, there were strong correlations between act and ag measurements. As aforementioned (see section 5.1) sTm fragments isolated from plasma are known to possess activities ranging from full Tm cofactor activity to zero (Ishii et al., 1990) (Hosaka et al., 1998) (Takahashi et al., 1998) (Uehara et al., 2001). In addition, sTm molecules have been shown to possess anti-inflammatory properties (Uchiba et al., 1996) (Conway et al., 2002) (Abeyama et al., 2005). As such, they may provide some degree of protection against atherosclerosis and it complications. The development of an assay to measure sTm act in plasma may help elucidate the clinical importance of variation in circulating sTm fragments, and may facilitate the identification and investigation of Tm mutations with impaired Tm cofactor activity. A small proportion of patients with lower sTm cofactor act than sTm ag levels was identified in this cohort. It is possible that mutations are present that alter function of the Tm protein in a few individuals. There were also individuals within this patient group who had low levels of

both act and ag. Genetic analysis of those with a lower sTm act than ag levels and low levels of both act and ag was carried out (see Chapter 6), to determine whether mutations or variants in the Tm gene, or variants in genes regulating Tm levels, contribute to reduced circulating sTm.

A number of variables, which correlated well with all three plasma sTm measurements, when introduced into a univariate regression model made a significant contribution to variance in each sTm assay. The significant correlation with creatinine, urea, proteinuria and SBP suggests that impaired renal function is an important determinant of sTm plasma levels. This is plausible, as the liver and kidneys have been identified as the major sites of plasma sTm degradation and clearance, respectively (Kumada et al., 1988) (Takano et al., 1990). An increased body of evidence has shown that chronic kidney disease is associated with a high risk of cardiovascular disease. The ARIC study showed reduced kidney function to be an independent risk factor for cardiovascular disease (Weiner et al., 2004) and the HOPE study showed renal insufficiency to be an independent predictor of cardiovascular events (including cardiovascular death, MI and stroke) (Mann et al., 2002). Furthermore, a number of studies have indicated that high serum creatinine levels may be an independent predictor of all-cause and cardiovascular disease death (Shulman et al., 1989) (Matts et al., 1993) (Luno et al., 2002). Blood pressure, elevated triglyceride levels and low HDL-cholesterol levels have been found to accompany high levels of serum creatinine, pointing to an association between components of the metabolic syndrome and chronic renal failure (Luno et al., 2002). It has subsequently been suggested that serum creatinine may act as a marker for generalized vascular disease in the kidney. Blood urea, another marker of kidney function, has also been associated with adverse outcome and has been incorporated into MI risk predictor models (Luria et al., 1976) (Normand et al., 1996). In the OPUS-TIMI

trial, elevated levels of urea were associated with increased mortality amongst patients with acute coronary syndromes, independent of other biomarkers (Kirtane et al., 2005). As such, the high degree of correlation between both serum creatinine and blood urea, and all three sTm measurements, which itself is thought to serve as a marker of endothelial cell damage (Ishii and Majerus, 1985) (Takano et al., 1990) (Ishii et al., 1991) (Borawski et al., 2001), may point to the manifestation of generalised atherosclerosis.

A trend towards increased frequency of personal CHD was observed across the last three quartiles of sTm, though this only reached statistical significance for the DS sTm ag levels. Increased levels of sTm in the plasma of subjects with established CHD is perhaps not surprising. As mentioned in section 1.3.3.1, endothelial injury, leading to compromised endothelial integrity and function, is a crucial early event in the development of atherosclerosis (Ross, 1999). It has been proposed that Tm is cleaved from the endothelial cell surface as a consequence of endothelial damage (Ishii and Majerus, 1985) (Takano et al., 1990) (Ishii et al., 1991) (Borawski et al., 2001). Furthermore, pathological cleavage, induced by pro-inflammatory cytokines, may contribute to sTm circulating in human plasma (Takano et al., 1990) (Boehme et al., 1996). A number of studies have demonstrated elevated levels of pro-inflammatory cytokines in individuals with clinically overt type 2 diabetes (Dovio and Angeli, 2001) (Spranger et al., 2003). The observed small but significant correlation, in the current study, between IH sTm ag level and CRP levels would support the suggestion that pro-inflammatory cytokines may contribute to circulating sTm through pathological cleavage. This pathological cleavage may result in elevated sTm levels, and reduced Tm on the surface of vascular endothelial cells. Although sTm might provide some degree of protection against atherosclerosis due to its proposed anti-inflammatory and anti-

coagulant properties (Uchiba et al., 1996) (Hosaka et al., 1998) (Mohri et al., 1998) (Takahashi et al., 1998) (Conway et al., 2002), the endothelial cell surface is thought to be the major physiological site for Tm function. Reduced Tm on the endothelial cell surface would seriously compromise the anti-thrombotic and anti-inflammatory potential. High levels of sTm may thus indicate an on-going pro-thrombotic or inflammatory state.

When family history of CHD was analysed across sTm quartiles of each sTm distribution, the percentage of individuals with a family history of CHD was highest in the lowest quartile for each sTm measurement. If family history of CHD had no effect upon sTm levels, 25% would be expected in each quartile. 10% more than expected were identified in the lowest sTm quartile. This apparently anomalous finding suggests that low levels of sTm may carry a risk of CHD, and may imply a genetic nature to CHD risk conferred by low sTm levels. As mentioned in section 1.3.2, a family history of early CHD is well known to be a good predictor of atherosclerosis, not completely explained by classical cardiovascular risk factors (Goldbourt and Neufeld, 1986). The observation that cardiovascular risk tends to cluster in affected families, demonstrates the importance of genetic factors in the aetiology of CHD (Grant, 2003). A previous study that evaluated the relationship between CHD and sTm in a nested case-control study, taken from a prospective study (ARIC), demonstrated an inverse association between incident CHD and circulating levels of plasma Tm (Salomaa et al., 1999). These results would extend the observations made by Salomma *et al.*, and support the suggestion that clinically significant variants may be present either within the Tm gene or other genes that influence gene expression, which in turn is reflected by plasma sTm levels.

Evidence suggests that factors acting to shed sTm from the endothelium also upregulate expression (Dittman et al., 1989) (Bartha et al., 1993) (Ma et al., 1997); see section 1.4.5. In health it would, therefore, be expected that as sTm is cleaved from the endothelium, more Tm is expressed to compensate. High levels of circulating Tm may imply an on-going pro-thrombotic or inflammatory state, while very low levels may indicate an inability for the Tm expression machinery to respond following thrombotic or inflammatory challenge. Animal models and *ex-vivo* samples from human atherosclerosis support a role for reduced endothelial Tm as risk for CHD (Weiler-Guettler et al., 1998) (Waugh et al., 1999) (Laszik et al., 2001); see section 1.4.5.

As aforementioned (see section 1.4.7.2), variants within the Tm promoter region have been shown to downregulate Tm expression (Li et al., 2000) (Nakazawa et al., 2002). A consequence of this would be reduced Tm on the surface of vascular endothelium, leading to reduced anti-thrombotic and anti-inflammatory potential. A number of variants associated with increased risk for cardiovascular diseases or identified in families with MI, have been reported in the Tm gene to date (Ireland et al., 1997) (Norlund et al., 1997a) (Doggen et al., 1998b) (Kunz et al., 2000) (Li et al., 2000) (Kunz et al., 2002) (Li et al., 2002). Few studies have, however, assessed the association between sTm levels and family history of CHD or the effect of variants upon circulating Tm levels (Ohlin and Marlar, 1995) (Kunz et al., 2000) (Li et al., 2000) (Kunz et al., 2002).

Subjects within the EDSC study were genotyped for the -1208/09TT>delTT and A455V Tm variants and the effects of these variant sites upon plasma sTm levels assessed. In both EW and IA within the EDSC study, the rare allele frequency for the A455V variant was similar to that noted in the case-control (see Chapter 3) and prospective studies (see

Chapter 4), and agreed with previous reports (Norlund et al., 1997a) (Wu et al., 2001). In contrast, a lower frequency was noted for the 455V allele in AC subjects within EDSC. This result is in agreement with previously published data, where a lower 455V allele frequency was reported in healthy Black Americans compared to healthy White Americans (Wu et al., 2001). In EW and AC subjects, the rare allele frequency for the -1208/09TT>delTT polymorphism was similar to that noted in the HIFMECH (see Chapter 3) and NPHSII studies (see Chapter 4), and agreed with previous studies (Le Flem et al., 2001). In contrast, a higher frequency was noted for the -1208/09delTT allele in IA subjects within EDSC. Differences in allele frequencies between populations have been reported for other Tm promoter variants. The -33G>A variant, for example, is extremely rare in the Caucasian population, but has been identified at much higher frequency in Indian Asian, Korean and Taiwanese populations (Ireland et al., 1997) (Li et al., 2002). The observation that differing variant frequencies occur in different populations highlights the importance of stratifying by population. The similar allele frequency for -1208/09delTT in EW in NPHSII and EDSC suggests no selection by diabetes status.

The -1208/09TT>delTT variant was found to be in strong LD with A455V, in all ethnic groups, in agreement with the findings in the HIFMECH (see Chapter 3) and NPHSII studies (see Chapter 4) and reports in previous studies (Le Flem et al., 2001). Due to the differences in relative allele frequency of the two variants between EW, IA and AC, the haplotype structure is likely to differ in the three ethnic groups. In IA patients, the 455V allele will occur on the -1208/09delTT allele in the majority of cases, due to the high proportion of individuals with a V/delTT allele. In AC patients, the -1208/09TT allele is likely to occur on the A455 allele in most cases, due to the low proportion of individuals with a V/TT allele. Furthermore, these findings would suggest that, unlike the situation

in EW and IA, genotyping for the A455V variant in AC will essentially reflect the V/delTT haplotype effect.

When the association between Tm genotype and sTm levels was assessed in the three different ethnic groups within EDSC, no statistically significant differences were noted in plasma sTm measurements by either -1208/09TT>delTT or A455V genotype alone in any of the ethnic groups. Similarly, there was no evidence to suggest differences in any of the sTm plasma measurements by the combined Tm genotype (V/delTT) in the three ethnic groups within EDSC. Upon further analysis, however, the percentage of individuals who were heterozygous and homozygous for both rare alleles was higher in those within the lowest 5th percentile of the sTm sTm IH act distribution, compared to those in the the remaining 95 percentiles. In the ag assays, the percentage of individuals who were heterozygous and homozygous for the rare allele at both sites did not differ significantly in the lowest 5th percentile of the distribution compared to the remaining 95 percentiles. These results may suggest that the A455V variant, which lies in the 6th EGF domain of Tm (van der Velden et al., 1991) (a region with a key role in thrombin binding and activation of protein C (Kurosawa et al., 1988) (Hayashi et al., 1990) (Tsiang et al., 1992) (Ye et al., 1992), may compromise the activity of the Tm protein. Firm conclusions cannot, however, be drawn from these results as observations are based on small numbers. As such the possibility for chance findings cannot be excluded. Analysis of large studies is required to expand the results obtained here and to investigate further the potential contribution the A455V variant makes, in the context of the V/delTT Tm haplotype, to CHD risk.

There are mixed reports for associations between polymorphic sites identified in the Tm gene and circulating sTm levels. Several studies have reported no association between

the A455V Tm variant and circulating sTm levels (Norlund et al., 1997a) (Wu et al., 2001) (Aleksic et al., 2002). In contrast, lower sTm levels were observed in CAD patients with the -33G>A mutation, whereas no difference in sTm levels were observed in control subjects with and without the -33G>A variant (Li et al., 2000). In another study, a frameshift mutation (insT 1689), which predicts an elongated protein, was identified in the Tm gene of a patient with MI and was associated with significantly reduced sTm levels in the plasma of family members carrying the mutation (Kunz et al., 2000).

In summary, these results suggest that atherosclerotic processes cause increase shedding of sTm from the endothelial surface, with high sTm levels indicating an on-going pro-thrombotic or inflammatory state. However, some individuals may not be able to react appropriately to this atherosclerotic challenge due to low expression of Tm.

CHAPTER SIX

SCREENING THE CODING REGION OF TM FOR GENETIC VARIATION BY SEQUENCING

6. Screening the coding region of Tm for genetic variation by sequencing

6.1 Introduction

As described previously (see Chapter 5), circulating levels of sTm antigen and activity were measured in a large cohort of patients with type 2 diabetes. Upon analysis, a small proportion of patients with lower sTm cofactor activity than sTm antigen levels was identified in this cohort. It is possible that mutations are present that alter function of the Tm protein in a few individuals. In addition, individuals who had low levels of both activity and antigen were also identified in this patient group.

6.2 Aim

The purpose of the work described in this section was to screen the Tm coding region for genetic variation, in those individuals (from the EDSC study; see Chapter 5) previously shown to have either lower sTm activity than antigen levels or low levels of both activity and antigen, to determine whether mutations or variants within the Tm gene contribute to reduced circulating sTm levels.

6.3 Methods

6.3.1 Screening

DNA from 44 diabetic patients (EDSC study), with lower sTm cofactor activity than sTm antigen levels or low levels of both activity and antigen (see Chapter 5), was screened for variation by DNA sequencing. The calculation of an sTm act:ag ratio

helped identify the subgroup of patients with lower sTm cofactor activity than antigen levels. Eighteen subjects who fell within the lowest 5th percentile of the sTm act:ag ratio distribution and twenty six subjects who fell within the lowest 5th percentile of the sTm antigen distributions were screened for variation within the Tm gene.

A strategy of screening of Tm gene fragments by PCR amplification and sequencing was used. The coding region (1.7 kb) of the Tm gene (spanning the sequence between +267 and +1960; nucleotides numbered from transcription start site) was screened for variants. Three overlapping fragments were amplified from patient genomic DNA. The sequence and position of the primers used for amplification, together with the length of the fragments and the annealing temperatures for each fragment have been defined in section 2.2.5. All PCR products were run on a 1% low melting point agarose (stained with EtBr; see section 2.2.4.1), with the inclusion of a DNA size marker, to make sure the correct fragment size was obtained. Examples of agarose gel electrophoresis for amplified fragments are given in Figure 6.1. Samples were subsequently purified using the GFX PCR DNA and Gel Bans Purification Kit (Amersham Biosciences (UK) Ltd. Buckinghamshire, UK), according to the manufacturer's protocol (see section 2.2.4.3). Direct sequencing was done by the chain termination sequencing method (Sanger and Coulson, 1975) using the ABI Big Dye Terminator v 3.1 Cycle Sequencing reagents and an automated sequencer type 377 XL (Applied Biosystems. CA, USA), as described in section 2.2.5. The samples were sequenced in both directions using the same primers as the ones used to generate the PCR products; see Table 2.4. Clear chromatograms were obtained for all the gene fragments sequenced.

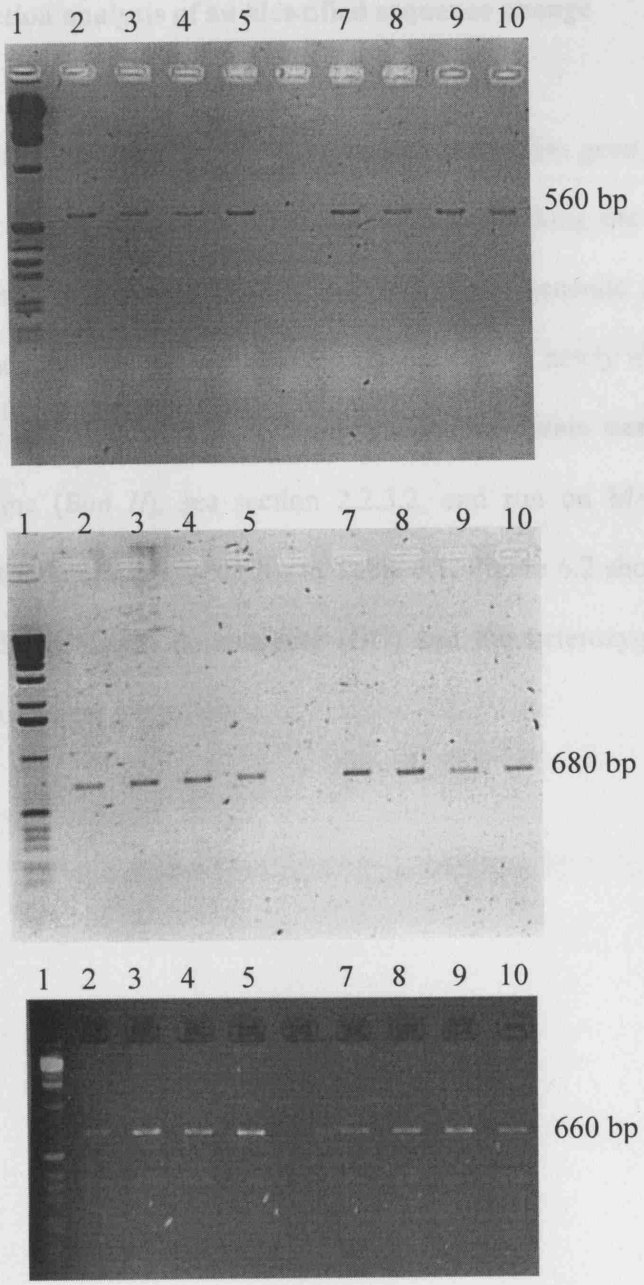


Figure 6.1 Agarose gel electrophoresis of PCR amplification products for sequencing.

Examples of a typical agarose gel electrophoresis of 10 µl of 8 amplification products for the 3 overlapping fragments used to sequence the entire 1.7 kb coding region of the Tm gene. The positions of the primers used for amplification, the length of the fragments and the annealing temperatures for each fragment have been defined in section 2.2.5. The panels show amplification from 8 different genomic DNAs (lanes 2-5 & 7-10) and a 1kb DNA size marker (lane 1). The size of the product is given next to the photograph.

6.3.2 Restriction analysis of an identified sequence change

A sequence variant identified in the coding region of the Tm gene altered a naturally occurring restriction enzyme recognition site. Primers flanking the DNA sequence of interest were designed and used in PCR amplification to generate a 250 bp fragment. The primer sequences and PCR conditions used to study the newly identified variant are shown in Table 6.1. Following PCR amplification, fragments were digested using a restriction enzyme (*Ban II*), see section 2.2.3.2, and run on MADGE, see section 2.2.4.2. The restriction profile is shown in Table 6.1. Figure 6.2 shows a representative gel indicating the wild-type homozygote (GG) and the heterozygote (GA). No rare homozygotes (AA) were identified.

Variant	Primer Pair	PCR Product (bp)	Restriction Enzyme	Restriction Pattern	
1261G>A (E364E)	5'-TTCGAGTGCCACTGCTACC-3' 5'-CTTCAGGGCACTCACAGCTA-3'	250	*Ban II* (2 U)	**G allele (bp) 194, 56**	**A allele (bp) 250**

Table 6.1 Primer pair and restriction pattern for the 1261 G>A variant.
PCR reaction: DNA was amplified as described in section 2.2.3.1. Cycling conditions consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, and a final extension of 7 min at 72°C. **Restriction digest:** 8 µl of each PCR product were added to 5 µl of digestion mix containing 2 U of enzyme and 1.3 µl of 1x appropriate restriction buffer in a final volume of 13 µl.

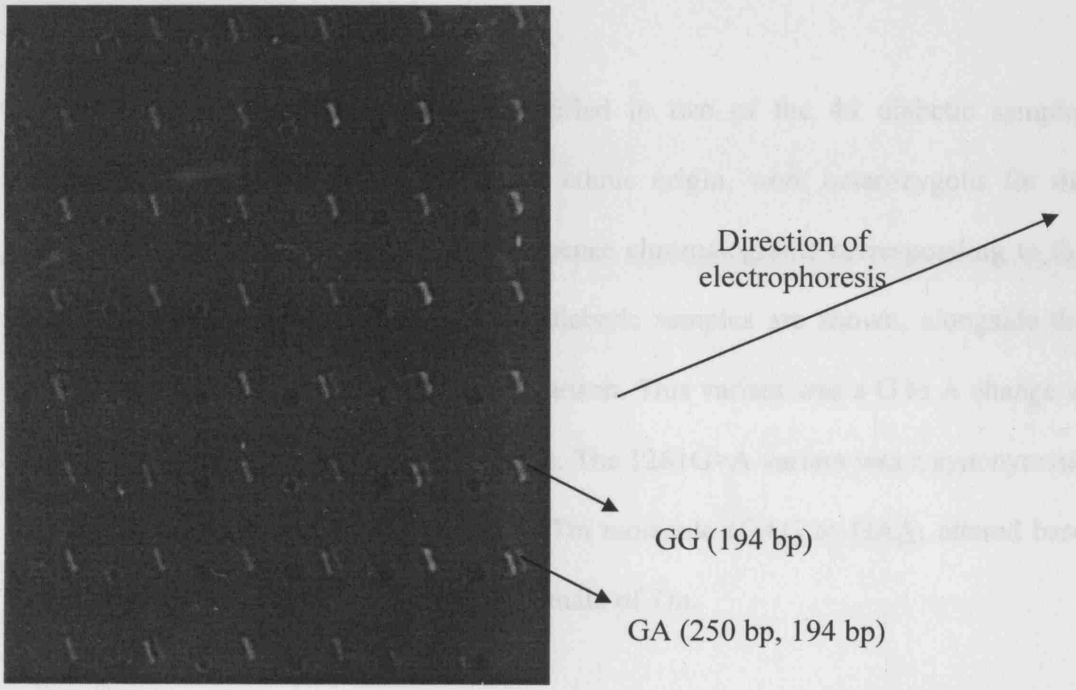


Figure 6.2 Band pattern for the 1261G>A (E346E) Tm coding region variant.
Presence of the 1261 G allele can be identified following restriction digest with *Ban II*, resulting in the production of two fragments of 194 bp and 56 bp. The latter fragment cannot be seen as it is run off the gel as a result of the small size. The 1261 A allele can be identified by the uncut fragment of 250 bp.

6.4 Results

6.4.1 Direct sequencing analysis of Tm coding region

A total of 264 fragments (3 overlapping fragments for each of the 44 DNA samples; forward and reverse primer) were sequenced. 31.8% of the diabetic subjects analysed were carrying at least one variant allele for the A455V Tm polymorphic site (n=13 heterozygotes; n=1 homozygote rare). These sequencing results were in 100% agreement with previous genotyping analysis for A455V.

Only one novel point mutation was identified in two of the 44 diabetic samples analysed. Both subjects, who were of AC ethnic origin, were heterozygous for the newly identified variant. In Figure 6.3, sequence chromatograms corresponding to the mutated region identified in two separate diabetic samples are shown, alongside the corresponding wild-type sequence for comparison. This variant was a G to A change at position 1261 from the transcription start site. The 1261G>A variant was a synonymous base change at position 364 of the mature Tm molecule (GA<u>G</u> to GA<u>A</u>; altered base underlined, Glu>Glu), within the 4th EGF domain of Tm.

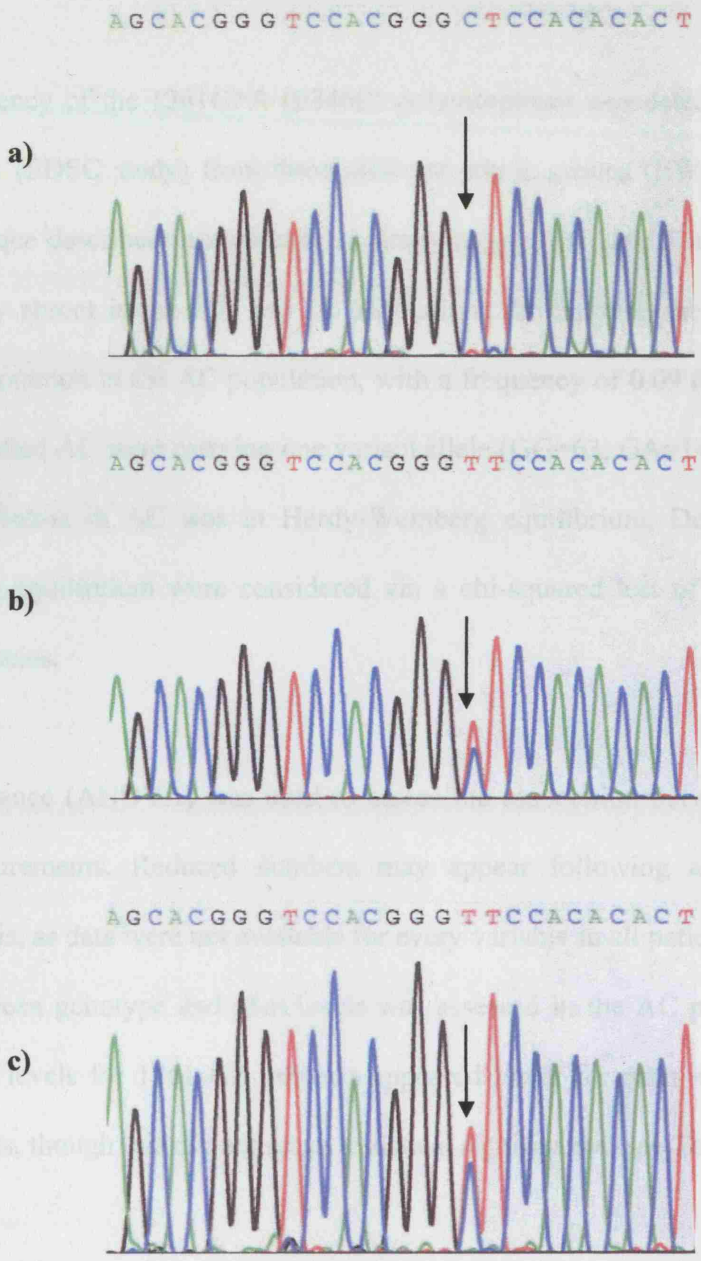


Figure 6.3 Sequencing chromatograms of normal DNA and 1261G>A.
a) Wild-type genomic DNA, **b)** Genomic DNA of 1st subject heterozygous for mutation, **c)** Genomic DNA of 2nd subject heterozygous for mutation. Sequence obtained using reverse primer presented.

6.4.2 Allele frequency of detected variant (E346E)

The allele frequency of the 1261G>A (E346E) polymorphism was determined in 100 diabetic subjects (EDSC study) from three different ethnic groups (EW, IA and AC) using the technique described in section 6.3.2. Interestingly, the 1261A allele appeared to be completely absent in the EW and IA populations. In contrast, the variant allele appeared to be common in the AC population, with a frequency of 0.09 (CI 0.07-0.11). 18.2% of the studied AC were carrying one variant allele (GG=63, GA=14, AA=0). The frequency distribution in AC was in Hardy-Weinberg equilibrium. Deviations from Hardy-Weinberg equilibrium were considered via a chi-squared test of observed and expected frequencies.

Analysis of variance (ANOVA) was used to assess the association between genotype and sTm measurements. Reduced numbers may appear following adjustment for statistical analysis, as data were not available for every variable in all patients. When the association between genotype and sTm levels was assessed in the AC population, the geometric mean levels for 1261 GA subjects appeared lower for each assay, than for 1261 GG subjects, though this did not reach statistical significance; see Table 6.2.

	1261G>A (E346E)		P-value
	GG	GA	
IH sTm act (SEq/ml)*	3.9 ± 1.2 n=39	3.3 ± 0.75 n=7	0.139
IH sTm ag (SEq/ml)*	5.5 ± 2.4 n=37	5.1 ± 3.0 n=7	0.655
DS sTm ag (ng/ml)*	58.5 ± 20.7 n=40	47.45 ± 9.0 n=7	0.188

Table 6.2 sTm measurements by 1261 G>A (E346E) genotype in AC subjects. Geometric mean ± approximate SD shown. Analysed by ANOVA after appropriate transformation. *Ln-transformed.

6.5 Discussion

In this section, the coding region of the Tm gene was sequenced to determine whether mutations or variants within the Tm gene contribute to reduced circulating sTm levels. 31.8% of the diabetic subjects analysed in this study were carrying at least one variant allele for the A455V polymorphic site (n=13 heterozygotes; n=1 homozygote rare). These sequencing results were in 100% agreement with previous genotyping analysis for A455V. The 'screening programme' involved 44 type 2 diabetic patients from the EDSC study (see Chapter 5) who were previously shown to have either a lower sTm activity than antigen level or low levels of both activity and antigen.

A single base pair change was identified in two of the 44 diabetic samples analysed. The variant occurred in two subjects of AC origin. The novel point mutation identified was a G to A substitution at position 1261 (from the transcription start site). This novel variant alters the codon at position 364 of the mature Tm molecule (GA<u>G</u> to GA<u>A</u>; altered base underlined), which is located in the 4th EGF domain of Tm, a region known to play a key role in the binding of thrombin and activation of protein C (Hayashi et al., 1990) (Tsiang et al., 1992). Despite the fact that the 1261G>A variant alters a codon in a functionally important region of the Tm gene, it does not alter the amino acid, since both GAG and GAA code for the amino acid glutamic acid. Many codons are redundant, meaning that two or more codons code for the same amino acid. Degenerate codons usually differ in their third position. The degeneracy of the genetic code accounts for the existence of silent mutations. The silent G to A substitution at nucleotide position 1261 is unlikely to affect the function of the final protein product directly, but it could be in LD with another variant site of functional consequence.

The frequency of the 1261G>A (E346E) polymorphism was determined in 100 diabetic subjects (EDSC study) from three different ethnic groups (EW, IA and AC). Interestingly, the 1261A allele appeared to be absent in the EW and IA populations investigated, which would suggest that in EW and IA subjects, the prevalence of the 1261G>A polymorphism in the Tm gene, if any, must be extremely low. In contrast, the variant allele was relatively common in the AC population (rare allele frequency 0.09). Differences in allele frequencies between populations have been reported for other Tm gene variants identified to date, as previously discussed (Ireland et al., 1997) (Wu et al., 2001) (Li et al., 2002); see section 1.4.7.2.

When the association between genotype and sTm levels was assessed in the AC population, the mean levels for AC patients heterozygous for Tm 1261G>A appeared lower for each assay, compared to those homozygous for the common allele, though this did not reach statistical significance. As aforementioned (see Chapter 5), there are mixed reports for associations between Tm variants and circulating sTm levels (Norlund et al., 1997a) (Kunz et al., 2000) (Li et al., 2000) (Wu et al., 2001) (Aleksic et al., 2002).

The newly identified 1261G>A Tm variant is unlikely to be having a direct impact on protein function, as it does alter the final amino acid (E346E). In addition, this variant site appears to be unique to subjects of AC origin, with a very low prevalence expected in EW and IA populations. Finally, there was no evidence to suggest that this variant site contributes to altered circulating sTm levels although it should be noted that there were small numbers of heterozygotes for 1261G>A (n=7). Analysis of a large cohort would be useful to confirm this, particularly as significantly lower levels were identified overall for AC compared to EW and IA patients; see Table 5.1.

CHAPTER SEVEN

IN VITRO REPORTER GENE ANALYSIS DIRECTED TO EXPLAINING THE EFFECT OF TM PROMOTER VARIANTS ON CHD: EXPRESSION UNDER BASAL AND SUPPRESSED CONDITIONS

7. *In vitro* reporter gene analysis directed to explaining the effect of Tm promoter variants on CHD: expression under basal and suppressed conditions

7.1 Introduction

Several reports have suggested an association between variants within the proximal Tm promoter region and risk of CHD (Ireland et al., 1997) (Li et al., 2000) (Li et al., 2002). These proximal promoter variants, which lie in close proximity to consensus sequences for transcriptional control elements of the Tm gene, have been shown to alter Tm gene transcriptional activity (Li et al., 2000) (Nakazawa et al., 2002). The distal promoter region of the Tm gene is also known to contain several important consensus regulatory elements including a silencer element, a putative SSRE and four retinoic acid response elements that could modulate Tm promoter activity. Potentially functional variants, which alter or lie in close proximity to these regulatory elements, have been previously identified in this region of the Tm gene (Le Flem et al., 2001), but have received relatively little attention to determine their functionality.

Contribution to risk for CHD by these Tm distal promoter variants was assessed previously; see Chapters 3 & 4. The high frequency -1748G>C polymorphism, which creates a putative Sp1-like transcription factor binding site, appeared to be neutral with regards to CHD risk in the HIFMECH case-control study; see Chapter 3. Risk associated with the -1166G>A variant site could not be assessed in the HIFMECH case-control study because numbers were too small; see Chapter 3. However, this particular site alters the core binding sequence of a putative SSRE. Exposure of endothelial cells to shear stress has been shown to alter Tm mRNA and antigen levels (Malek et al.,

1994) (Takada et al., 1994), which may be mediated by this SSRE. As such, the -1166G>A variant site is a strong candidate for modulating risk for CHD, but would require extremely large cohorts to test it. A haplotype comprising the rare alleles for two common Tm variants (-1208/09TT>delTT & A455V) contributed to the risk of MI, in the North of Europe (HIFMECH), particularly in individuals with obesity, metabolic syndrome or inflammation; see Chapter 3. These findings were subsequently extended in a large prospective study (NPHSII), where a trend towards increased CHD risk was observed in those with increased BMI and a significant risk interaction also with increased triglyceride levels was identified; see Chapter 4. An associated increased inflammatory response was also noted for V/delTT carriers in both studies. Tm -1208/09TT>delTT lies between a DR4 sequence (-1531 to –1516) and SSRE (-1163 to -1168); see sections 1.4.4 & 1.4.7. As such, it is possible that the deletion variant disrupts nuclear protein binding.

The mechanism through which Tm genotype contributes to CHD risk may be through an interaction with mediators of metabolic syndrome. As mentioned in section 1.3.2, the metabolic syndrome is characterised by a clustering of insulin resistance with established cardiovascular risk factors, such as increased triglyceride levels, increased SBP, decreased HDL-cholesterol levels, obesity and hyperglycemia (Kaplan, 1989) (Haffner et al., 1992). Obese individuals have a higher risk of developing CHD compared to lean individuals (Lamarche, 1998) (Stevens et al., 1998) and there is increasing evidence that adipose tissue in general and abdominal adiposity in particular, is a key player in the inflammatory process (Yudkin, 1999). Adipose tissue has been demonstrated to secrete pro-inflammatory cytokines (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999). Inflammatory mediators contribute to progression of atherosclerosis by several mechanisms; see section 1.3.3.1. In relation

to haemostasis, they have been shown to stimulate TF expression (responsible for the initiation of the coagulation cascade) and to cause natural anti-coagulant and fibrinolytic deregulation (Nawroth et al., 1986) (Moore et al., 1987) (Gregory et al., 1989) (Esmon et al., 1991) (Nemerson, 1995) (Orbe et al., 1999) (Speiser et al., 2001). In this context, inflammatory mediators have been shown to have a profound effect on Tm expression; see section 1.4.5. Furthermore, pathological cleavage, induced by pro-inflammatory cytokines, may contribute to sTm circulating in human plasma (Takano et al., 1990) (Boehme et al., 1996).

In this section, previously identified potentially functional variants in the distal promoter region of the Tm gene were assessed for their effect upon the promoter driving expression of a reporter gene. Basal and suppressed conditions (TNF-α & IL-1β) were evaluated using a dual *luciferase* reporter gene assay.

Previous reporter gene analyses of the Tm gene have assessed only small sections of the promoter region. A large stretch of the Tm promoter was studied in an attempt to keep the system as physiological as possible. As such a 2056 bp Tm promoter fragment which contains both the distal and proximal promoter regions as they occur in the gene, and including a silencer element removed in other published reporter gene studies, was cloned into the pGL-3-Basic firefly *luciferase* reporter vector (Promega Co. Madison, WI, USA).

7.2 Aim

The purpose of the work described in this section was to determine the strength of the Tm promoter in driving gene expression under basal conditions and in response to biochemical agonists (TNF-α & IL-1β), and to determine whether variants within the Tm promoter alter basal and suppressed gene expression.

7.3 Methods

7.3.1 RT-PCR

Mammalian cells (HUVECs & EA.hy 926) were grown in 12-well plates at 37°C, 5% CO_2 in a humidified incubator; see section 2.3.1. Total RNA was extracted from endothelial cells using TRIzol (Gibco-BRL Ltd, Paisley, UK); see section 2.4.6. Following extraction, RNA was DNase digested (see section 2.4.7), and reverse transcribed using SUPERSCRIPT II reverse transcriptase and random hexamer primers (Gibco-BRL Ltd, Paisley, UK); see section 2.4.8. 100-150 ng cDNA was amplified by PCR using Tm and GAPDH specific primers; see section 2.4.8.1. PCR products were run on an agarose gel, visualised under UV light and photographed using a Syngene Trans-illuminator and the Gene-Snap Syngene software (Syngene, Cambridge, UK); see section 2.2.4.1.

7.3.2 Cloning and mutagenesis

A fragment (2056 bp) containing the 5' UTR of the Tm gene was amplified by PCR from genomic DNA from an individual homozygous for the common alleles at all variant sites (-1748G>C, -1208/09TT>delTT & -1166G>A); see section 2.4.1. The fragment, shown in Figure 2.9, was cloned into the pGL-3-Basic firefly *luciferase* reporter vector (Promega Co. Madison, WI, USA); see section 2.2.6.1.

Constructs containing the desired single point mutations were generated by site-directed mutagenesis using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, UK); see section 2.4.2. Large scale preparation of plasmid DNA was done using the Gene Elute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich Company Ltd. Dorset, UK) following transformation into *E.Coli* XL1-Blue competent cells (Stratagene. CA, USA); see sections 2.2.2.2 and 2.2.6.2, respectively. All clones were confirmed by automated sequencing using an ABI PRISM 377 sequencer, as described in section 2.2.5. The primers used to sequence the Tm promoter constructs are listed in Table 2.6.

7.3.3 Assay of promoter activity

Tm promoter constructs were transfected into HUVECs or EA.hy926 cells grown in 96-well plates using the Lipofectamine™ 2000 method (Gibco-BRL Ltd, Paisley, UK); see section 2.4.3. Promoter activity was assayed using a dual *luciferase* assay kit (Promega Co. Madison, WI, USA), as described in section 2.4.5. Tm promoter activity was assessed under basal conditions and in response to the inflammatory cytokines TNF-α and IL-1β; see sections 2.4.3 and 2.4.4. Wild-type and mutant constructs were assessed in quadruplicate and the experiment repeated five times. The activity of the mutant

allele containing constructs relative to the activity of the co-transfected *Renilla* construct is given in appendices 3-5. Promoter activity was taken as the ratio of firefly luminescence to *Renilla* luminescence, thus adjusting for transfection efficiency. This normalised ratio was then averaged to give a mean ratio for each construct. The mean of each construct was then normalised against the pGL-3-Basic reading and expressed relative to the value obtained for the wild-type construct under basal conditions; see section 2.4.5.

Initially, a range of agonist concentrations and times were used, to determine the optimum experimental conditions, see below (results 7.4).

i) Dose response

Following transfection of exponentially growing EA.hy 926 cells (see section 2.4.3), the serum free medium was removed and cells incubated with normal growth medium (EBM supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml BBE, and 500 µl Gentamicin sulphate amphotericin-B) for 24 hours to allow cells to recover before treatment. Transfected cells were subsequently treated by incubating them with normal growth medium supplemented with TNF-α (0 to 2 ng/ml) or IL-1β (0 to 1 ng/ml) for 6 hours. The experiment was performed in quadruplicate and performed on two different occasions. Reporter gene activity was measured using the *luciferase* assay; see section 2.4.5. Promoter activity was taken as the ratio of firefly luminescence to *Renilla* luminescence, thus adjusting for transfection efficiency (appendix 1). This normalised ratio was then averaged to give a mean ratio. The calculated values for the treated constructs were then expressed relative to the value obtained for the wild-type construct under basal conditions.

ii) Time course

Following transfection of exponentially growing EA.hy 926 cells (see section 2.4.3) the serum free medium was removed and cells incubated with normal growth medium (EBM supplemented with 10% FBS, 500 µl human recombinant epidermal growth factor, 2 ml BBE, and 500 µl Gentamicin sulphate amphotericin-B) for 24 hours to allow cells to recover prior to being treated. Transfected cells were subsequently treated by incubating them with normal growth medium supplemented with 2 ng/ml TNF-α or 1 ng/ml IL-1β for 0, 3, 6, 15 and 24 hours. This experiment was performed in duplicate and repeated two times. Reporter gene activity was measured using the *luciferase* assay; see section 2.4.5. Promoter activity was taken as the ratio of firefly luminescence to *Renilla* luminescence, thus adjusting for transfection efficiency (appendix 2). This normalised ratio was then averaged to give a mean ratio. The calculated values for the treated constructs were then expressed relative to the value obtained for the wild-type construct under basal conditions.

7.3.4 Statistical analysis

Data were entered into an EXCEL spreadsheet (Microsoft). Statistical analysis was carried out by myself, using SPSS version 12.0.1. (SPSS Inc., Chicago, USA). One sample t-Test was performed to compare differences in relative promoter activity by genotype under basal conditions. A paired sample t-Test was used to compare differences in relative promoter activity by genotype in response to the biochemical agonists. P-values <0.05 were considered as significant.

7.4 Results

7.4.1 Tm mRNA production by mammalian cells (HUVECs & EA.hy 926)

The RT-PCR method was used to investigate Tm mRNA production by the mammalian cells (HUVECs & EA.hy 926) utilised in the *in vitro* reporter gene analysis. Both HUVECs and EA.hy926 cells have previously been shown to express Tm (Yu et al., 1992), and this was confirmed in this experiment. For GAPDH, the primers were designed to span an intron. This allows confirmation of mRNA without contaminating DNA. As shown in Figure 7.1, the genomic DNA control gave a larger band due to the presence of an intron, spliced out in mRNA. This was not possible for Tm as this gene is intronless (Jackman et al., 1987). Thus mRNA and a genomic DNA gave the same size bands. As an added control step, primers complementary to DNA sequences within the promoter region of the Tm gene were incorporated in the same PCR mix to identify amplification of any contaminating genomic DNA. The resulting band (604 bp) was absent from the HUVECs and EA.hy 926 mRNA samples, as shown in Figure 7.1.

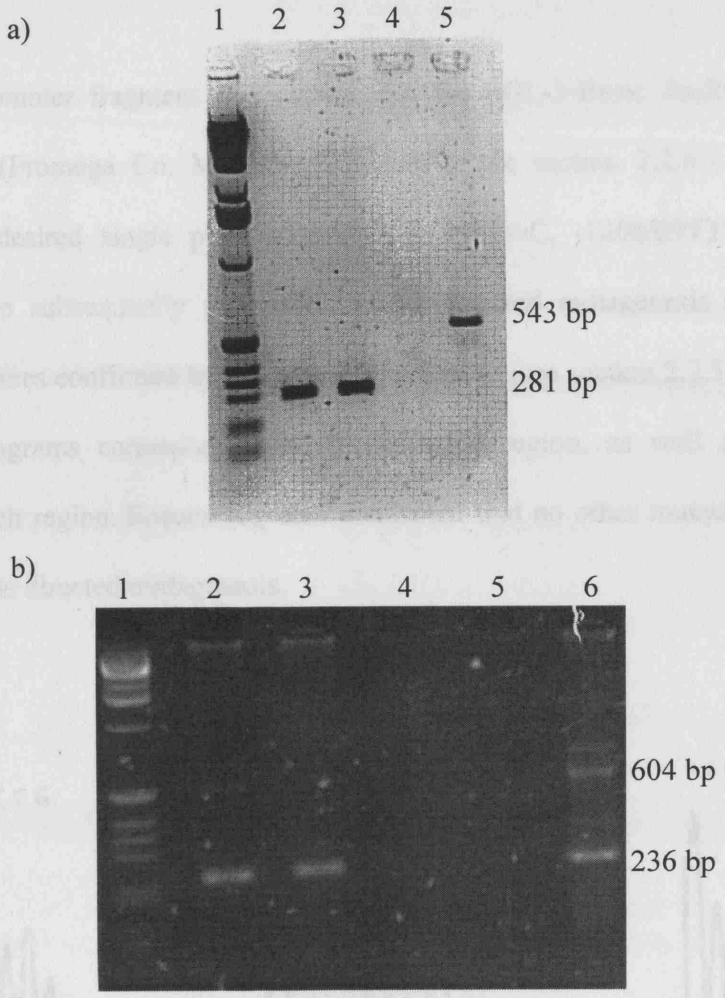


Figure 7.1 Agarose gel electrophoresis of Tm and GAPDH cDNA.
RT-PCR of total RNA extracted from HUVECs and EA.hy 926 cells was followed by the amplification of the GAPDH (panel a) and Tm (panel b) cDNA. Panel **a) GAPDH amplification**: *Lane 1*:1 kb DNA size marker, *Lane 2*: HUVEC cDNA, *Lane 3*: EA.hy 926 cDNA, *Lane 4*: -ve control, *Lane 5*: Genomic DNA. Panel **b) Tm amplification**: *Lane 1*:1 kb DNA size marker, *Lane 2*: HUVEC cDNA, *Lane 3*: EA.hy 926 cDNA, *Lane 4*: -ve control HUVEC, *Lane 5*: -ve control EA.hy 926, *Lane 6: Genomic DNA.*

7.4.2 Tm promoter-reporter gene fusion constructs

A large Tm promoter fragment was cloned into the pGL-3-Basic firefly *luciferase* reporter vector (Promega Co. Madison, WI, USA); see section 2.2.6.1. Constructs containing the desired single point mutations (-1748G>C, -1208/09TT>delTT and -1166G>A) were subsequently generated by site directed mutagenesis (see section 2.4.2) and all clones confirmed by automated sequencing (see section 2.2.5). Figure 7.2 shows chromatograms corresponding to the mutated region, as well as wild-type sequence for each region. Sequencing also confirmed that no other mutation had been introduced by site directed mutagenesis.

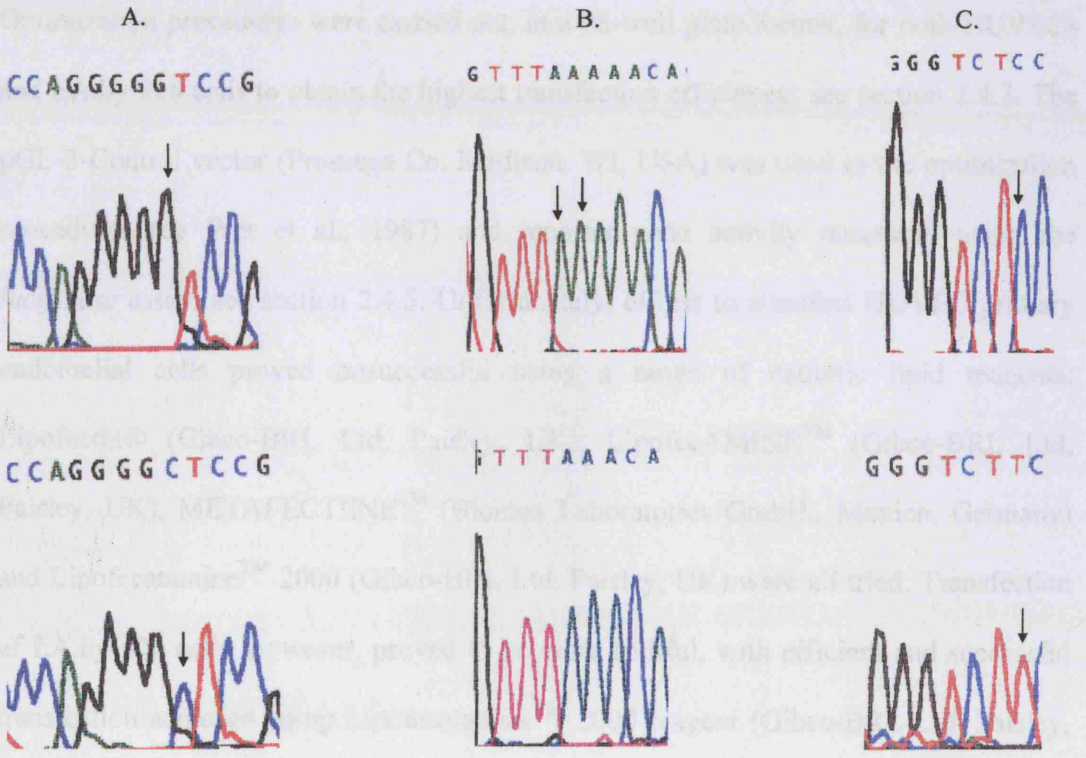


Figure 7.2 Sequencing chromatograms of mutated and wild-type promoter-reporter gene constructs.
The sequencing was performed as described in section 2.2.5 by the automated method using plasmid construct as template. For the region containing the introduced variant site (bottom sequence each panel) the corresponding wild-type region is also shown (top sequence each panel). The mutated nucleotides are indicated by arrows. Panel **A**: -1748G>C (forward primer); **B**: -1208/09TT>delTT (reverse primer); **C**: -1166G>A (reverse primer).

7.4.3 Transfection of mammalian cells (HUVECs & EA.hy 926)

Successful transfection of HUVEC and EA.hy 926 cells, using cationic lipid reagents, has previously been reported (Tazawa et al., 1993) (Le Flem et al., 1999) (Matsumura et al., 1999) (Gu et al., 2000a) (Nakazawa et al., 2002). These mammalian endothelial cells were chosen for these experiments because they have been previously used in experiments to identify key regulatory elements required for expression of the Tm gene in endothelial cells and to characterize Tm gene mutations in the proximal promoter region (Yu et al., 1992) (Tazawa et al., 1993) (Matsumura et al., 1999) (Nakazawa et al., 2002).

Optimization procedures were carried out, in a 96-well plate format, for both HUVECs and EA.hy 926 cells to obtain the highest transfection efficiency; see section 2.4.3. The pGL-3-Control vector (Promega Co. Madison, WI, USA) was used in the optimization procedures (de Wet et al., 1987) and reporter gene activity measured using the *luciferase* assay; see section 2.4.5. Unfortunately, efforts to transfect HUVEC primary endothelial cells proved unsuccessful using a range of cationic lipid reagents: Lipofectin® (Gibco-BRL Ltd, Paisley, UK), LipofecAMINE™ (Gibco-BRL Ltd, Paisley, UK), METAFECTENE™ (Biontex Laboratories GmbH., Munich, Germany) and Lipofecatamine™ 2000 (Gibco-BRL Ltd, Paisley, UK) were all tried. Transfection of EA.hy 926 cells, however, proved to be more fruitful, with efficient and successful transfection achieved using Lipofecatamine™ 2000 reagent (Gibco-BRL Ltd. Paisley, UK) at a DNA:Lipofecatamine™ 2000 reagent ratio of 1:2.5.

Once conditions that resulted in detectable reporter gene activity were established using the pGL-3-Control vector (Promega Co. Madison, WI, USA), an initial transfection experiment was carried out with the pTm WT construct. The pGL-3-Control vector (Promega Co. Madison, WI, USA) was used as a positive control, to assess the efficiency of the system. The pGL-3-Basic vector (Promega Co. Madison, WI, USA) was also used to provide a reading to which experimental firefly *luciferase* activity could be normalised. The pRL-TK *Renilla* luciferase vector was used as an internal control. Promoter activity was taken as the ratio of firefly luminescence to *Renilla* luminescence, thus adjusting for transfection efficiency. This normalised ratio was then averaged to give a mean ratio. The calculated values for constructs were then expressed relative to the value obtained for the pGL-3-Basic vector (set as 1.0). Results are shown in Figure 7.3.

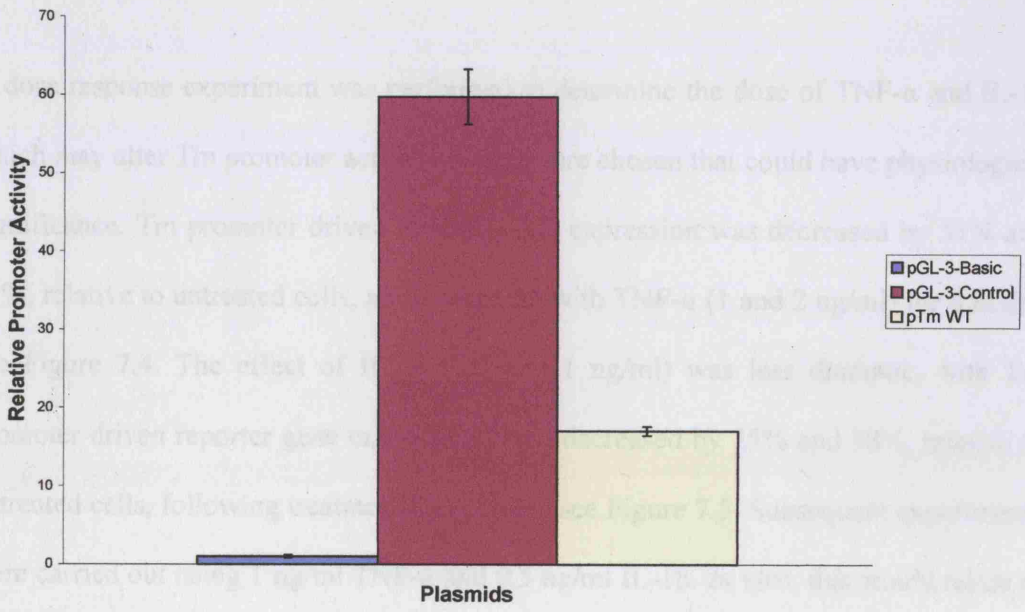


Figure 7.3 Induction of *luciferase* expression in EA.hy 926 cells.
EA.hy 926 cells were co-transfected with experimental firefly *luciferase* vectors (pGL-3-Basic, pGL-3-Control & pTm WT) and pRL-TK vector. Firefly *luciferase* activity was normalised to *Renilla* luciferase activity and expressed relative to pGL-3-Basic. Results expressed as means ± SE, n=4.

Transient transfection of the promoterless pGL-3-Basic vector (Promega Co. Madison, WI, USA) resulted in low *luciferase* activity. Highest *luciferase* activity was noted for the pGL-3-Control vector (Promega Co. Madison, WI, USA), which would be expected since the SV40 promoter/enhancer region is driving *luciferase* expression (de Wet et al., 1987). Transient transfection of the wild-type Tm construct (pTm WT) into EA.hy 926 cells resulted in an enhancement of *luciferase* activity relative to the pGL-3-Basic promoterless vector. As such detectable reporter gene activity was successfully established using the experimental Tm promoter-reporter gene fusion constructs. High basal activity for Tm promoter constructs in endothelial cells has been reported previously (von der Ahe et al., 1993).

7.4.4 Dose response curve for the pTm WT construct in relation to TNF-α and IL-1β concentration

A dose response experiment was performed to determine the dose of TNF-α and IL-1β which may alter Tm promoter activity. Doses were chosen that could have physiological significance. Tm promoter driven reporter gene expression was decreased by 51% and 74%, relative to untreated cells, after treatment with TNF-α (1 and 2 ng/ml) for 6 hours; see Figure 7.4. The effect of IL-1β (0.5 and 1 ng/ml) was less dramatic, with Tm promoter driven reporter gene expression being decreased by 25% and 38%, relative to untreated cells, following treatment for 6 hours; see Figure 7.5. Subsequent experiments were carried out using 1 ng/ml TNF-α and 0.5 ng/ml IL-1β. *In vivo*, this would relate to the plasma concentration found in a poorly controlled subject with diabetes. Doses of cytokines were chosen that did not completely inhibit wild-type Tm construct, to allow variation between the wild-type and variant constructs to be assessed.

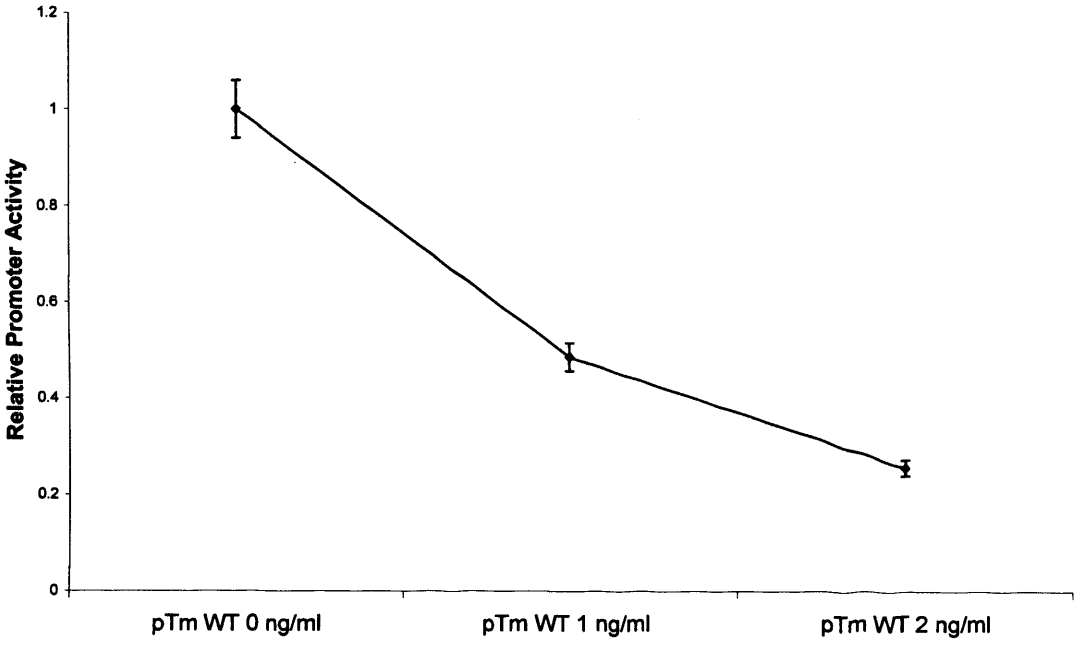


Figure 7.4 Dose response curve for pTm WT promoter activity with TNF-α concentration after 6 hour treatment.
Promoter activity is given relative to the wild-type construct under basal conditions. Results expressed as means ± SE, n=8.

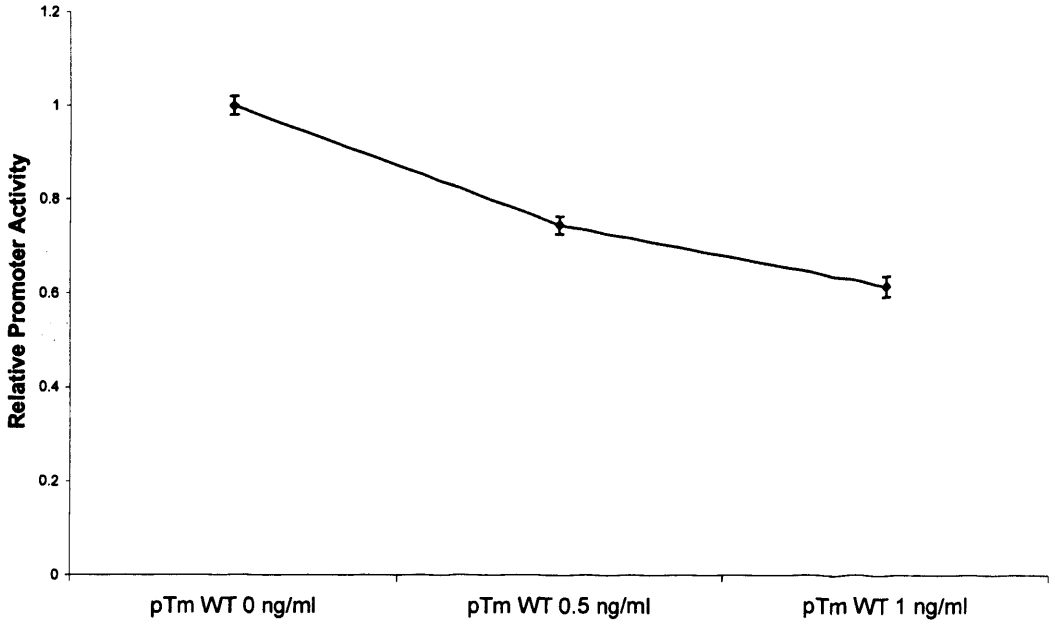


Figure 7.5 Dose response curve for pTm WT promoter activity with IL-1β concentration after 6 hour treatment.
Promoter activity is given relative to the wild-type construct under basal conditions. Results expressed as means ± SE, n=8.

7.4.5 Time course for TNF-α and IL-1β for the pTm WT construct

A time course experiment was also carried out to determine the optimum experimental conditions for TNF-α and IL-1β treatment. In response to TNF-α treatment, Tm promoter driven reporter gene expression declined to 65% of the untreated cells at 6 hours treatment and remained low until 24 hours. Maximum reduction was observed at 15 hours (25% of untreated). IL-1β treatment caused a more rapid response, being 45% of the untreated vector at 3 hours, further declining to 13% of untreated vector at 15 hours. In subsequent experiments, cells were treated for 15 hours; see Figures 7.6 and 7.7.

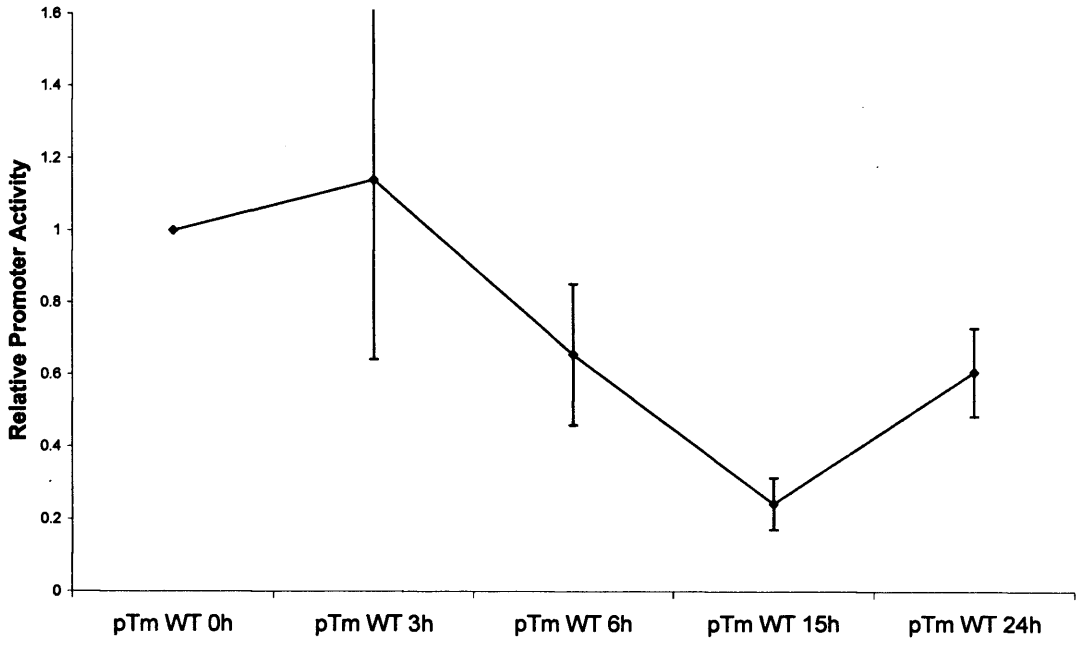


Figure 7.6 Time course for pTm WT promoter activity with 2 ng/ml TNF-α.
Promoter activity is given relative to the wild-type construct under basal conditions. Results expressed as means ± SE, n=4.

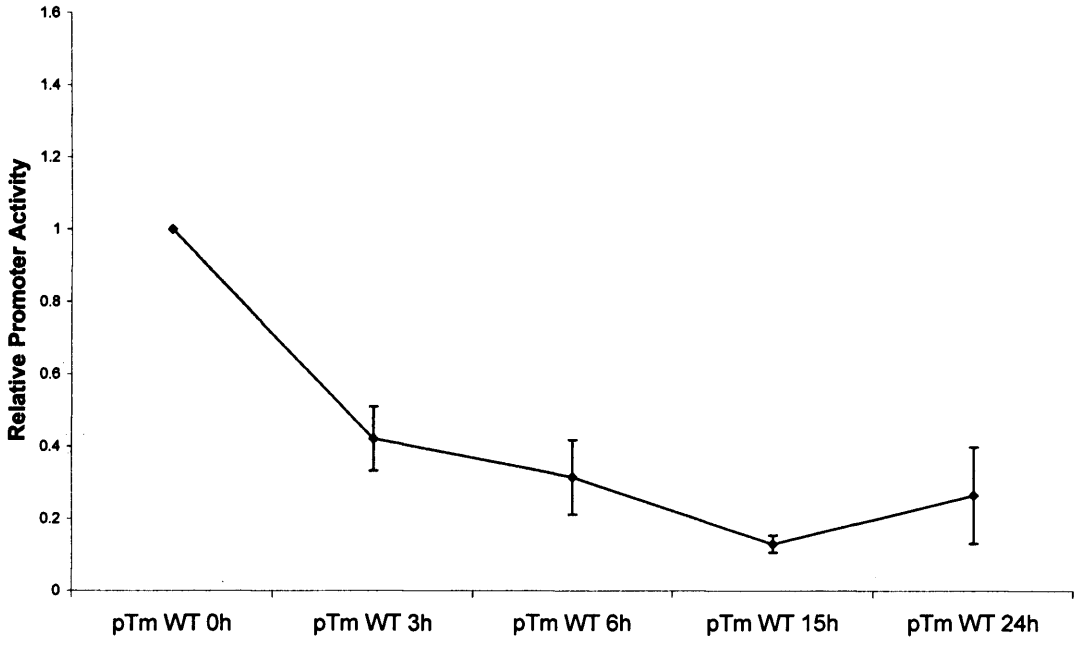
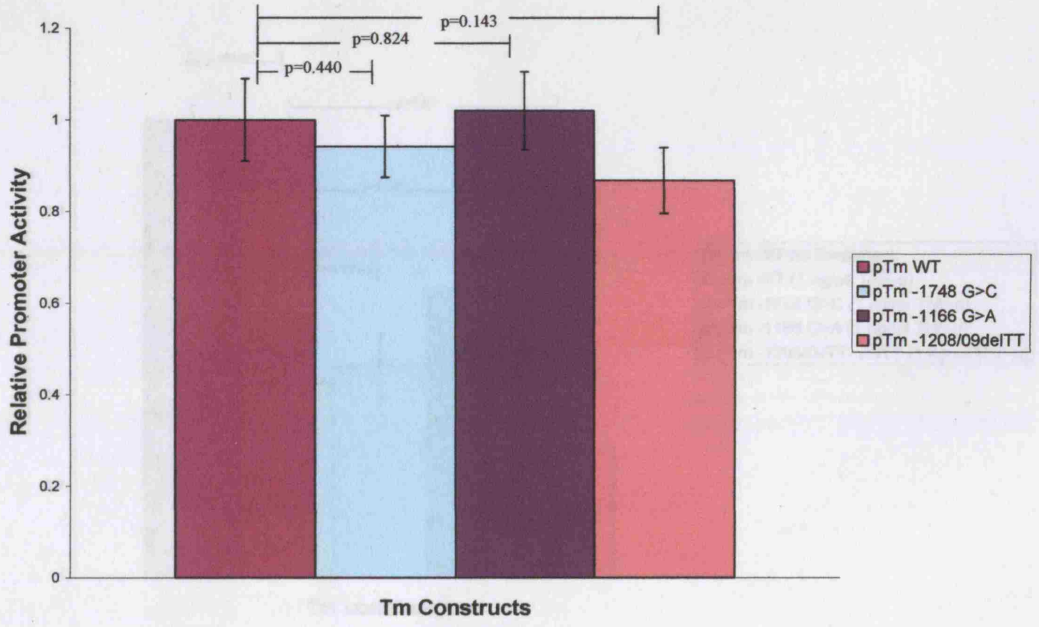


Figure 7.7 Time course for pTm WT promoter activity with 1 ng/ml IL-1β.
Promoter activity is given relative to the wild-type construct under basal conditions. Results expressed as means ± SE, n=4.

7.4.6 The effect of Tm promoter variants on reporter gene expression under basal conditions and in response to inflammatory cytokines

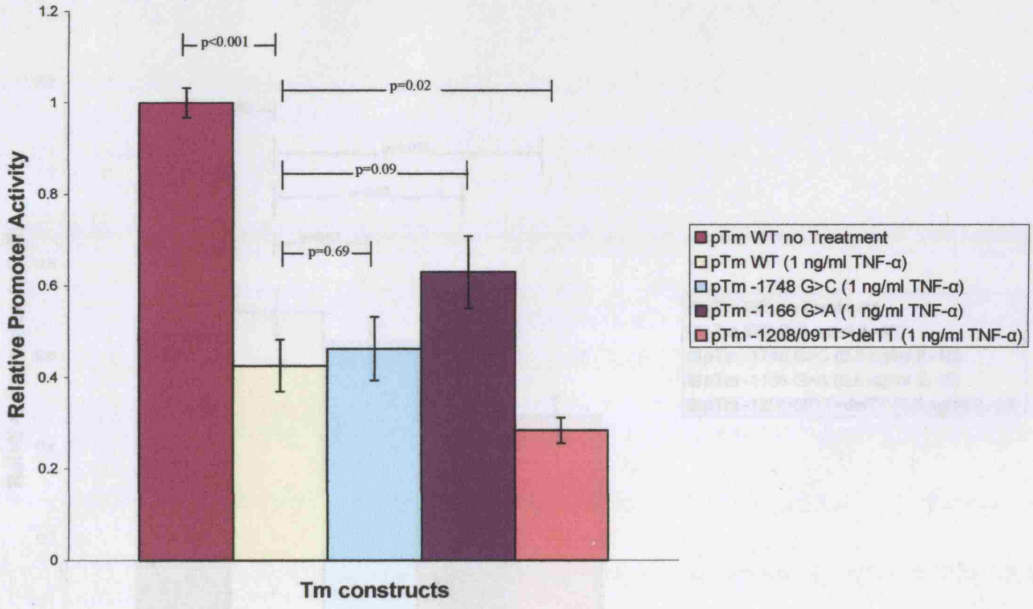
No statistically significant functional effect upon the transcriptional activity of the Tm promoter was observed for any of the distal promoter variants under basal conditions; see Figure 7.8. This work, therefore, suggests that the distal promoter variants assessed in this study are unlikely to be associated with altered Tm synthesis and expression under basal conditions.



Tm promoter variant	Relative promoter activity (% of wild-type)	P-value
-1748G>C	94 ± 6	0.440
-1166G>A	102 ± 8	0.824
-1208/09TT>delTT	87 ± 7	0.143

Figure 7.8 Relative promoter activities for pTm constructs under basal conditions. Wild-type and mutant allele containing Tm constructs were transiently transfected into EA.hy 926 cells. Promoter activity is given relative to the wild-type Tm construct (pTm WT). Results expressed as means ± SE, n=20. Statistical analysis performed by one sample t-test.

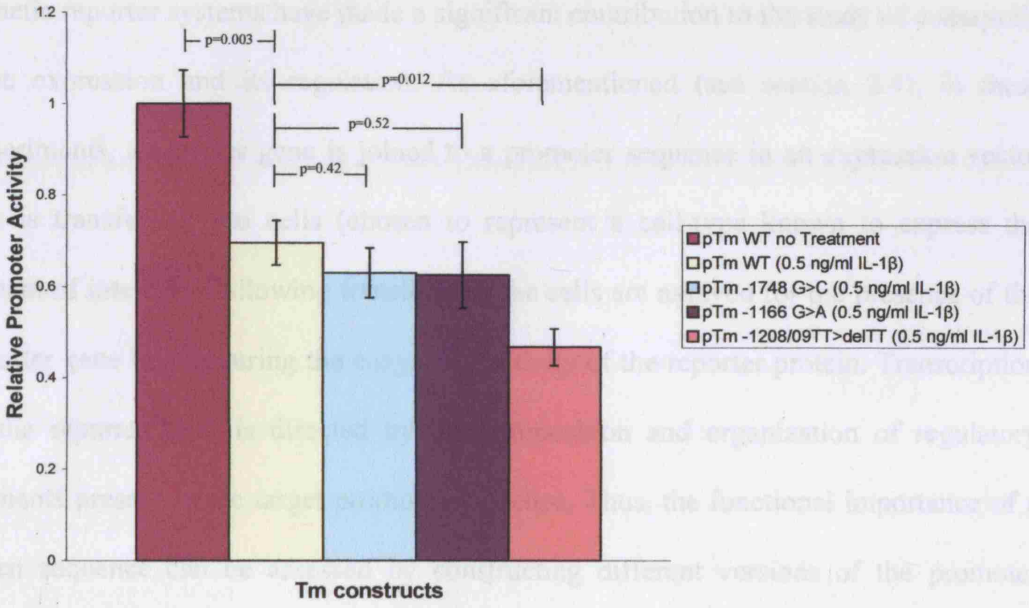
TNF-α (1 ng/ml) treatment was found to have a significant deleterious effect on the reporter gene activity driven by the Tm promoter region (42 ± 6% of wild-type untreated; p=0.001), see Figure 7.9. Relative promoter activity was further reduced for the -1208/09delTT allele following 1 ng/ml TNF-α treatment (p=0.02). In contrast, no significant alteration in relative promoter activity was noted for the -1748C or the -1166A allele constructs following addition of TNF-α, as compared to the wild-type treated construct, although a non-significant increase in relative promoter activity was noted for the -1166A allele construct.



Tm promoter variant	Relative promoter activity (% of wild-type untreated)	p value	Relative promoter activity (% of wild-type treated)	P-value
Wild-type	42 ± 6	0.001	Reference	
-1748G>C	46 ± 7	0.004	108 ± 7	0.69
-1166G>A	63 ± 8	0.009	148 ± 8	0.09
-1208/09TT>delTT	28 ± 7	<0.0001	67 ± 3	0.02

Figure 7.9 Relative promoter activities for pTm constructs in response to TNF-α treatment.
Wild-type and mutant allele containing Tm constructs were transiently transfected into EA.hy 926 cells. Transfected cells were treated with 1 ng/ml TNF-α, for 15 hours. Promoter activity is given relative to the untreated or treated wild-type Tm construct (pTm WT). Results expressed as means ± SE, n=20. Statistical analysis performed by paired t-test.

A similar effect for WT and delTT constructs, was observed following treatment with 0.5 ng/ml IL-1β; see Figure 7.10. IL-1β treatment had a significant deleterious effect on reporter gene expression for the wild-type construct (pTm WT), as compared to the wild-type construct under basal conditions (70 ± 5% of wild-type untreated; p=0.003), though the effect was milder than seen following the addition of 1 ng/ml TNF-α. The -1208/09TT>delTT variant was once again found to have a significant deleterious effect on reporter gene expression in response to 0.5 ng/ml IL-1β. Variants -1748G>C and -1166G>A did not have a detectable functional effect on reporter gene expression following addition of IL-1β.



Tm promoter variant	Relative promoter activity (% of wild-type untreated)	p value	Relative promoter activity (% of wild-type treated)	P-value
Wild-type	70 ± 5	0.003	Reference	
-1748G>C	63 ± 5	0.001	91 ± 5	0.42
-1166G>A	62 ± 7	0.007	90 ± 7	0.52
-1208/09TT>delTT	47 ± 4	<0.0001	67 ± 4	0.012

Figure 7.10 Relative promoter activities for pTm constructs in response to IL-1β treatment.
Wild-type and mutant allele containing Tm constructs were transiently transfected into EA.hy 926 cells. Transfected cells were treated with 0.5 ng/ml IL-1β, for 15 hours. Promoter activity is given relative to the untreated or treated wild-type Tm construct (pTm WT). Results expressed as means ± SE, n=20. Statistical analysis performed by paired t-test.

7.5 Discussion

The aim of this section was to investigate the potential mechanism through which previously identified variants in the distal promoter region of the Tm gene (-1748G>C, -1208/09TT>delTT and -1166G>A) (Le Flem et al., 2001) may be associated with CHD in an environment of metabolic syndrome, as shown in previous chapters (see Chapters 3 & 4). The *in vitro* dual *luciferase* reporter gene assay was used to evaluate the potential effects of these variants on the transcriptional activity of the promoter, under basal conditions and in response to inflammatory cytokines.

Genetic reporter systems have made a significant contribution to the study of eukaryotic gene expression and its regulation. As aforementioned (see section 2.4), in these experiments, a reporter gene is joined to a promoter sequence in an expression vector that is transfected into cells (chosen to represent a cell-type known to express the protein of interest). Following transfection, the cells are assayed for the presence of the reporter gene by measuring the enzymatic activity of the reporter protein. Transcription of the reporter gene is directed by the composition and organization of regulatory elements present in the target promoter sequence. Thus, the functional importance of a given sequence can be assessed by constructing different versions of the promoter region and comparing the relative efficiency of reporter gene expression.

In the current study, a 2056 bp fragment, containing the 5' UTR of the Tm gene, was cloned into the pGL-3-Basic firefly *luciferase* reporter vector (Promega Co. Madison, WI, USA). Reporter gene analysis, to date, has been done using small fragments of the Tm promoter (Le Flem et al., 1999) (Li et al., 2001) (Nakazawa et al., 2002). How these small fragments relate to the *in vivo* situation must be viewed sceptically. Indeed, in one

such reporter gene study, a variant in the proximal promoter region of the Tm gene was found to have an adverse effect upon reporter gene expression using a construct of 150 bp. When the construct size was increased to 300 bp, however, no differences were observed between the wild-type and mutant alleles (Le Flem et al., 1999). The entire region between the distal and proximal promoter regions, including a region previously defined as a silencer region, was thus kept in the test construct in the experiments here, in an attempt to keep the environment as physiological as possible.

The Tm promoter-reporter gene fusion constructs (bearing either wild-type or variants) were transfected into mammalian endothelial cells EA.hy 926. Transfection experiments were initiated in primary HUVECs but unfortunately efforts proved unsuccessful, despite previous reports of successful transfection using cationic lipid reagents (Tazawa et al., 1993) (Matsumura et al., 1999) (Nakazawa et al., 2002). The inability to transfect HUVECs despite an effort to fully optimise all the recommended conditions could not be fully explained. A possibility is that the cell stocks supplied to our laboratory had compromised viability, since four different lipid based delivery techniques failed to transfect this primary endothelial cell line properly. However, transient transfection of EA.hy 926 cells, an endothelial cell line generated from a fusion of HUVECs with a lung carcinoma (A549) cell line (Arnalich et al., 2000), proved fruitful. EA.hy 926 cells have been previously used in experiments to determine the transcriptional regulation of the Tm gene and to characterize Tm gene mutations in the 5' UTR region (Yu et al., 1992) (Le Flem et al., 1999). In addition, transformed cell lines are easier to grow and maintain in culture and tend to be more amenable to transfection than primary cells. Furthermore, EA.hy 926, like HUVECs, were shown to express Tm, in agreement with previous studies (Yu et al., 1992). An important consideration when carrying out reporter gene expression assays is to use a cell line that expresses the gene of interest.

This ensures the expression machinery regulating gene expression is intact and is thus able to drive the expression of the reporter gene fused to the promoter region under investigation.

In the reporter gene assays, none of the distal promoter variants assessed (-1748G>C, -1208/09TT>delTT and -1166G>A) showed an effect on the expression of the reporter gene under basal conditions, which would suggest that they do not effect the transcriptional activity of the Tm promoter.

The -1748G>C variant has previously been shown to create a putative Sp1-like transcription factor binding site (**G**$^{-1748}$TCCGCGTC; **bold underlined** base: modified base), with only one mismatch relative to the consensus sequence (C/AC/TCCGC/AT/CT/CC/A) (Le Flem et al., 2001). Sp1 is a transcription factor that has a zinc-finger as a DNA-binding domain and a glutamine-rich region as an activation domain (Courey and Tjian, 1988). Sp1 binds TFIID (transcription initiation factor) to initiate transcription, which is of particular importance in genes lacking a TATA-like sequence (Pugh and Tjian, 1990). The TATA-box positions the RNA polymerase II (responsible for transcription of all protein-coding genes) for correct transcription initiation. As such, the -1748G>C variant might be expected to have an effect on Tm gene expression. Sp1-like responsive elements, however, occur quite frequently in the genome, with only a limited number of Sp1 sites being transcriptionally active. Indeed, site directed mutation analysis of four potential Sp1 binding sites of the proximal promoter region of the Tm gene revealed that only two of the sites had positive functions for the activity of the Tm promoter (Tazawa et al., 1993). In addition, evidence has been presented suggesting that the spacing between Sp1 binding sites is important for gene expression, with Sp1 sites synergistically activating transcription

(Matsuzawa et al., 1991) (Pascal and Tjian, 1991) (Segal and Berk, 1991). Consequently, although the -1748G>C variant creates a putative Sp1 recognition site, the generated Sp1 site may not be functional, and may explain the lack of an effect on transcription by the -1748G>C variant. The lack of an effect on reporter gene expression would agree with the observed lack of an association between the -1748C allele and CHD risk in the HIFMECH case-control study; see Chapter 3.

There was no evidence to suggest an effect on the transcriptional activity of the Tm promoter by the -1208/09TT>delTT variant, under basal conditions. Although this polymorphic site lies in close proximity to regulatory elements, it does not form an integral part of any known consensus sequence, which may explain the lack of effect on transcription. In agreement, the -1208/09TT>delTT gene polymorphism was not associated with altered Tm expression in monocytes under basal conditions (Reny et al., 2005).

The -1166A allele failed to show an effect on Tm promoter transcriptional activity. The -1166G>A variant lies within the core binding sequence of a putative SSRE (GA**G**$^{-1166}$ACC; **bold underlined** base: modified base) (Le Flem et al., 2001). As such, the -1166G>A variant site may alter the level of Tm expression under shear stress conditions. However, the reporter gene analysis carried out here, was performed under basal, static conditions. The lack of an effect for this polymorphism in the current study is perhaps not surprising. The SSRE consensus sequence (GGTCTC), or its complementary sequence (GAGACC), has been identified in the 5'UTR region of several endothelial genes, many of which have been shown to be responsive to shear stress (Nakada et al., 2000) (Resnick et al., 2000). Previous studies have also demonstrated that mutations altering the SSRE consensus sequence abolish shear stress

responsiveness (Resnick et al., 1993). Conflicting results have been published concerning the effect of shear stress on Tm expression. Application of fluid stress in the physiological range of magnitude (15 and 36 dynes/cm^2) on BAECs, caused a short lived increase followed by a decrease in Tm mRNA levels (Malek et al., 1994). In contrast, exposure of HUVECs to moderate shear stress levels (15 dynes/cm^2) caused a dramatic increase in Tm mRNA levels, which preceded the increase in surface Tm antigen levels, suggesting transcriptional control (Takada et al., 1994). Tm response to fluid shear stress in the physiological range may be mediated by the SSRE. As such, the -1166G>A variant site may alter Tm expression under shear stress conditions and is a potential candidate for modulating risk for CHD. The effect of the -1166G>A variant on the level of Tm expression under shear stress condition, by reporter gene assay, should help address this possibility.

EA.hy 926 cells transfected with Tm promoter-reporter gene fusion constructs (bearing or not bearing promoter mutations) were also exposed to inflammatory cytokines (TNF-α & IL-1β), to assess how variants within the Tm promoter affect expression under inflammatory conditions. Both TNF-α and IL-1β are central mediators of inflammatory reactions. They are members of cytokine networks implicated in pro-inflammatory processes and have been shown to contribute to the progression of atherosclerosis by several mechanisms; see section 1.3.3.1. With regards to haemostasis, immunological mediators, such as TNF-α and IL-1β, are capable of inducing a pro-coagulant shift in endothelial cells by altering the levels of various blood coagulation enzymes, TF and natural anti-coagulants (Gregory et al., 1989) (Esmon et al., 1991) (Gu et al., 2000b) (Shimokawa et al., 2000). Specifically, the expression of Tm on the surface of endothelial cells exposed to TNF-α *in vitro* has been shown to decrease, and the intradermal application of TNF-α was also found to downregulate the expression of Tm

on the surface of human endothelial cells (Lentz et al., 1991) (Speiser et al., 2001) (Nan et al., 2005). *In vitro*, IL-1 was found to cause a decrease in Tm expression and activity on the cell surface, and intravenous infusion of IL-1 was also found to decrease Tm cell surface co-factor activity (Nawroth et al., 1986) (Archipoff et al., 1991).

Inflammatory cytokines mediate many of their effects on transcription via activation of the NF-κβ pathway; reviewed in (Rothwarf and Karin, 1999). NF-κβ, a transcription factor, is composed of homo- and hetero-dimers of the Rel family of proteins, which include p50, p52, p65 (RelA), RelB and c-Rel. NF-κβ is predominantly found as a heterodimer of p65 and p50 (Ghosh et al., 1998). The NF-κβ dimers form a cytoplasmic complex with the inhibitory protein IκBα (Henkel et al., 1993) (Baeuerle, 1998). Pro-iflammatory mediators, like TNF-α and IL-1β, cause the dissociation and subsequent proteolytic degradation of IκBα, thus allowing the translocation of the NF-κβ dimers to the nucleus (Ghosh et al., 1998). Binding of the NF-κβ dimers to specific consensus sequences in the promoter region of target genes activates or suppresses transcription (Li and Stashenko, 1993) (Kouba et al., 1999) (Gires et al., 2001).

In the current study, both TNF-α (1 ng/ml, 15 h) & IL-1β (0.5 ng/ml, 15 h) had a significant deleterious effect on the transcriptional activity of the Tm promoter region (TNF-α: 42% of wild-type untreated; $p=0.001$, IL-1β: 70% of wild-type untreated; $p<0.0001$). Previous studies, using short promoter-reporter gene fusion constructs have demonstrated a decrease in Tm promoter activity following TNF-α treatment (Yu et al., 1992) (von der Ahe et al., 1993). How these small fragments relate to the *in vivo* situation must be viewed sceptically. The observed detrimental effect on transcription, in response to inflammatory cytokines is likely mediated by activation of NF-κβ (Sohn et al., 2005). NF-κβ activation is not thought to mediate cytokine-induced repression of

Tm through direct association with the Tm promoter, as the 5' UTR lacks a classic NF-κβ consensus site (Yu et al., 1992) (Tazawa et al., 1993). NF-κβ, rather, appears to exert its effect on Tm gene expression, indirectly, by competition for the transcription co-activator p300/CBP (cyclic adenosine monophosphate response element binding protein - binding protein) (Sohn et al., 2005). Evidence suggests that full transcriptional activity of NF-κβ requires physical interaction with p300/CBP (Sheppard et al., 1999), which modulates transcription factor activity through its histone acetyltransferase activity (Chan and La Thangue, 2001) (Vo and Goodman, 2001). NF-κβ has been shown to inhibit the activities of certain transcription factors by competing for the limited pools of p300/CBP in the nucleus (Ravi et al., 1998) (Speir et al., 2000) (Gires et al., 2001). With regards to the Tm gene, Van der Ahe *et al.* reported that TNF-α sensitivity of the Tm gene, in endothelial cells, is in part dependent on three Ets core motifs (GGAA) located within a distinct region in the Tm promoter, just upstream of the TATA-box (-76 to -30; relative to the transcription start site) (von der Ahe et al., 1993). Although binding of Ets transcription factors to the Ets consensus sequence has previously been shown to mediate Tm expression interaction with the closely related p300/CBP transcription coactivator is required for full transcriptional activity of the Ets family of proteins (von der Ahe et al., 1993) (Jayaraman et al., 1999). Thus, by competing for the stable, but limited pools of p300/CBP within the nucleus, NF-κβ may inhibit the activity of the Ets-like transcription factors, thus suppressing expression of the Tm gene (Sohn et al., 2005).

Variants -1748G>C and -1166G>A, did not show a statistically significant effect on reporter gene expression when subjected to inflammatory cytokine treatment, as compared to the wild-type treated construct. Interestingly, reporter gene expression was further repressed for the -1208/09TT>delTT variant, following treatment with either

TNF-α or IL-1β (67% of wild-type treated; $p \leq 0.02$). These results suggest that only the -1208/09TT>delTT variant is likely to result in reduced transcription of the gene, following an inflammatory stimulus.

Although the -1208/09TT>delTT polymorphic site does not form an integral part of a known consensus sequence within the 5' UTR of the Tm gene, the double T deletion variant creates an NF-κβ-like sequence motif (**GGGTGTTT**<u>tt</u>**AAA**CAGTT; <u>underlined</u> bases: modified bases; **bold** bases: NF-κβ core sequence (Pierce et al., 1988)), which differs in a few nucleotides from a canonical NF-κβ binding site (GGGRNNYYCC; where R=purine & Y=pyrimidine) (Sen and Baltimore, 1986). The various dimeric combinations of the aforementioned NF-κβ subunits show slightly different preferences for different DNA binding motifs (Ghosh et al., 1998). Weak binding of non-canonical NF-κβ-like sequences that diverge from the consensus NF-κβ binding site have been demonstrated (Zabel et al., 1991). In addition, certain NF-κβ subunits (p50 and p52) have been found to lack the carboxy-terminal transcriptional activation domain of the other Rel members. In concordance, p50 and p52 homodimers have been shown to lack transcriptional activity (Ghosh et al., 1998). Furthermore, studies have demonstrated that binding of uncommon (not the predominant p65/p50 heterodimer) NF-κβ dimers to non-canonical NF-κβ-like sequence interferes with transcription, thus repressing promoter transcriptional activity (Li and Stashenko, 1993) (Kouba et al., 1999) (Iber et al., 2000). As such, the observed deleterious effect of the -1208/09delTT allele on transcriptional activity of the Tm promoter in response to inflammatory cytokines may be due to the binding of atypical NF-κβ species to the newly generated NF-κβ-like sequence motif, causing transcriptional interference and thus reducing transcription of the Tm gene. Alternatively, due to the proximity of the -1208/09TT>delTT variant to known regulatory elements [-1208/09TT>delTT lies between a DR4 sequence (-1531 to

–1516) and SSRE (-1163 to -1168); see sections 1.4.4 & 1.4.5], it is possible that the deletion variant disrupts nuclear protein binding. The DR4 site, in particular, has been shown to play an important role in regulating Tm gene expression in response to biochemical agonists and antagonists, through interaction with the RAR-RXRα heterodimer (Horie et al., 2001) (Ishii et al., 2003).

The work presented here suggests that the -1208/09TT>delTT variant may be more sensitive to downregulation by inflammatory cytokines. This finding would suggest a mechanism for the association of this variant site with CHD in metabolic syndrome, a condition in which increased levels of inflammatory cytokines have been identified.

CHAPTER EIGHT

A GENOTYPE:GENOTYPE APPROACH TO CHD RISK:

TM -1208/09TT>DELTT AND TNF-A -308G>A

8. A genotype:genotype approach to CHD risk: Tm -1208/09TT>delTT and TNF-α -308G>A

8.1 Introduction

A haplotype comprising the rare alleles for two common Tm variants (-1208/09TT>delTT & A455V) contributed to the risk of MI, in the North of Europe (HIFMECH), particularly in individuals with obesity, metabolic syndrome or inflammation (see Chapter 3), and was confirmed in a large prospective study (NPHSII; see Chapter 4).

In vitro reporter gene analysis (see Chapter 7) demonstrated increased sensitivity of the -1208/09TT>delTT Tm variant to downregulation by inflammatory cytokines, including TNF-α. *In vivo*, increased TNF-α levels are likely, therefore, to adversely affect Tm expression in individuals with the -1208/09delTT allele. Elevated levels of pro-inflammatory cytokines, including TNF-α, have been reported in diabetes and metabolic syndrome (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999) and is a potential mechanism explaining the gene-environment interaction observed for the V/delTT haplotype. While TNF-α measurements have not been made in either NPHSII, HIFMECH or EDSC, a literature search revealed a variant in the promoter of the TNF-α gene, -308G>A, for which increased TNF-α production has been shown for the rare allele (Kroeger et al., 1997) (Kroeger et al., 2000) (Vendrell et al., 2003). TNF-α genotyping was therefore carried out for the NPHSII and EDSC cohorts (Appendix 6) to determine the effect of the combined Tm and TNF-α genotypes upon risk for CHD, sTm levels, thrombin generation (F1+2) and inflammation (CRP).

8.2 Aim

The purpose of the work described in this section was to assess contribution to CHD risk by the -1208/09TT>delTT Tm variant, in combination with the TNF-α -308G>A variant. Analysis was initially carried out in diabetics within NPHSII. A cross sectional study of type 2 diabetes (EDSC) was also used to further assess contribution to CHD risk. The effect of the -1208/09TT>delTT Tm variant, in combination with the TNF-α -308G>A variant upon thrombin generation (F1+2), a marker of inflammation (CRP) and sTm measurements was also assessed.

8.3 Methods

8.3.1 Description of study subjects (NPHSII & EDSC)

Both NPHSII and EDSC have been described earlier in this thesis; see Chapters 4 & 5. Individuals, within the prospective NPHSII study (n=3012) who developed type 2 diabetes over 15 years follow-up (n=212) were analysed for both Tm and TNF-α variants. 927 patients within the EDSC study [EW, n=331; IA, n=503; AC n=93)] were analysed for both Tm and TNF-α variants. Reduced numbers may appear in the text and tables following adjustment for statistical analysis, since data were not available for every variable in all patients. In NPHSII, CRP levels had been measured in 587 randomly selected baseline samples using a commercial assay (Cordia High Sensitivity CRP). Prothrombin fragment F1+2 had been measured previously in 2442 individuals (Cooper et al., 2000). Baseline measurements were used for analysis. In EDSC, circulating levels of sTm were measured, using activity (act) and antigen (ag) assays; see section 2.2.1. F1+2 and CRP levels had been measured using ELISA assays (Dade

Behring, Marburg, Germany & DiaMed, Switzerland, respectively). F1+2 measurements had been made in subjects not taking lipid lowering drugs.

8.3.2 Genotyping for polymorphisms in Tm and TNF-α

Tm genotyping for the Tm variant sites under investigation (-1208/09TT>delTT and A455V) was performed as described in Chapter 2. PCR conditions and restriction enzyme digestion conditions are summarised in Tables 2.1-2.3. Band patterns for these two Tm polymorphisms studied are shown in Figures 2.5 and 2.7. Genotyping for TNF-α -308G>A is shown in appendix 6, and was carried out by Julia Grizenkova.

8.3.3 Statistical analysis

Data were entered into an EXCEL spreadsheet (Microsoft). Statistical analysis was carried out by myself with help from Jackie Cooper. Statistical analysis was conducted using 'Intercooled STATA' version 7.0 (College Station, Texas) and SPSS version 12.0.1. (SPSS Inc., Chicago, USA). In NPHSII, F1+2 and CRP levels were ln-transformed before analysis. Normality was considered graphically with QQ-norm plots and formally via a Kolmogorov-Smirnov test. Continuous variables were assessed by analysis of variance (ANOVA) on normally distributed data, or after appropriate transformation. Results are presented as mean ± standard deviation where appropriate. Association of Tm genotype with risk of CHD was assessed using Cox's proportional hazards model, with significance assessed by the likelihood ratio test. Results are presented as Relative Risk (RR) with 95% CI. Results were adjusted for age and practice unless otherwise stated (NPHSII). This was carried out to allow for differences in baseline data according to age and practice. Age was included as a covariate in the

model and data stratified by practice (using the strata option in STATA). Interaction between Tm genotype and other clinical characteristics was tested by a model including an interaction term. In *EDSC*, assessment of a normal distribution was considered graphically with QQ-norm plots, and formally via a Kolmogorov-Smirnov test. Ln-transformation was required for BMI, urea, glucose, HbA_{1C}, SBP, triglyceride, prothrombin F1+2, CRP, IH sTm act, IH sTm ag and DS sTm ag levels, whereas square root-transformation was required to normalise creatinine, DBP, HDL-cholesterol and LDL-cholesterol levels. Results are presented as mean ± standard deviation where appropriate. For data that were not normally distributed, results are presented as the geometric mean ± approximate standard deviation or median (interquartile range), as indicated in the results. Continuous variables were assessed by analysis of variance (ANOVA) on normally distributed data, or after appropriate transformation. Risk of CHD was assessed via OR using logistic regression and its statistical significance by likelihood ratio test using those individuals with complete data for the required analysis. P-values <0.05 were considered significant.

8.4 Results

8.4.1 Clinical and biochemical details

Clinical details for NPHSII and EDSC were presented earlier in this thesis; see sections 4.4.1 & 5.4.1, respectively.

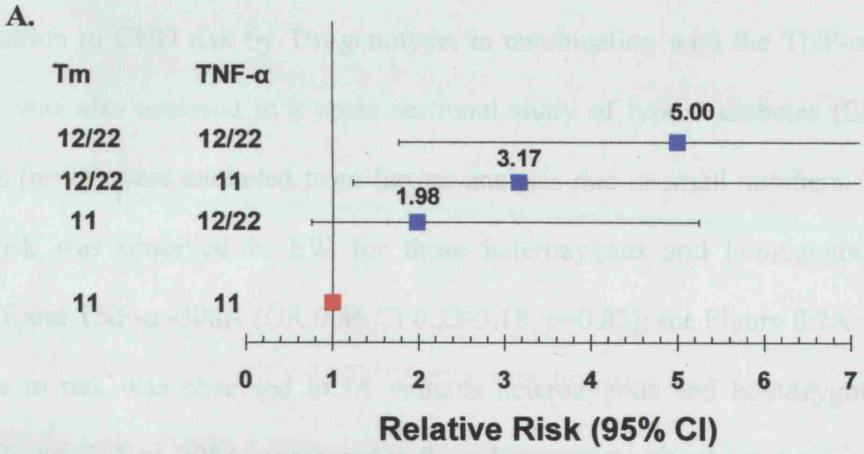
8.4.2 Contribution to CHD risk by Tm genotype, in combination with the TNF-α -308G>A variant.

Since the -1208/09TT>delTT Tm variant was found to compromise Tm expression in response to TNF-α (see Chapter 7), the contribution to CHD risk by Tm genotype was assessed in combination with the TNF-α -308G>A variant, which has been associated with higher TNF-α production (Kroeger et al., 1997) (Kroeger et al., 2000) (Vendrell et al., 2003). This analysis was carried out to try to expand knowledge of the mechanism through which Tm genotype may decrease protection against CHD. Due to the low number of rare homozygotes identified for each of the variants studied, subjects with one and two rare alleles were combined and compared to those with two common alleles at each variant site. The previous analysis of NPHSII identified a risk-interaction with factors present in metabolic syndrome. For this later analysis, only those individuals within NPHSII who had diabetes at baseline or developed it during 15 years of follow up were analysed. These data had not been available at the time the previous analysis was performed.

In NPHSII, risk was assessed in men heterozygous and homozygous for Tm V/delTT and TNF-α -308G>A, compared to those homozygous for the common allele at all sites. When risk was assessed in diabetic men within NPHSII, carriers of both Tm V/delTT and TNF-α -308A had a 5-fold increased risk of CHD (RR 5.00 CI 1.77-14.07; p=0.002) compared to those with the common allele at all sites, after adjustment for age and practise. V/delTT carriers who were homozygous for TNF-α -308G had a 3-fold increased risk of CHD (RR 3.17 CI 1.23-8.20; p=0.02), compared to those with the common allele at all sites. There was no evidence to suggest a significant contribution to CHD risk for TNF-α -308A alone; see Figure 8.1A.

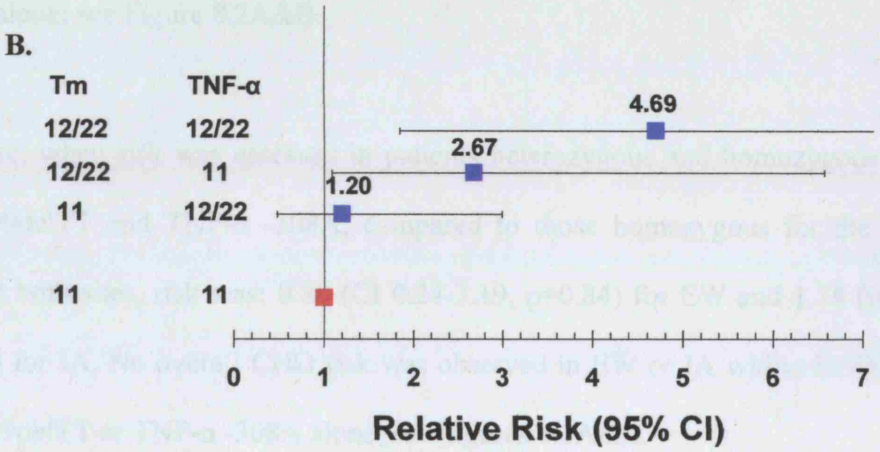
Similar results were obtained when risk was assessed for the -1208/09TT>delTT Tm variant alone, as opposed to the V/delTT Tm combined genotype. Carriers of both Tm -1208/09delTT and TNF-α -308A had an almost 5-fold increased risk of CHD (RR 4.69 CI 1.84-12.00; p=0.001), compared to those with the common allele at both sites, after adjustment for age and practise. RR was 2.67 (CI 1.09-6.56; p=0.03) for -1208/09delTT carriers who were homozygous for TNF-α -308G, compared to those with the common allele at both sites. Once again, TNF-α -308A alone did not contribute significantly to CHD-risk; see Figure 8.1B.

A.



Tm V/delTT	TNF-α -308G>A	Total N	No. of events (%)	RR (95% CI)	P-value
11	11	108	12 (11.1)	1.00	-
	12/22	33	7 (21.2)	1.98 (0.75-5.24)	0.17
12/22	11	23	8 (34.8)	3.17 (1.23-8.20)	0.02
	12/22	14	6 (42.9)	5.00 (1.77-14.07)	0.002

B.



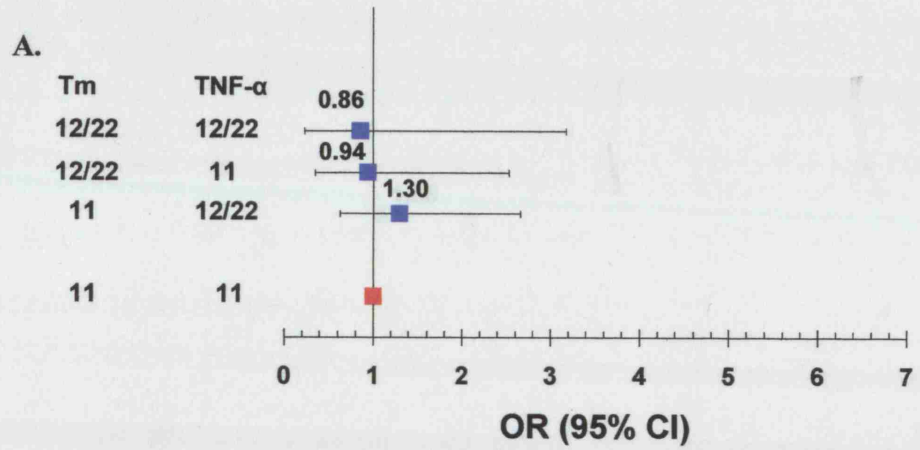
Tm -1208/09TT>delTT	TNF-α -308G>A	Total N	No. of events (%)	RR (95% CI)	P-value
11	11	125	17 (13.6)	1.00	-
	12/22	45	7 (15.6)	1.20 (0.48-2.99)	0.70
12/22	11	26	8 (30.8)	2.67 (1.09-6.56)	0.03
	12/22	16	7 (43.8)	4.69 (1.84-12.00)	0.001

Figure 8.1 CHD risk analysis in NPHSII diabetics.
A. CHD risk trend in carriers of Tm both Tm V/delTT and TNF-α -308A, compared to those homozygous for the common allele at all sites. **B.** CHD risk trend in carriers of Tm both Tm -1208/09delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites. Results adjusted for age and practice. **Tm V/delTT: AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **Tm -1208/09TT>delTT: TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A: GG** (11), **GA** (12), **AA** (22).

Contribution to CHD risk by Tm genotype, in combination with the TNF-α -308G>A variant, was also assessed in a cross sectional study of type 2 diabetes (EDSC). AC subjects (n=93) were excluded from further analysis due to small numbers. No overall CHD risk was observed in EW for those heterozygous and homozygous for Tm V/delTT and TNF-α -308A (OR 0.86 CI 0.23-3.18, p=0.82); see Figure 8.2A. A 1.7 fold increase in risk was observed in IA patients heterozygous and homozygous for Tm V/delTT and TNF-α -308A, compared to those homozygous for the common allele at all sites, although this failed to reach statistical significance (OR 1.76 CI 0.71-4.38, p=0.23); see Figure 8.2B. In either EW or IA patients within EDSC, there was no evidence to suggest an association with CHD risk for either Tm V/delTT or TNF-α -308A alone; see Figure 8.2A&B.

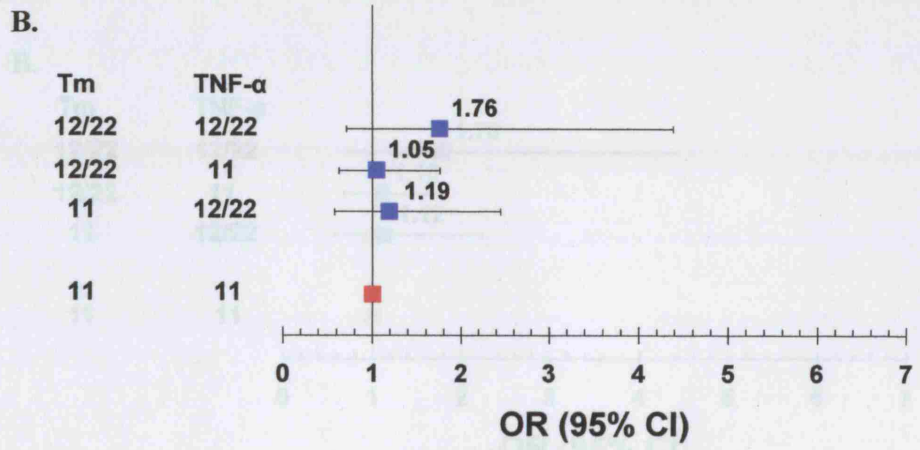
Likewise, when risk was assessed in patients heterozygous and homozygous for both -1208/09delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites, risk was: 0.88 (CI 0.24-3.19, p=0.84) for EW and 1.78 (0.72-4.40, p=0.22) for IA. No overall CHD risk was observed in EW or IA within EDSC for Tm -1208/09delTT or TNF-α -308A alone; see Figures 8.3A&B.

A.



Tm V/delTT	TNF-α -308G>A	Total (n)	No CHD (%)	CHD (%)	OR (95% CI)	P-value
11	**11**	129	107 (49.8)	22 (46.8)	1.00	-
	12/22	76	60 (27.9)	16 (34.0)	1.30 (0.63-2.66)	0.47
12/22	**11**	37	31 (14.4)	6 (12.8)	0.94 (0.35-2.53)	0.90
	12/22	20	17 (7.9)	3 (6.4)	0.86 (0.23-3.18)	0.82

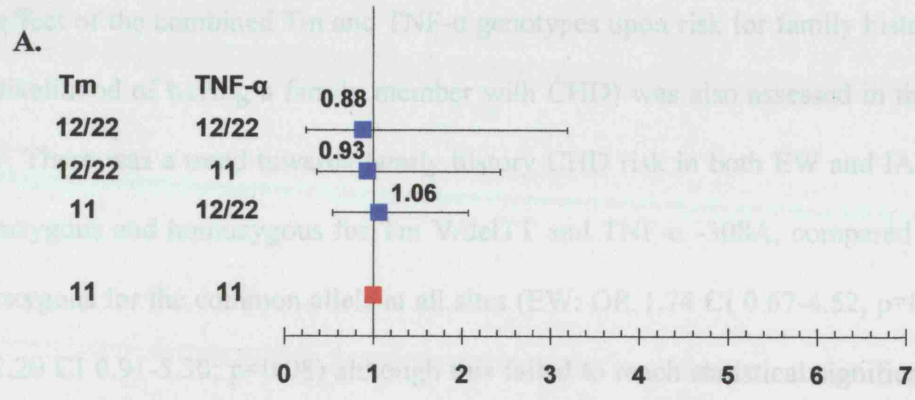
B.



Tm V/delTT	TNF-α -308G>A	Total (n)	No CHD (%)	CHD (%)	OR (95% CI)	P-value
11	**11**	269	203 (62.1)	66 (58.4)	1.00	-
	12/22	43	31 (9.5)	12 (10.6)	1.19 (0.58-2.45)	0.64
12/22	**11**	106	79 (24.2)	27 (23.9)	1.05 (0.63-1.76)	0.85
	12/22	22	14 (4.3)	8 (7.1)	1.76 (0.71-4.38)	0.23

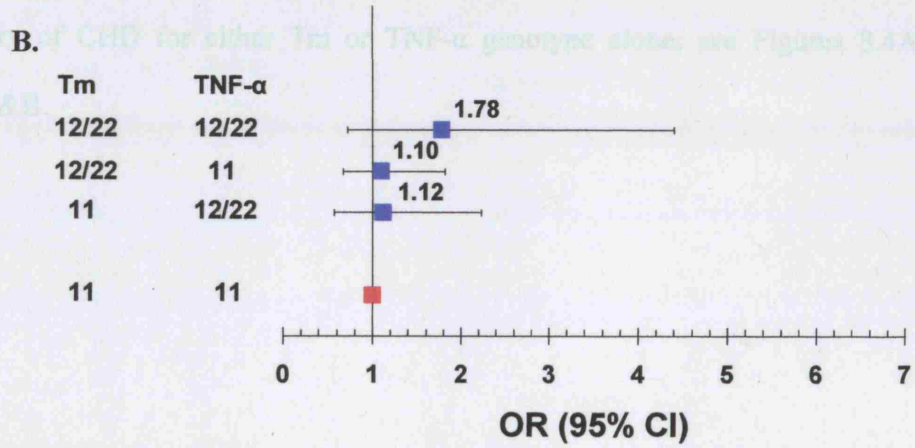
Figure 8.2 CHD risk analysis in EDSC.
CHD risk trend in carriers of Tm both Tm V/delTT and TNF-α -308A, compared to those homozygous for the common allele at all sites. **A.** EW. **B.** IA. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

A.



Tm -1208/09 TT>delTT	TNF-α -308G>A	Total (n)	No CHD (%)	CHD (%)	OR (95% CI)	P-value
11	11	173	144 (53.7)	29 (53.7)	1.00	-
	12/22	91	75 (28.0)	16 (29.6)	1.06 (0.54-2.07)	0.87
12/22	11	38	32 (11.9)	6 (11.1)	0.93 (0.36-2.43)	0.88
	12/22	20	17 (6.3)	3 (5.6)	0.88 (0.24-3.19)	0.84

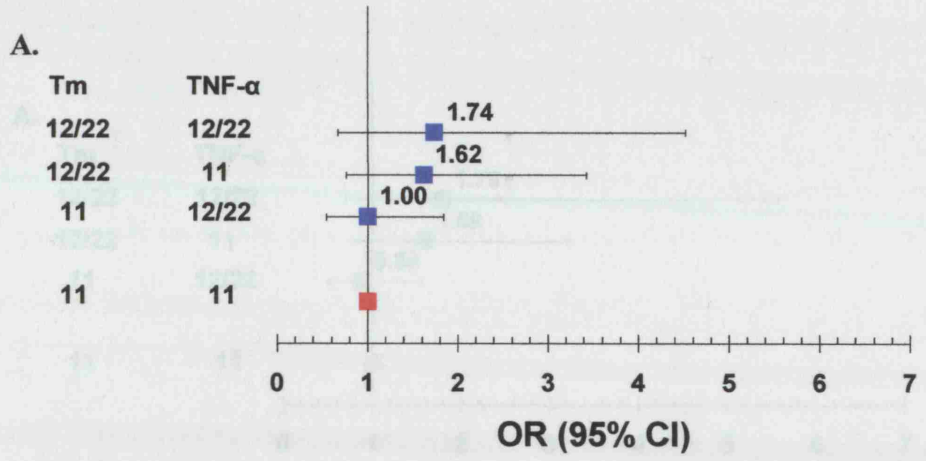
B.



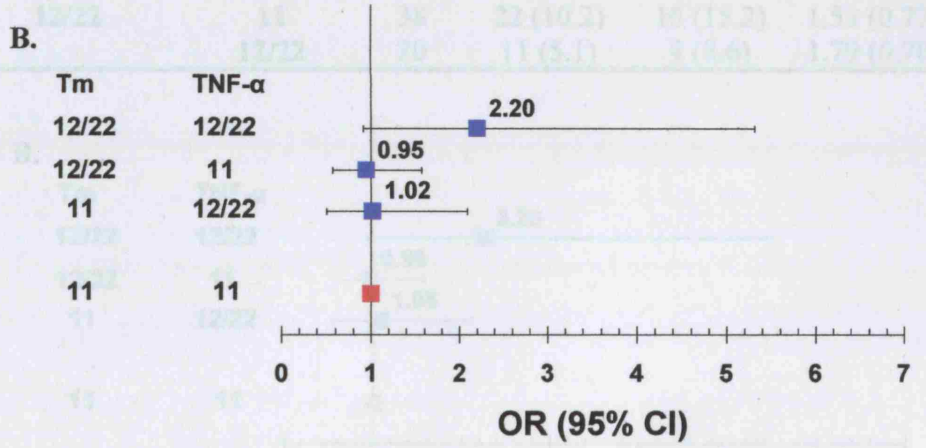
Tm -1208/09 TT>delTT	TNF-α -308G>A	Total (n)	No CHD (%)	CHD (%)	OR (95% CI)	P-value
11	11	304	230 (64.1)	74 (60.2)	1.00	-
	12/22	49	36 (10.0)	13 (10.6)	1.12 (0.57-2.23)	0.74
12/22	11	107	79 (22.0)	28 (22.8)	1.10 (0.67-1.82)	0.71
	12/22	22	14 (3.9)	8 (6.5)	1.78 (0.72-4.40)	0.22

Figure 8.3 CHD risk analysis in EDSC.
CHD risk trend in carriers of Tm both Tm -1208/09delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites. **A.** EW. **B.** IA. **Tm -1208/09TT>delTT**: **TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

The effect of the combined Tm and TNF-α genotypes upon risk for family history CHD (i.e. likelihood of having a family member with CHD) was also assessed in the EDSC study. There was a trend towards family history CHD risk in both EW and IA patients heterozygous and homozygous for Tm V/delTT and TNF-α -308A, compared to those homozygous for the common allele at all sites (EW: OR 1.74 CI 0.67-4.52, p=0.26; IA: OR 2.20 CI 0.91-5.30; p=0.08) although this failed to reach statistical significance; see Figure 8.4A&B. Risk was similar when -1208/09delTT Tm genotype and TNF-α -308A were compared to those homozygous for the common allele at both sites, risk was: 1.78 (CI 0.72-4.4, p=0.23) for EW and 2.26 (0.94-5.42, p=0.07) for IA; see Figure 8.5A&B. Again this effect failed to reach statistical significance. In both EW and IA patients within EDSC, there was no evidence to suggest an association with risk for family history of CHD for either Tm or TNF-α genotype alone; see Figures 8.4A&B and 8.5A&B.

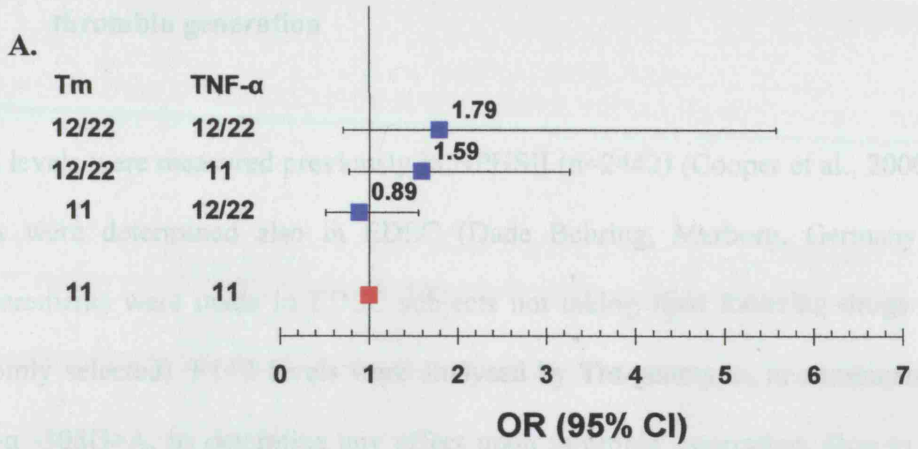


Tm V/delTT	TNF-α -308G>A	Total (n)	No family history (%)	Family history (%)	OR (95% CI)	P-value
11	11	129	87 (51.2)	41 (45.6)	1.00	-
	12/22	76	51 (30.0)	24 (26.7)	1.00 (0.54-1.84)	1.0
12/22	11	37	21 (12.4)	16 (17.8)	1.62 (0.76-3.42)	0.21
	12/22	20	11 (6.5)	9 (10.0)	1.74 (0.67-4.52)	0.26

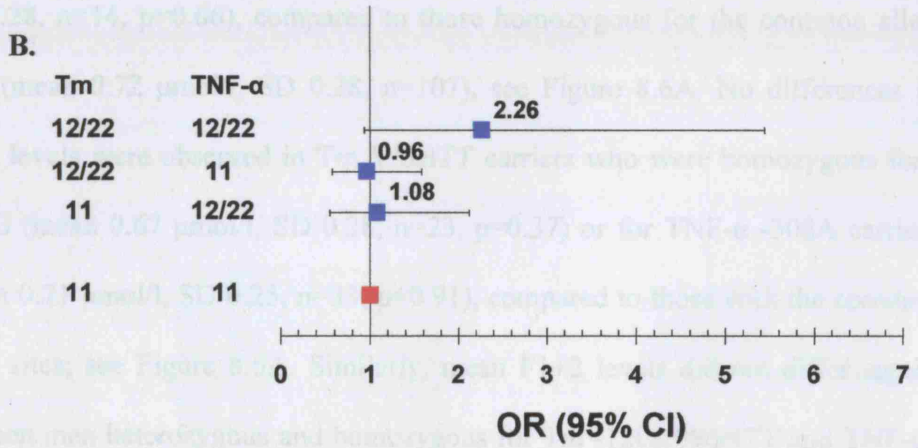


Tm V/delTT	TNF-α -308G>A	Total (n)	No family history (%)	Family history (%)	OR (95% CI)	P-value
11	11	269	195 (61.7)	74 (59.7)	1.00	-
	12/22	43	31 (9.8)	12 (9.7)	1.02 (0.50-2.09)	0.96
12/22	11	106	78 (24.7)	28 (22.6)	0.95 (0.57-1.57)	0.83
	12/22	22	12 (3.8)	10 (8.1)	2.20 (0.91-5.30)	0.08

Figure 8.4 Risk for family history CHD in EDSC.
Family history CHD risk trend in carriers of Tm both Tm V/delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites. **A.** EW. **B.** IA. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).



Tm -1208/09 TT>delTT	TNF-α -308G>A	Total (n)	No family history (%)	Family history (%)	OR (95% CI)	P-value
11	11	172	118 (54.9)	54 (51.4)	1.00	-
	12/22	90	64 (29.8)	26 (24.8)	0.89 (0.51-1.55)	0.68
12/22	11	38	22 (10.2)	16 (15.2)	1.59 (0.77-3.26)	0.21
	12/22	20	11 (5.1)	9 (8.6)	1.79 (0.70-4.57)	0.23



Tm -1208/09 TT>delTT	TNF-α -308G>A	Total (n)	No family history (%)	Family history (%)	OR (95% CI)	P-value
11	11	304	222 (63.8)	82 (61.2)	1.00	-
	12/22	49	35 (10.1)	14 (10.5)	1.08 (0.55-2.12)	0.82
12/22	11	107	79 (22.7)	28 (20.9)	0.96 (0.58-1.58)	0.87
	12/22	22	12 (3.5)	10 (7.5)	2.26 (0.94-5.42)	0.07

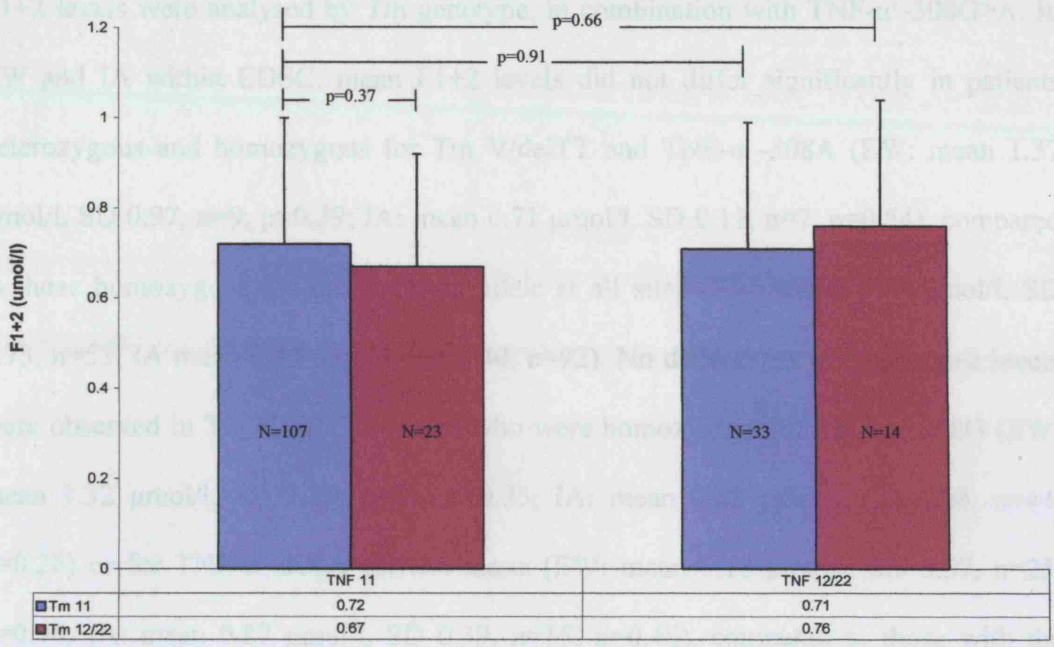
Figure 8.5 Risk for family history CHD in EDSC.
Family history CHD risk trend in carriers of Tm both Tm -1208/09delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites. **A.** EW. **B.** IA. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

8.4.3 Effects of Tm genotype, in combination with TNF-α -308G>A upon thrombin generation

F1+2 levels were measured previously in NPHSII (n=2442) (Cooper et al., 2000). F1+2 levels were determined also in EDSC (Dade Behring, Marburg, Germany). F1+2 measurements were made in EDSC subjects not taking lipid lowering drugs (n=316; randomly selected). F1+2 levels were analysed by Tm genotype, in combination with TNF-α -308G>A, to determine any effect upon thrombin generation. Due to the low numbers of rare homozygotes, these were grouped with heterozygotes for analysis.

In NPHSII diabetics, mean baseline F1+2 levels did not differ significantly in men heterozygous and homozygous for Tm V/delTT and TNF-α -308A (mean 0.76 μmol/l, SD 0.28, n=14, p=0.66), compared to those homozygous for the common allele at all sites (mean 0.72 μmol/l, SD 0.28, n=107), see Figure 8.6A. No differences in mean F1+2 levels were observed in Tm V/delTT carriers who were homozygous for TNF-α -308G (mean 0.67 μmol/l, SD 0.28, n=23, p=0.37) or for TNF-α -308A carriers alone (mean 0.71 μmol/l, SD 0.25, n=33, p=0.91), compared to those with the common allele at all sites; see Figure 8.6A. Similarly, mean F1+2 levels did not differ significantly between men heterozygous and homozygous for Tm -1208/09delTT and TNF-α -308A (mean 0.69 μmol/l, SD 0.29, n=16, p=0.75), compared to those homozygous for the common allele at both sites (mean 0.72 μmol/l, SD 0.27, n=124); see Figure 8.6B. No significant differences in mean F1+2 levels were observed for Tm and TNF-α genotypes alone (Tm -1208/09TT>delTT: mean 0.71 μmol/l, SD 0.40, n=26, p=0.86; TNF-α -308G>A: mean 0.68 μmol/l, SD 0.25, n=45, p=0.41), compared to those homozygous for the common allele at both sites; see Figure 8.6B. Numbers were too small to analyse similarly for cases and controls separately.

A.



B.

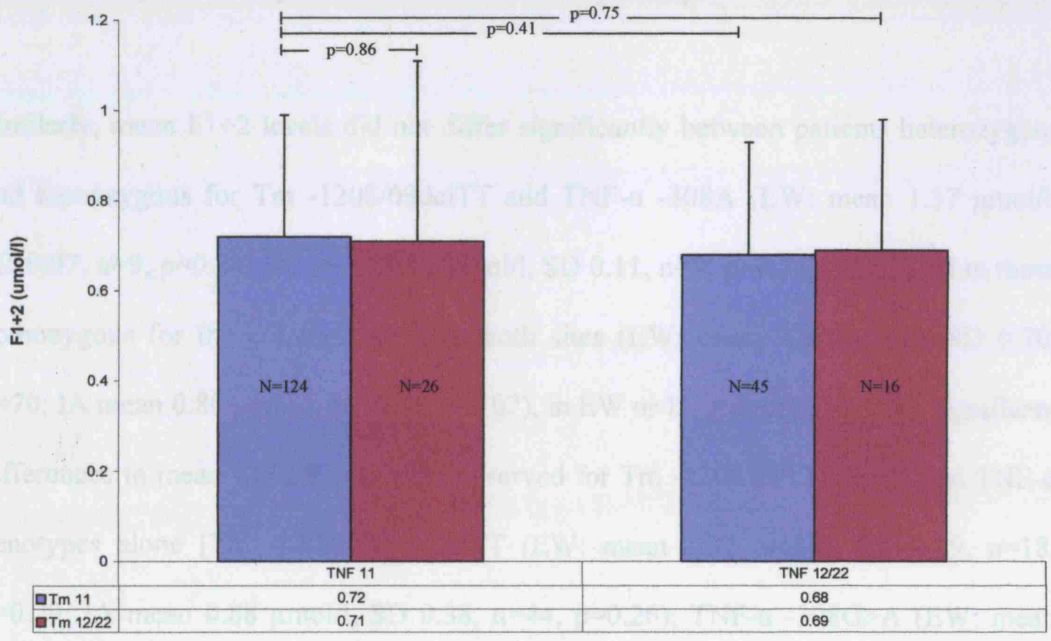


Figure 8.6 Geometric mean F1+2 levels (± approximate SD) across the combined Tm and TNF-α genotype groups in NPHSII diabetics.
A. Tm V delTT. **B.** Tm -1208/09TT>delTT. Analysis performed on ln-transformed data. **Tm V/delTT: AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **Tm -1208/09TT>delTT: TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A: GG** (11), **GA** (12), **AA** (22).

A similar picture to the one seen in NPHSII diabetics was observed in EDSC, when F1+2 levels were analysed by Tm genotype, in combination with TNF-α -308G>A. In EW and IA within EDSC, mean F1+2 levels did not differ significantly in patients heterozygous and homozygous for Tm V/delTT and TNF-α -308A (EW: mean 1.37 μmol/l, SD 0.97, n=9, p=0.39; IA: mean 0.71 μmol/l, SD 0.11, n=7, p=0.54), compared to those homozygous for the common allele at all sites (EW: mean 1.10 μmol/l, SD 0.73, n=53; IA mean 0.80 μmol/l, SD 0.40, n=92). No differences in mean F1+2 levels were observed in Tm V/delTT carriers who were homozygous for TNF-α -308G (EW: mean 1.32 μmol/l, SD 1.29, n=18, p=0.35; IA: mean 0.88 μmol/l, SD 0.38, n=44, p=0.26) or for TNF-α -308A carriers alone (EW: mean 1.10 μmol/l, SD 0.57, n=25, p=0.98; IA: mean 0.87 μmol/l, SD 0.39, n=15, p=0.46), compared to those with the common allele at all sites; see Table 8.1. As was the case for NPHSII, numbers were too small to analyse similarly for cases and controls separately.

Similarly, mean F1+2 levels did not differ significantly between patients heterozygous and homozygous for Tm -1208/09delTT and TNF-α -308A (EW: mean 1.37 μmol/l, SD 0.97, n=9, p=0.34; IA: mean 0.71 μmol/l, SD 0.11, n=7, p=0.51), compared to those homozygous for the common allele at both sites (EW: mean 1.10 μmol/l, SD 0.70, n=70; IA mean 0.80 μmol/l, SD 0.38, n=107), in EW or IA within EDSC. No significant differences in mean F1+2 levels were observed for Tm -1208/09TT>delTT and TNF-α genotypes alone [Tm -1208/09TT>delTT (EW: mean 1.32 μmol/l, SD 1.29, n=18, p=0.29; IA mean 0.88 μmol/l, SD 0.38, n=44, p=0.26); TNF-α -308G>A (EW: mean 1.13 μmol/l, SD 0.57, n=21, p=0.81; IA mean 0.90 μmol/l, SD 0.40, n=16, p=0.35)], as compared to those homozygous for the common allele at both sites; see Table 8.2.

		EW		IA		EW+IA	
Tm V/delTT	**TNF-α -308G>A**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**
11	**11**	1.10 ± 0.73 n=53	Reference	0.80 ± 0.40 n=92	Reference	0.80 ±0.46 n=145	Reference
	12/22	1.10 ± 0.57 n=25	0.98	0.87 ± 0.39 n=15	0.46	0.82 ± 0.40 n= 40	0.80
12/22	**11**	1.32 ± 1.29 n=18	0.35	0.88 ± 0.38 n=44	0.26	0.90 ± 0.57 n=62	0.16
	12/22	1.37 ± 0.97 n=9	0.39	0.71 ± 0.11 n=7	0.54	0.86 ± 0.47 n=16	0.64

Table 8.1 Geometric mean F1+2 levels (± approximate SD) across the combined Tm V/delTT and TNF-α -308G>A genotype groups in EW and IA within EDSC.

Analysis performed on ln-transformed data. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

		EW		IA		EW+IA	
Tm -1208/09TT>delTT	**TNF-α -308G>A**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**	**F1+2 µmol/l Geometric mean±SD (n)**	**P-value**
11	**11**	1.10 ± 0.70 n=70	Reference	0.80 ± 0.38 n=107	Reference	0.80 ± 0.43 n=177	Reference
	12/22	1.13 ± 0.57 n=21	0.81	0.90 ± 0.40 n=16	0.35	0.84 ± 0.40 n=47	0.55
12/22	**11**	1.32 ± 1.29 n=18	0.29	0.88 ± 0.38 n=44	0.26	0.90 ± 0.57 n=62	0.14
	12/22	1.37 ± 0.97 n=9	0.34	0.71 ± 0.11 n=7	0.51	0.86 ± 0.47 n=16	0.62

Table 8.2 Geometric mean F1+2 levels (± approximate SD) across the combined Tm -1208/09TT>delTT and TNF-α -308G>A genotype groups in EW and IA within EDSC.

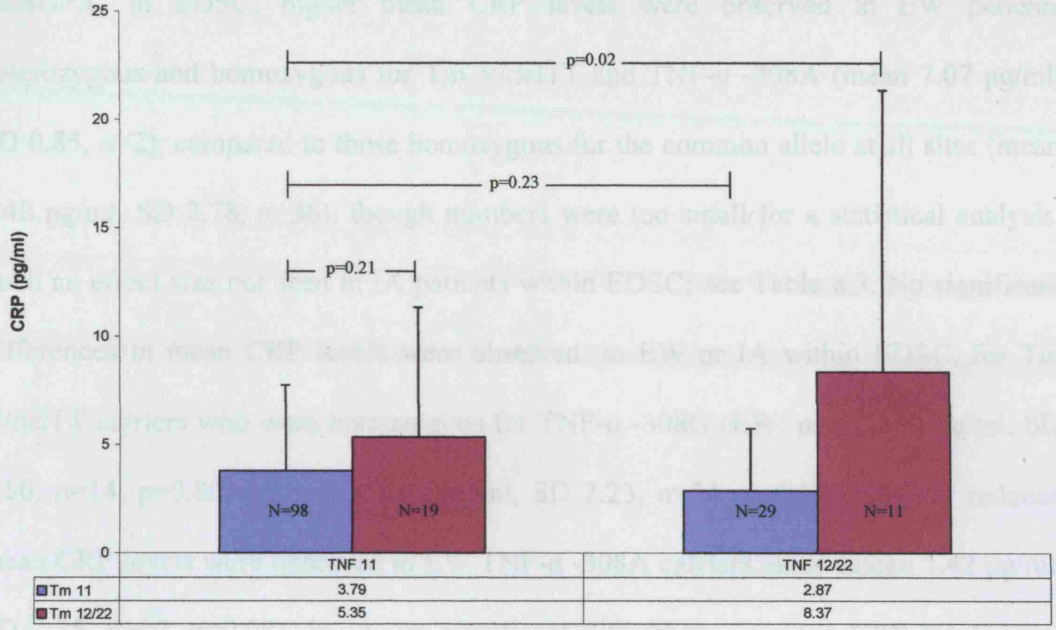
Analysis performed on ln-transformed data. **Tm -1208/09TT>delTT**: **TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

8.4.4 Association of Tm genotype, in combination with TNF-α -308G>A, with a marker of inflammation

In NPHSII, CRP levels had been measured in 587 randomly selected baseline samples, using a commercial assay (Cordia High Sensitivity CRP). CRP levels had been determined also in randomly selected EDSC samples (n=212), using a commercial assay (DiaMed, Switzerland). CRP levels were also analysed by Tm and TNF-α genotype. Due to the low numbers of rare homozygotes, these were grouped with heterozygotes for analysis.

In NPHSII diabetics, mean CRP levels were significantly higher in men heterozygous and homozygous for Tm V/delTT and TNF-α -308A (mean 8.37 pg/ml, SD 12.99, n=11, p=0.02), compared to those homozygous for the common allele at all sites (mean 3.79 pg/ml, SD 3.96, n=98); see Figure 8.7A. No significant differences in mean CRP levels were observed in Tm V/delTT carriers who were homozygous for TNF-α -308G (mean 5.35 pg/ml, SD 5.99, n=19, p=0.21) or in TNF-α -308A carriers alone (mean 2.87 pg/ml, SD 2.88, n=29, p=0.23), compared to those with the common allele at all sites; see Figure 8.7A. Similarly, significantly higher mean CRP levels were noted in Tm -1208/09delTT and TNF-α -308A carriers (mean 8.17 pg/ml, SD 11.80, n=13, p=0.02), compared to those homozygous for the common allele at both sites (mean 3.89 pg/ml, SD 4.05, n=115); see Figure 8.7B. No significant differences in mean CRP levels were observed for Tm and TNF-α genotypes alone (Tm -1208/09TT>delTT: mean 4.74 pg/ml, SD 5.92, n=21, p=0.45; TNF-α -308G>A: mean 2.95 pg/ml, SD 2.91, n=41, p=0.16), compared to those homozygous for the common allele at both sites; see Figure 8.7B. Numbers were too small to analyse similarly for cases and controls separately.

A.



B.

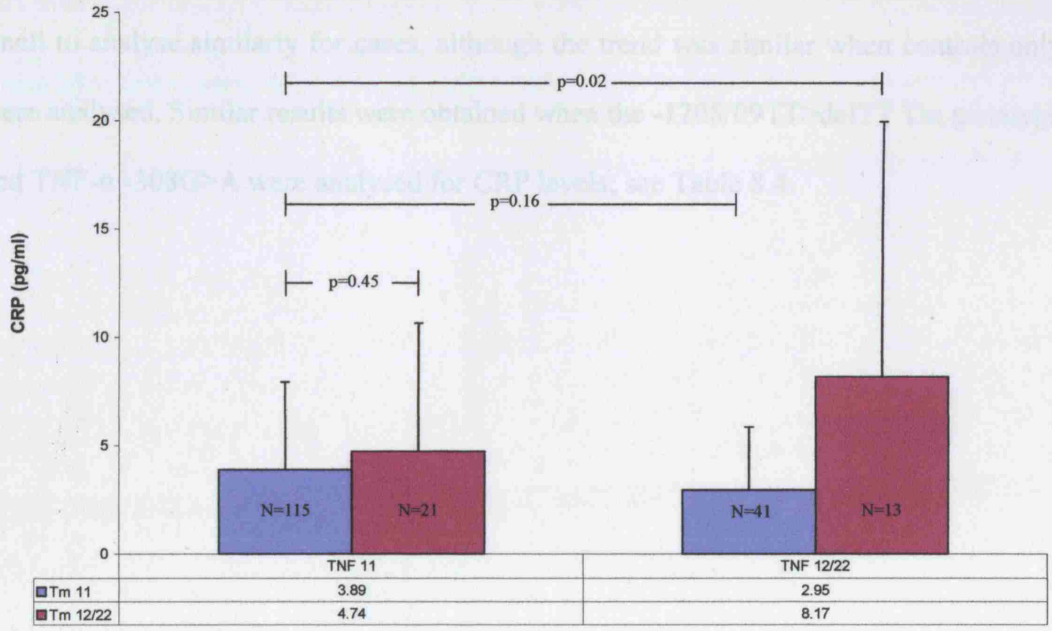


Figure 8.7 Geometric mean CRP levels (± approximate SD) across the combined Tm and TNF-α genotype groups in NPHSII diabetics.
A. Tm V/delTT. **B.** Tm -1208/09TT>delTT. Analysis performed on ln-transformed data. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **Tm -1208/09TT>delTT**: **TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

When CRP levels were analysed by Tm genotype, in combination with TNF-α -308G>A, in EDSC, higher mean CRP levels were observed in EW patients heterozygous and homozygous for Tm V/delTT and TNF-α -308A (mean 7.07 pg/ml, SD 0.85, n=2), compared to those homozygous for the common allele at all sites (mean 2.40 pg/ml, SD 2.78, n=36), though numbers were too small for a statistical analysis. Such an effect was not seen in IA patients within EDSC; see Table 8.3. No significant differences in mean CRP levels were observed, in EW or IA within EDSC, for Tm V/delTT carriers who were homozygous for TNF-α -308G (EW: mean 2.62 pg/ml, SD 2.50, n=14, p=0.80; IA: mean 1.69 pg/ml, SD 2.23, n=34, p=0.19). Slightly reduced mean CRP levels were observed in EW TNF-α -308A carriers alone (mean 1.42 pg/ml, SD 1.35, n=19, p=0.08). In IA, no significant differences in mean CRP levels were observed for TNF-α -308A carriers alone (mean 2.45 pg/ml, SD 1.57, n=10, p=0.93), compared to those with the common allele at all sites; see Table 8.3. Numbers were too small to analyse similarly for cases, although the trend was similar when controls only were analysed. Similar results were obtained when the -1208/09TT>delTT Tm genotype and TNF-α -308G>A were analysed for CRP levels; see Table 8.4.

		EW		IA		EW+IA	
Tm V/delTT	**TNF-α -308G>A**	**CRP pg/ml Geometric mean±SD (n)**	**P-value**	**CRP pg/ml Geometric mean±SD (n)**	**P-value**	**CRP pg/ml Geometric mean±SD (n)**	**P-value**
11	**11**	2.40 ± 2.78 n=36	Reference	2.36 ± 2.86 n=70	Reference	2.29 ± 2.72 n=106	Reference
	12/22	1.42 ± 1.35 n=19	0.08	2.45 ± 1.57 n=10	0.93	1.59 ± 1.44 n=29	0.16
12/22	**11**	2.62 ± 2.50 n=14	0.80	1.69 ± 2.23 n=34	0.19	1.86 ± 2.27 n=48	0.30
	12/22	7.07 ± 0.85 n=2	-	1.65 ± 1.43 n=3	-	2.82 ± 2.71 n=5	-

Table 8.3 Geometric mean CRP levels (± approximate SD) across the combined Tm V/delTT and TNF-α -308G>A genotype groups, in EW and IA within EDSC.

Analysis performed on ln-transformed data. **Tm V/delTT**: **AA/TT** (11) = homozygous for both common alleles. **V/delTT** (12/22) = heterozygous or homozygous for both rare alleles. **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

		EW		IA		EW+IA	
Tm -1208/09TT>delTT	**TNF-α -308G>A**	**CRP pg/ml Gemotric mean±SD (n)**	**P-value**	**CRP pg/ml Geometric mean±SD (n)**	**P-value**	**CRP pg/ml Geometric mean±SD (n)**	**P-value**
11	**11**	2.55 ± 2.91 n=44	Reference	2.31 ± 2.68 n=78	Reference	2.30 ± 2.64 n=122	Reference
	12/22	1.59 ± 1.68 n=21	0.11	2.63 ± 1.72 n=11	0.73	1.76 ± 1.71 n=32	0.25
12/22	**11**	2.62 ± 2.50 n=14	0.93	1.68 ± 2.91 n=35	0.18	1.85 ± 2.24 n=49	0.26
	12/22	7.07 ± 0.85 n=2	-	1.65 ± 1.43 n=3	-	2.82 ± 2.71 n=5	-

Table 8.4 Geometric mean CRP levels (± approximate SD) across the combined Tm -1208/09TT>delTT and TNF-α -308G>A genotype groups, in EW and IA within EDSC.
Analysis performed on ln-transformed data. **Tm -1208/09TT>delTT**: **TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22).

8.4.5 Effects of genotype on sTm measurements (EDSC)

In EDSC, circulating levels of sTm were measured, using activity (act) and antigen (ag) assays, see section 2.2.1. sTm levels were not available in NPHSII. *In vitro* reporter gene analysis, presented in this thesis (see Chapter 7), demonstrated increased sensitivity for the -1208/09TT>delTT variant to downregulation by inflammatory cytokines. As such the effect of the -1208/09TT>delTT variant, in combination with TNF-α -308G>A, upon sTm levels was assessed in the current study. The hypothesis was: Exacerbated downregulation of Tm, in individuals with the -1208/09delTT genotype, in response to inflammatory cytokines, may contribute to reduced Tm on the surface of vascular endothelial cells, which may in turn be reflected by reduced plasma sTm levels. As shown in Table 8.5, no significant differences were observed in plasma sTm measurements by combined Tm and TNF-α genotype in either ethnic group, following adjustment for creatinine. Numbers were too small to analyse in cases and controls separately. As mentioned earlier (see Chapter 5), sTm levels correlated well with creatinine levels in the EDSC study ($r>0.5$, $p<0.001$) and contributed significantly to variance in all three plasma sTm assays ($p<0.001$). Elevated creatinine levels may indicate impaired renal function or may point to generalized vascular disease, both of which have been shown to influence circulating levels of sTm (Ishii and Majerus, 1985) (Kumada et al., 1988) (Takano et al., 1990) (Ishii et al., 1991) (Inukai et al., 1996) (Borawski et al., 2001). As such the effect of the -1208/09TT>delTT variant, in combination with TNF-α -308G>A, upon sTm levels was analysed in those subjects with low creatinine levels ($\leq$ 83 μmol/l; median creatinine), in an attempt to account for these adverse effects. Again, there was no evidence to suggest an effect of the combined Tm and TNF-α genotype on plasma sTm measurements in those with low creatinine levels.

	Tm -1208/09TT>delTT	TNF-α -308G>A	DS sTm ag (ng/ml) Geometric mean±SD (n)	P-value	IH sTm ag (SEq/ml) Geometric mean±SD (n)	P-value	IH sTm act (SEq/ml) Geometric mean±SD (n)	P-value
EW	11	11	54.9 ± 17.3 n=104	Ref.	4.74 ± 1.64 n=93	Ref.	3.37 ± 0.93 n=100	Ref.
		12/22	55.4 ± 14.3 n=55	0.86	4.94 ± 1.32 n=43	0.52	3.54 ± 0.77 n=55	0.27
	12/22	11	54.7 ± 18.9 n=27	0.95	5.45 ± 2.63 n=23	0.09	3.32 ± 0.96 n=26	0.81
		12/22	58.3 ± 20.7 n=16	0.47	4.58 ± 1.17 n=15	0.72	3.20 ± 0.89 n=15	0.48
IA	11	11	49.6 ± 16.2 n=182	Ref.	4.36 ± 1.31 n=170	Ref.	3.30 ± 0.83 n=194	Ref.
		12/22	47.0 ± 13.5 n=29	0.44	4.27 ± 1.09 n=29 p	0.76	3.22 ± 0.79 n=32	0.58
	12/22	11	54.6 ± 23.1 n=64	0.15	4.81 ± 1.96 n=68	0.13	3.37 ± 0.92 n=75	0.59
		12/22	48.9 ± 9.9 n=13	0.89	4.30 ± 1.00 n=15	0.87	3.35 ± 0.73 n=15	0.84
EW+IA	11	11	51.8 ± 16.8 n=286	Ref.	4.43 ± 1.41 n=263	Ref.	3.32 ± 0.87 n=294	Ref.
		12/22	51.2 ± 13.6 n=84	0.80	4.54 ± 1.20 n=72	0.60	3.40 ± 0.77 n=87	0.47
	12/22	11	54.9 ± 22.3 n=91	0.13	4.91 ± 2.12 n=91	0.11	3.33 ± 0.95 n=101	0.88
		12/22	51.1 ± 12.9 n=29	0.83	4.27 ± 0.99 n=30	0.56	3.23 ± 0.78 n=30	0.59

Table 8.5 sTm measurements by Tm -1208/09TT>delTT in combination with TNF-α in EW and IA subjects within EDSC.
Adjusted for creatinine. Geometric mean ± approximate SD shown. Analysed by ANOVA after ln-transformation. **Tm -1208/09TT>delTT**: **TT/TT** (11), **TT/delTT** (12), **delTT/delTT** (22). **TNF-α -308G>A**: **GG** (11), **GA** (12), **AA** (22). Ref: Reference.

8.5 Discussion

The aim of this section was to assess contribution to CHD risk by Tm genotype (V/delTT or -1208/09TT>delTT alone), in combination with the TNF-α -308G>A variant, in an attempt to expand the mechanism through which Tm genotype may decrease protection against CHD, particularly in metabolic syndrome. Analysis was carried out in type 2 diabetic subjects due to the clinically detrimental effect of Tm -1208/09delTT in an environment of metabolic syndrome; see Chapters 3 & 4.

Carriers of both Tm V/delTT and TNF-α -308A had a 5-fold increased risk for CHD in individuals within the prospective NPHSII study (n=3012) who developed type 2 diabetes over 15 years follow-up (n=212). V/delTT carriers homozygous for TNF-α -308G had a 3-fold increased risk of CHD, whereas TNF-α -308A alone did not appear to contribute to CHD risk. Similarly, increased risk was observed in Tm -1208/09delTT and TNF-α -308A carriers, and increased risk was identified for Tm -1208/09delTT alone. Increased TNF-α production, associated with the TNF-α -308G>A variant (Kroeger et al., 1997) (Kroeger et al., 2000), may contribute to a deleterious hyperinflammatory state, which may explain the increased risk for CHD in carriers of both Tm and TNF-α genotype compared to carriers of Tm genotype alone. These data would suggest that in the presence of an inflammatory challenge, Tm genotype contributes to CHD, and that the risk effect is mediated in part by the promoter variant.

Contribution to CHD risk by Tm genotype, in combination with TNF-α -308G>A, was also assessed in a cross sectional study of type 2 diabetes (EDSC). The lack of a significant contribution to CHD risk in EW and IA within the cross sectional study of diabetes

(EDSC), appears to conflict with the data in NPHSII. However, case-control cross sectional studies are prone to intrinsic bias, for example due to altered rates of disease progression, subsequent progression of secondary phenotypes, or genotype associations with death or treatment changes. An example of this would be if the Tm/TNF-α combined genotype was associated with increased risk for CHD. Tm/TNF-α individuals might have a higher mortality in association with clinical and biochemical phenotypes associated with diabetes. The lack of an effect might also be due to the use of pharmacological treatments known to alter progression of CHD, such as lipid lowering drugs and aspirin. Such influences are well documented confounders (Risch, 2000) (Humphries et al., 2003) (Sing et al., 2003).

There was tentative evidence to suggest risk for family history of CHD conferred by Tm genotype in combination with TNF-α -308G>A, in both EW and IA within EDSC. EW and IA patients heterozygous and homozygous for Tm V/delTT and TNF-α -308A exhibited an increased risk for family history of CHD. An analogous increase in family history of CHD was observed in patients heterozygous and homozygous for both -1208/09delTT and TNF-α -308A, compared to those homozygous for the common allele at both sites. This finding would support a contribution to CHD by variants present in the Tm gene. These results have to be interpreted with caution. The statistical power of the cross sectional study is limited by its size. Although apparently suggestive, no firm conclusion about risk for family history of CHD associated with Tm genotype in combination with TNF-α can be made solely on the basis of this study.

There was no evidence to suggest an effect of Tm genotype, when considered alone or in combination with TNF-α -308G>A, upon F1+2 levels in either diabetics within the prospective study (NPHSII) or the cross sectional study of type 2 diabetes (EDSC). The

lack of any effect of Tm genotype, upon thrombin generation (F1+2 levels) would suggest that coagulation activation status is not influenced by this Tm variant. Tm on the surface of vascular endothelial cells is perhaps not rate limiting for thrombin inhibition or the small changes caused by the promoter variant does not impact upon thrombin generation. It should also be noted that plasma F1+2 levels may not reflect the total amount of thrombin being produced. Coagulation enzymes tend to associate with molecules present in plasma or receptors on cell membranes. Thrombin for instance binds to antithrombin in plasma (Rosenberg et al., 1975) (Stead et al., 1976) (Eisele and Lamy, 1998) (Mammen, 1998), Tm on endothelial cells (Owen and Esmon, 1981) (Esmon et al., 1982b) (Esmon, 1989) and PAR on numerous cells, including platelets and endothelial cells (Chung et al., 1990) (Rasmussen et al., 1991) (Vu et al., 1991). Similarly, F1+2 fragments can associate with cell membranes (Spronk et al., 2004).

On the other hand, elevated CRP levels were observed in diabetics (within the prospective NPHSII study) heterozygous and homozygous for Tm V/delTT and TNF-α -308A. Similarly, higher CRP levels were noted in Tm -1208/09delTT and TNF-α -308A carriers. A similar trend was observed in EW within EDSC, though numbers for the rare alleles were too small for a statistical analysis. An associated increased inflammatory response was noted for V/delTT carriers earlier in this thesis; see Chapters 3 & 4. These data would suggest that in response to an inflammatory challenge, Tm genotype might operate on CHD risk by compromising the direct anti-inflammatory potential of Tm, as well as through long-term affects on APC and its roles in vascular inflammation (Esmon, 2001) (Esmon, 2002); see section 1.4.3. Firm conclusions cannot, however, be drawn from these results as observations are based on small numbers. As such the possibility for chance findings

cannot be excluded. Interestingly, no genotype effect on CRP levels was seen in IA patients.

The effect of Tm genotype, in combination with TNF-α -308G>A, upon sTm levels was assessed in EDSC to determine whether downregulation of Tm, in individuals with the -1208/09delTT genotype, in response to inflammatory cytokines, may contribute to reduced Tm on the surface of vascular endothelial cells, which may in turn be reflected by plasma sTm levels. No significant differences were, however, observed in plasma sTm measurements by combined Tm and TNF-α genotype in either ethnic group. As such conclusions as to whether plasma sTm levels reflect Tm expression on the endothelium cannot be made on the basis of the current results.

The causes for lack of an effect by Tm genotype (shown to reduce Tm expression *in vitro*; see Chapter 7) on sTm levels in the current study may be multifactorial. Numerous environmental factors are known to contribute to circulating plasma sTm levels, including pro-inflammatory cytokines and endothelial cell injury (a crucial early event in the development of atherosclerosis) (Takano et al., 1990) (Ishii et al., 1991) (Boehme et al., 1996). Elevated sTm levels have been identified in various clinical conditions including cardiovascular disease and diabetes (Inukai et al., 1996) (Ohlin et al., 1996). The extent to which sTm levels reflect Tm reduced expression, including any genetic effects, on the endothelium may be hindered by these environmental factors causing shedding of sTm. The presence of other variant sites either in the Tm gene itself or in genes regulating Tm expression may also influence the ability to detect an association between Tm genotype and levels. Haplotype analysis across the gene, including variants specific to different ethnic groups, will help address this.

Reasons for the differences in effect of Tm genotype in EW and IA are not entirely clear. IA have an increased risk for type 2 diabetes and CHD compared to White populations (Balarajan, 1991) (Balarajan, 1995) (Chaturvedi, 2003). Discrepancies between gene-gene and gene-environment interactions may differ between IA and EW populations, with variants only showing their effects in populations with a particular genetic or environmental background (Talmud and Humphries, 2002). Further studies are required to address these issues.

In summary, these results suggest that in the presence of an inflammatory challenge, Tm genotype contributes to risk for CHD, and that the risk effect is mediated in part by the promoter variant. An associated increased inflammatory response, measured by CRP levels, would suggest that downregulation of Tm expression is likely to compromise the anti-inflammatory potential on the surface of vascular endothelium, thus contributing to risk for CHD.

CHAPTER NINE

DISCUSSION AND FUTURE WORK

9. Discussion and future work

9.1 Thesis conclusion

Inflammatory mediators play a key role in the initiation, progression and thrombotic complications of atherosclerotic plaques. The clinical manifestation of CHD results from the development of atherosclerotic plaques in the coronary arteries feeding the heart, which when ruptured lead to occlusive thrombus formation. The blood coagulation cascade, triggered by inflammatory events, plays a major role in coronary thrombosis, but the way in which perturbed coagulation contributes to disease remains uncertain. Evidence has been presented supporting the concept that compromised production, function and availability of haemostatic factors, such as fibrinogen, FVII, vWF, PAI-1 and t-PA, alter the physiological haemostatic balance, favouring thrombosis, thus contributing to atherothrombotic events (Wilhelmsen et al., 1984) (Meade et al., 1986) (Ridker and Hennekens, 1991). A pro-coagulant imbalance would favour formation of excessive amounts of thrombin, the pivotal enzyme of the coagulation cascade. In addition to promoting clot formation (Gailani and Broze, Jr., 1991) (Brummel et al., 1999), thrombin acts as a mitogen for lymphocytes, fibroblasts and vascular SMCs (Chen and Buchanan, 1975), stimulates human platelet activation and aggregation (Harmon and Jamieson, 1986) (Bizios et al., 1986) (Huang and Detwiler, 1987), is chemotactic to monocytes (Bar-Shavit et al., 1983a) (Bar-Shavit et al., 1983b) and neutrophils (Sehnal and Slany, 2002), and induces the expression of leukocyte adhesion molecules and cytokines on the surface of vascular endothelial cells (Colotta et al., 1994) (Ueno et al., 1996). By triggering these inflammatory and proliferative processes, which are central to the development of atherosclerosis, thrombin is likely to play a central role in development of arterial thrombosis (Coughlin et al., 1992).

Control of its activity and generation forms an important haemostatic function preventing occlusive blood clot formation. Thus genes coding for key proteins in the modulation of thrombin and fibrin formation are strong candidates for CHD risk factors. One of these proteins, Tm, is expressed at the endothelial cell surface and has the ability to minimize pro-coagulant, pro-inflammatory and growth-promoting effects of thrombin. Moreover, it has the ability to accelerate PC activation, which in turn downregulates the propagation of the coagulation cascade. Furthermore, Tm has direct anti-inflammatory properties (Conway et al., 2002); see section 1.4.3.4. As such, Tm may have a role in modifying susceptibility to occlusive thrombotic disease, particularly in the microvasculature (such as the coronaries) where the density of Tm expression is highest. The main aim of this thesis was to investigate whether genetic variation in Tm could influence the risk for CHD and to assess the molecular mechanisms of the effect of the variants showing strong effects on risk or plasma phenotypes, to further our understanding of the role of Tm in the pathogenesis of CHD.

The analysis of several previously identified, potentially functional Tm variants in a case-control Caucasian cohort (HIFMECH) and a prospective Caucasian study (NPHSII) yielded interesting results. A haplotype comprising the rare alleles for two common Tm variants (-1208/09TT>delTT & A455V; V/delTT) contributed to the risk of MI, in the North of Europe, particularly in individuals with obesity, metabolic syndrome or inflammation; see Chapter 3. These findings were subsequently extended in a large prospective study (NPHSII), where a trend towards increased risk was observed in those with increased BMI and a significant interaction with increased triglyceride levels was identified; see Chapter 4.

While a suggestion for increased risk for 455V alone (i.e V/TT), in both Northern and Southern Europeans, was identified in the case-control, HIFMECH (OR≥1.36), but did not reach statistical significance, no suggestion for increased risk was observed in the prospective study, NPHSII (RR 0.9). It is possible that both studies were underpowered to determine any significant effect. However, these results may suggest that risk for V/delTT is acting through either the combination of 455V and -1208/09delTT or through the distal promoter variant alone.

Past association studies of the effect of A455V genotype have shown both risk and protection associated with the 455V allele in different populations (Norlund et al., 1997a) (Wu et al., 2001). In a study of 159 healthy Swedish volunteers and 97 patients who had suffered an acute MI event before the age of 50, the A455 allele was more frequent amongst the MI survivors than controls (Norlund et al., 1997a). By contrast, a study of 376 incident CHD cases and 461 healthy controls demonstrated that the presence of the 455V allele was associated with a 6.1-fold increased risk of CHD in the Black but not the Caucasian population (Wu et al., 2001). It is possible that this discrepancy is due to differences in the haplotype structure in different populations.

The -1208/09TT>delTT variant was found to be in strong LD with A455V, in the HIFMECH (see Chapter 3), NPHSII (see Chapter 4) and all ethnic groups within EDSC, in agreement with previous reports (Le Flem et al., 2001). Haplotype structure has been demonstrated to differ, in the current analysis, in the three ethnic groups. This is shown clearly in Table 5.3. In EW, there were approximately equal proportions of individuals having a V/TT (18%) and V/delTT (15%) allele. In IA, only 8% had a V/TT allele, compared to 23% with V/delTT. AC had very few individuals with a V/TT allele (3.4%),

but a similar proportion of individuals with a V/delTT allele (12.5%) as shown for EW. These findings would suggest that unlike the situation in EW and IA, genotyping for the A455V variant in AC will essentially reflect the V/delTT haplotype effect. Furthermore, it can be proposed that the elevation in CHD risk reported previously for the 455V allele in Black Americans (Wu et al., 2001) is detecting the Tm haplotype (V/delTT) effect.

Reporter gene analysis was used to investigate the effect of the promoter variants on transcriptional activity, under basal conditions and in response to inflammatory cytokines (TNF-α & IL-1β), to elucidate the molecular mechanisms responsible for the clinical effects observed. Interestingly, the -1208/09TT>delTT variant was found to have a significant deleterious effect on reporter gene expression following treatment with either TNF-α or IL-1β. Thus, the -1208/09TT>delTT variant may be more sensitive to downregulation by inflammatory cytokines. This finding would suggest a mechanism for the association of this variant site with CHD in metabolic syndrome, a condition in which increased levels of inflammatory cytokines have been identified (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999).

sTm levels were measured in a type 2 diabetic cohort (EDSC) to investigate the relationship of sTm with CHD. sTm levels were determined using in-house (ag and act) and commercial (ag) assays. Environmental contributors to sTm assay variance and effects of disease complications were evaluated. There was good correlation between sTm ag measurements and between sTm act and both sTm ag assays. Main contributors to variance for all sTm assays were creatinine, urea, proteinuria and SBP, which may indicate impaired renal function or the presence of generalized vascular disease. A small proportion of patients with lower sTm cofactor act than sTm ag levels was identified. There were also individuals

within this patient group who had low levels of both act and ag. These results may indicate that variants are present either within the Tm gene or other genes that influence Tm gene expression. When sTm act and ag levels were analysed across quartiles of their distributions, there was a trend towards an increase in the percentage of individuals within each quartile who had CHD themselves, across the last 3 quartiles for each sTm distribution, but this only reached significance for the DS sTm ag levels. Conversely, a statistically significant higher percentage of individuals with family history of CHD was identified in the lowest quartile for each sTm measurement, which decreased across quartiles reaching the lowest percentage in the 4^{th} quartile. This finding extends the findings of a previous study that evaluated the relationship between CHD and sTm in a nested case-control study, taken from a prospective study (ARIC), which demonstrated an inverse association between incident CHD and circulating levels of plasma Tm (Salomaa et al., 1999).

It has been suggested that plasma sTm levels, in healthy individuals, may result from the constitutive cleavage of membrane bound Tm, and may thus reflect the levels of Tm synthesized and expressed in endothelial cells and may be, in part, genetically determined (Salomaa et al., 1999) (Kunz et al., 2000). Firm conclusions as to whether plasma sTm levels are genetically determined cannot be made on the basis of the current results. No statistically significant differences were noted in plasma sTm measurements for a novel Tm variant (1261G>A; E364E), identified in the Tm coding region of individuals shown to have either lower sTm activity than antigen levels or low levels of both activity and antigen, although numbers were small for the rare allele. No other coding region mutation was identified by sequencing, for those with the lowest levels of sTm.

Whereas no overall significant effect of Tm genotype (V/delTT) upon sTm levels could be determined, the percentage of individuals who were heterozygous and homozygous for both rare alleles was higher in those within the lowest 5th percentile of the sTm sTm IH act distribution, compared to those in the the remaining 95 percentiles. These results may suggest that the A455V variant, which lies in the 6th EGF domain of Tm (van der Velden et al., 1991) (a region with a key role in thrombin binding and activation of protein C (Kurosawa et al., 1988) (Hayashi et al., 1990) (Tsiang et al., 1992) (Ye et al., 1992), may compromise the activity of the Tm protein. Firm conclusions cannot, however, be drawn from these results as observations are based on small numbers. As such the possibility for chance findings cannot be excluded. Analysis of large studies is required to expand the results obtained here and to investigate further the contribution A455V makes to altered function and CHD risk, in the context of the V/delTT Tm haplotype.

The lack of any association of the Tm -1208/09TT>delTT genotype, shown to reduce transcription of the gene in response to inflammatory cytokines, with altered sTm ag levels questions the capacity of sTm ag levels to reflect Tm expression on the endothelium. Numerous environmental factors are known to contribute to circulating plasma sTm levels, including pro-inflammatory cytokines and endothelial cell injury (a crucial early event in the development of atherosclerosis) (Takano et al., 1990) (Ishii et al., 1991) (Boehme et al., 1996). Elevated sTm levels have been identified in various clinical conditions including cardiovascular disease and diabetes (Inukai et al., 1996) (Ohlin et al., 1996). In particular, IL-1β and TNF-α, while shown to further down-regulate the Tm promoter in these studies, also increase shedding of Tm (Takano et al., 1990) (Boehme et al., 1996). The extent to which sTm levels reflect Tm expression, including any genetic effects, on the endothelium may be hindered by these environmental factors. The presence of other variant sites either

in the Tm gene itself or in genes regulating Tm expression may also influence the ability to detect an association between Tm genotype and levels. These gene-gene and gene-environment interactions may affect the capacity to detect any significant association. This may only be elucidated using Tm haplotype analysis across the gene and by extending sequencing of those with low sTm levels to the promoter and 3'UTR regions. This analysis was extended in part in this thesis by assessment in combination with a TNF-α genotype, see below.

The contribution to CHD risk by Tm genotype (V/delTT and -1208/09TT>delTT alone), in combination with the TNF-α -308G>A variant, was assessed in an attempt to expand the mechanism through which Tm genotype may decrease protection against the increased concentration of cytokines found in metabolic syndrome. TNF-α -308G>A has previously been associated with increased TNF-α production (Kroeger et al., 1997) (Kroeger et al., 2000) (Vendrell et al., 2003). The analysis of Tm genotype (V/delTT and -1208/09TT>delTT alone) in carriers of TNF-α -308G>A in individuals within the prospective NPHSII study who developed type 2 diabetes over 15 years follow-up, and a cross sectional study of type 2 diabetes (EDSC) yielded interesting results. Tm genotype (V/delTT & -1208/09TT>delTT) was associated with increased CHD risk in diabetics within the prospective study, particularly in carriers of the TNF-α -308A allele. These data would suggest that in the presence of an inflammatory challenge, Tm V/delTT contributes to CHD, potentially reflecting downregulation of the V/delTT allele by increased TNF-α.

When the effect upon sTm levels was assessed for Tm genotype in combination with TNF-α -308G>A in a cross sectional study of type 2 diabetes (EDSC), no significant differences were observed in the different ethnic groups. Neither was there evidence to

suggest an effect of Tm genotype, when considered alone or in combination with TNF-α -308G>A, upon thrombin generation (F1+2 levels) in either diabetics within the prospective study (NPHSII), or in the diabetic study (EDSC). Tm genotype in combination with TNF-α -308G>A was, however, associated with an increased inflammatory response in White diabetic individuals (NPHSII & EDSC), as measured by CRP levels.

The Tm-protein C pathway has both anti-thrombotic and anti-inflammatory characteristics. As such, downregulation of Tm expression may lead to increased thrombin generation and exacerbation of inflammation. The lack of any effect of the Tm haplotype V/delTT on thrombin generation (F1+2 levels) would suggest that the ability to regulate thrombin is not influenced by this Tm variant, although the haplotype contributed to CHD. It could be argued perhaps that changes in thrombin generation would not be expected in a healthy population, by a genotype in the PC pathway. However, this group has shown that a variant identified in another endothelial cell surface protein (EPCR; Ser219Gly), which participates in the PC pathway, has a significant effect upon F1+2 levels, in baseline samples from NPHSII (Ireland et al., 2005). As such, perhaps small changes in Tm expression on the surface of vascular endothelial cells may not be detrimental for thrombin generation, or perhaps Tm may not be rate limiting for thrombin inhibition. The suggestion of an increased inflammatory response (CRP) on the other hand would suggest that V/delTT might operate on CHD risk by compromising the direct anti-inflammatory potential of Tm.

In light of the current findings, one potential mechanism contributing to the clinically detrimental effect of the Tm gene variation in an environment of metabolic syndrome may be through an interaction with elevated levels of pro-inflammatory adipocytokines, reported in metabolic syndrome. As mentioned in section 1.3.2, the metabolic syndrome is

characterised by the clustering of insulin resistance with established cardiovascular risk factors, such as increased triglyceride levels, increased systolic blood pressure, HDL-cholesterol levels, obesity and hyperglycemia (Kaplan, 1989) (Haffner et al., 1992). Obese individuals have a higher risk of developing CHD compared to lean individuals (Lamarche, 1998) (Stevens et al., 1998) and there is increasing evidence that adipose tissue in general and abdominal adiposity in particular, is a key player in the inflammatory process (Yudkin, 1999). Adipose tissue has been demonstrated to secrete pro-inflammatory cytokines (Hotamisligil et al., 1995) (Kern et al., 1995) (Fried et al., 1998) (Yudkin et al., 1999). Interaction between Tm genotype and other aspects of the metabolic syndrome, however, may also contribute to CHD risk.

In conclusion, the CHD risk association data and subsequent functional work completed in this thesis suggests a contribution to CHD risk caused by variants or mutations in the Tm gene. Evidence is also presented that, at least in patients with type 2 diabetes, low sTm levels contribute to a genetic tendency towards CHD, although the genetic causes could not be fully elucidated. Specifically, a haplotype comprising the rare alleles for two common variants (V/delTT) in the Tm gene influences risk for CHD in an environment of metabolic syndrome through an interaction with elevated levels of pro-inflammatory cytokines. Furthermore, evidence has been presented in this thesis that this Tm haplotype may lead to an increased inflammatory response. These findings suggest a cycle of events whereby Tm variant plus inflammation leads to Tm downregulation and further inflammation. There is tentative evidence that this Tm haplotype, through the A455V, site may compromise the activity of the Tm protein. The increased percentage of individuals with a family history of CHD in the lowest quartile for each sTm measurement may imply a genetic nature to CHD risk conferred by low sTm levels.

9.2 Study limitations

An inherent limitation of any case-control study, and thus HIFMECH and EDSC, is that only survivors can be studied. This could lead to an underestimation of risk associated with factors having a greater impact on survival, as subjects with these factors will not be available for analysis. However, because higher numbers of cases can be studied than is possible for prospective studies, they are useful for the first analysis of a candidate gene. This limitation was addressed in part by subsequent analysis of a prospective study (NPHSII), where individuals who have not yet had the outcome event in question are monitored over several years. Results in HIFMECH and NPHSII cannot be generalized to women and other ethnic groups since these studies used only Caucasian middle-aged men. Further, it is recognized that in EDSC, the problems inherent in case-control studies may be exacerbated. However, EDSC was useful to assess plasma protein phenotypes and a family history of CHD, the latter of which will be less affected by survival bias. Furthermore, determining risk for two variant sites within the Tm gene does not capture all relevant variation within the gene. The possibility also that the Tm haplotype (V/delTT) is marking additional functional variants important in CHD either within or outside the Tm gene can thus not be excluded.

Concerning the genetic analysis carried out to determine whether variants in the Tm gene contribute to altered circulating sTm levels, it should be noted that screening was confined to the coding region of the Tm gene. Therefore, it cannot be excluded that novel variants, contributing to altered sTm levels, may occur in the promoter or 3'UTR regions of the Tm gene. Similarly, the possibility that variants present in other genes regulating Tm expression contribute to altered circulating sTm cannot be excluded either.

As far as the reporter gene analysis is concerned, results must be extrapolated with care to the *in vivo* situation, as they can be confounded by a number of factors. These factors include the type of cells used, whether cells are at basal state or stimulated by exogenous agents, and by the presence or absence of additional positive or negative regulatory elements contained within the sequence tested. Furthermore, varied lengths of DNA are used in constructs, which are divorced from their chromatin context and the influence of enhancer or inhibitory regulatory elements that would further influence gene transcription (Daley and Cargill, 2001).

The lack of adjustment for multiplicity of testing might be considered a weakness in the statistical analysis. Correction for multiple comparisons was not applied to the results, because the study design was predominantly 'hypothesis testing'. It is recognised that adjusting statistical significance for the number of tests that have been performed on study data (the Conferring method) may create more problems than it solves (Rothman, 1990) (Perneger, 1998). The Conferring method is concerned with the general null hypothesis (that all null hypotheses are true simultaneously), which is rarely of interest or use to researchers exploring novel hypotheses in medical research. Furthermore, Conferring adjustments do not guarantee a 'prudent' interpretation of results (Perneger, 1998). The likelihood of missing truly important differences is also increased.

9.3 Future work

As aforementioned, determining risk for two variant sites within the Tm gene does not capture all relevant variation within the gene. Haplotype analysis, using further SNPs, would allow associations of Tm with risk for CHD to be deduced fully. Very recently Auro et al. published results from a Tm haplotype structure and CHD in Finnish populations. Unfortunately, their study design did not allow them to report genotype-environment or genotype-phenotype associations, as small nested case-control studies were used (Auro et al., 2006). The study did, however, suggest risk for CHD associated with a haplotype containing the -1208/09delTT allele. The information available on the SNPper database could be used to generate tagging SNPs to cover 95% of the variation within the promoter (proximal and distal), coding region and 3'UTR in order to capture maximal variation within Tm in subsequent genotyping. Once tagging SNPs have been selected, haplotypes could be inferred by genotyping a small cohort of 50 people. The association of haplotypes with risk for CHD, as well as gene-environment interaction in relation to CHD risk and effects on intermediate phenotypes, would further the understanding of the role of Tm in the pathogenesis of CHD.

Pathological cleavage, induced by pro-inflammatory cytokines, may contribute to sTm circulating in human plasma (Takano et al., 1990) (Boehme et al., 1996), as well as causing downregulation of the Tm gene (Nawroth et al., 1986) (Archipoff et al., 1991) (Lentz et al., 1991) (von der Ahe et al., 1993) (Speiser et al., 2001) (Nan et al., 2005) (Sohn et al., 2005). As such, the determination of TNF-α and IL-1β measurements in EDSC would be of interest since it would allow the effect of inflammatory cytokines on sTm levels to be

assessed. This may help shed more light and further the understanding of the gene-environment interaction observed for Tm genotype.

In vitro investigation of HUVECs or monocytes selected by Tm haplotype for variant specific effects in response to inflammatory cytokines (TNF-α & IL-1β) would also be of interest. sTm act and ag in the supernatant, total Tm in the cell lysates, mRNA and total cell protein could be determined using this approach. Functional assessment of Tm activity on the surface of cells, selected by Tm haplotype, could be assessed by the ability of the variant site to activate PC in the presence of thrombin. In addition, flow cytometry could be used to assess cell surface expression. Monocytes have been shown to express Tm (McCachren et al., 1991), so it is possible that Tm expression on the surface of monocytes reflects that on the endothelium. These *in vitro* studies would help further the understanding of the mechanism for CHD risk interaction between Tm genotype and metabolic syndrome.

Reporter gene analysis to investigate the effect of the -1208/09TT>delTT promoter variant on transcriptional activity, in response to ox-LDL, would also be of interest. As mentioned earlier, Tm -1208/09TT>delTT lies in close proximity to a DR4 sequence (-1531 to –1516), which has been shown to play an important role in the regulation of Tm gene expression in response to ox-LDL (Ishii et al., 2003). Future investigations need also to examine the possibility that the -1208/09TT>delTT variant alters the binding of nuclear proteins to the 5'UTR of the Tm gene. This can be done using electrophoretic mobility shift assays (EMSA), a method that has been widely used in the study of sequence-specific DNA-binding proteins such as transcription factors. EMSA would help determine whether nuclear proteins bind to the promoter fragment of interest and assess whether the Tm deletion variant alters nuclear protein binding.

Finally, further work should also examine Tm expression in response to anti-inflammatory drugs. This will reveal whether anti-inflammatory treatments are able to counteract inflammatory cytokine-mediated downregulation, indicating a less pro-coagulant state of the endothelium, thus modifying susceptibility to occlusive thrombotic disease.

CONTRIBUTIONS TO THIS WORK

Contributions to this work by other researches are as follows:

1. Genomic DNA from participants of the HIFMECH, NPHSII and EDSC studies had been isolated previously by co-workers in the CVG laboratory.

2. Genotyping for TNF-α -308G>A was carried out by Julia Grizenkova, a Technician working with Dr Helen Ireland, BHF Grant Code: PG/04/018/16754.

3. Statistical analysis was performed with help from Emma Hawe and Jackie Cooper, both statisticians with the Centre for Cardiovascuar Genetics (CVG), UCL.

4. In house assays for Tm antigen and the cofactor activity in protein C activation were developed and measurements of plasma sTm levels in participants of the EDSC study performed (EDSC) by our collaborators in Sweden (Dr Ann Kristin Ohlin).

Appendix

Appendix 1

Ratio of firefly luminescence to *Renilla* luminescence for TNF-α and IL-1β dose response curves

Experiment	pTm Construct	Treatment	Relative Ratio
1	pTm WT	Untretated (TNF-α)	1.31
1	pTm WT	Untretated (TNF-α)	1.04
1	pTm WT	Untretated (TNF-α)	2.05
1	pTm WT	Untretated (TNF-α)	1.19
1	pTm WT	1 ng/ml TNF-α	0.28
1	pTm WT	1 ng/ml TNF-α	0.36
1	pTm WT	1 ng/ml TNF-α	0.52
1	pTm WT	1 ng/ml TNF-α	0.22
1	pTm WT	2 ng/ml TNF-α	0.76
1	pTm WT	2 ng/ml TNF-α	0.24
1	pTm WT	2 ng/ml TNF-α	0.54
1	pTm WT	2 ng/ml TNF-α	0.19
2	pTm WT	Untreated (TNF-α)	0.50
2	pTm WT	Untreated (TNF-α)	0.85
2	pTm WT	Untreated (TNF-α)	0.39
2	pTm WT	Untretated (TNF-α)	0.43
2	pTm WT	1 ng/ml TNF-α	0.20
2	pTm WT	1 ng/ml TNF-α	1.04
2	pTm WT	1 ng/ml TNF-α	0.29
2	pTm WT	1 ng/ml TNF-α	1.51
2	pTm WT	2 ng/ml TNF-α	0.19
2	pTm WT	2 ng/ml TNF-α	0.37
2	pTm WT	2 ng/ml TNF-α	0.25
2	pTm WT	2 ng/ml TNF-α	0.10
1	pTm WT	Untreated (IL-1β)	0.89
1	pTm WT	Untreated (IL-1β)	0.99
1	pTm WT	Untreated (IL-1β)	1.49
1	pTm WT	Untreated (IL-1β)	1.66
1	pTm WT	0.5 ng/ml IL-1β	1.45
1	pTm WT	0.5 ng/ml IL-1β	0.97
1	pTm WT	0.5 ng/ml IL-1β	0.75
1	pTm WT	0.5 ng/ml IL-1β	0.97
1	pTm WT	1 ng/ml IL-1β	1.17
1	pTm WT	1 ng/ml IL-1β	0.70
1	pTm WT	1 ng/ml IL-1β	0.50
1	pTm WT	1 ng/ml IL-1β	0.58
2	pTm WT	Untreated (IL-1β)	1.50
2	pTm WT	Untreated (IL-1β)	0.97
2	pTm WT	Untreated (IL-1β)	1.04
2	pTm WT	Untreated (IL-1β)	0.71
2	pTm WT	0.5 ng/ml IL-1β	0.97
2	pTm WT	0.5 ng/ml IL-1β	0.87
2	pTm WT	0.5 ng/ml IL-1β	1.26

2	pTm WT	0.5 ng/ml IL-1β	0.93
2	pTm WT	1 ng/ml IL-1β	0.86
2	pTm WT	1 ng/ml IL-1β	0.90
2	pTm WT	1 ng/ml IL-1β	0.20
2	pTm WT	1 ng/ml IL-1β	0.41

Appendix 2

Ratio of firefly luminescence to *Renilla* luminescence for TNF-α and IL-1β time course

Experiment	pTm Construct	Treatment	Relative Ratio
1	p Tm WT	Unreated (TNF-α)	5.88
1	p Tm WT	Unreated (TNF-α)	8.27
1	p Tm WT	2 ng/ml TNF-α (3 h)	9.63
1	p Tm WT	2 ng/ml TNF-α (3 h)	11.53
1	p Tm WT	2 ng/ml TNF-α (6 h)	7.43
1	p Tm WT	2 ng/ml TNF-α (6 h)	4.00
1	p Tm WT	2 ng/ml TNF-α (15 h)	2.27
1	p Tm WT	2 ng/ml TNF-α (15 h)	1.70
1	p Tm WT	2 ng/ml TNF-α (24 h)	4.37
1	p Tm WT	2 ng/ml TNF-α (24 h)	6.71
2	p Tm WT	Unreated (TNF-α)	11.66
2	p Tm WT	Unreated (TNF-α)	9.09
2	p Tm WT	2 ng/ml TNF-α (3 h)	10.35
2	p Tm WT	2 ng/ml TNF-α (3 h)	8.29
2	p Tm WT	2 ng/ml TNF-α (6 h)	4.25
2	p Tm WT	2 ng/ml TNF-α (6 h)	7.04
2	p Tm WT	2 ng/ml TNF-α (15 h)	2.48
2	p Tm WT	2 ng/ml TNF-α (15 h)	2.03
2	p Tm WT	2 ng/ml TNF-α (24 h)	3.88
2	p Tm WT	2 ng/ml TNF-α (24 h)	6.19
1	p Tm WT	Unreated (IL-1β)	9.46
1	p Tm WT	Unreated (IL-1β)	9.14
1	p Tm WT	2 ng/ml IL-1β (3 h)	3.93
1	p Tm WT	2 ng/ml IL-1β (3 h)	5.03
1	p Tm WT	2 ng/ml IL-1β (6 h)	3.57
1	p Tm WT	2 ng/ml IL-1β (6 h)	1.43
1	p Tm WT	2 ng/ml IL-1β (15 h)	0.97
1	p Tm WT	2 ng/ml IL-1β (15 h)	1.69
1	p Tm WT	2 ng/ml IL-1β (24 h)	2.89
1	p Tm WT	2 ng/ml IL-1β (24 h)	3.03
2	p Tm WT	Unreated (IL-1β)	13.55
2	p Tm WT	Unreated (IL-1β)	9.34
2	p Tm WT	2 ng/ml IL-1β (3 h)	4.56
2	p Tm WT	2 ng/ml IL-1β (3 h)	3.99
2	p Tm WT	2 ng/ml IL-1β (6 h)	4.81
2	p Tm WT	2 ng/ml IL-1β (6 h)	4.09
2	p Tm WT	2 ng/ml IL-1β (15 h)	1.26
2	p Tm WT	2 ng/ml IL-1β (15 h)	1.46
2	p Tm WT	2 ng/ml IL-1β (24 h)	2.32
2	p Tm WT	2 ng/ml IL-1β (24 h)	2.77

Appendix 3

Ratio of firefly luminescence to *Renilla* luminescence for pTm constructs under basal conditions

Experiment	Construct	Relative Ratio
1	pGL-3-Basic	0.02
1	pGL-3-Basic	0.01
1	pGL-3-Basic	0.01
1	pGL-3-Basic	0.01
1	pTm WT	1.01
1	pTm WT	0.56
1	pTm WT	0.52
1	pTm WT	0.33
1	pTm -1748G>C	0.42
1	pTm -1748G>C	0.60
1	pTm -1748G>C	0.46
1	pTm -1748G>C	0.57
1	pTm -1166G>A	0.62
1	pTm -1166G>A	0.51
1	pTm -1166G>A	0.91
1	pTm -1166G>A	0.79
1	pTm -1208/09TT>delTT	0.82
1	pTm -1208/09TT>delTT	0.51
1	pTm -1208/09TT>delTT	0.72
1	pTm -1208/09TT>delTT	0.66
2	pGL-3-Basic	0.00
2	pGL-3-Basic	0.00
2	pGL-3-Basic	0.01
2	pGL-3-Basic	0.01
2	pTm WT	1.37
2	pTm WT	1.62
2	pTm WT	1.64
2	pTm WT	2.25
2	pTm -1748G>C	1.70
2	pTm -1748G>C	1.77
2	pTm -1748G>C	1.70
2	pTm -1748G>C	1.86
2	pTm -1166G>A	1.50
2	pTm -1166G>A	1.66
2	pTm -1166G>A	2.10
2	pTm -1166G>A	2.35
2	pTm -1208/09TT>delTT	1.47
2	pTm -1208/09TT>delTT	1.14
2	pTm -1208/09TT>delTT	1.40
2	pTm -1208/09TT>delTT	1.04
3	pGL-3-Basic	0.01
3	pGL-3-Basic	0.01
3	pGL-3-Basic	0.01
3	pGL-3-Basic	0.01
3	pTm WT	0.63
3	pTm WT	0.91

3	pTm WT	1.01
3	pTm WT	1.62
3	pTm -1748G>C	0.58
3	pTm -1748G>C	0.41
3	pTm -1748G>C	0.37
3	pTm -1748G>C	0.51
3	pTm -1166G>A	0.65
3	pTm -1166G>A	0.42
3	pTm -1166G>A	0.64
3	pTm -1166G>A	0.58
3	pTm -1208/09TT>delTT	0.88
3	pTm -1208/09TT>delTT	0.45
3	pTm -1208/09TT>delTT	0.39
3	pTm -1208/09TT>delTT	0.29
4	pGL-3-Basic	0.00
4	pGL-3-Basic	0.00
4	pGL-3-Basic	0.00
4	pGL-3-Basic	0.00
4	pTm WT	0.41
4	pTm WT	0.32
4	pTm WT	0.54
4	pTm WT	0.40
4	pTm -1748G>C	0.48
4	pTm -1748G>C	0.42
4	pTm -1748G>C	0.60
4	pTm -1748G>C	0.37
4	pTm -1166G>A	0.40
4	pTm -1166G>A	0.40
4	pTm -1166G>A	0.36
4	pTm -1166G>A	0.43
4	pTm -1208/09TT>delTT	0.37
4	pTm -1208/09TT>delTT	0.37
4	pTm -1208/09TT>delTT	0.52
4	pTm -1208/09TT>delTT	0.33
5	pGL-3-Basic	0.02
5	pGL-3-Basic	0.02
5	pGL-3-Basic	0.02
5	pGL-3-Basic	0.02
5	pTm WT	0.70
5	pTm WT	0.88
5	pTm WT	1.02
5	pTm WT	0.86
5	pTm -1748G>C	1.39
5	pTm -1748G>C	-
5	pTm -1748G>C	1.29
5	pTm -1748G>C	1.48
5	pTm -1166G>A	1.63
5	pTm -1166G>A	1.50
5	pTm -1166G>A	1.42
5	pTm -1166G>A	1.60
5	pTm -1208/09TT>delTT	1.46
5	pTm -1208/09TT>delTT	0.94
5	pTm -1208/09TT>delTT	1.39
5	pTm -1208/09TT>delTT	0.78

Appendix 4

Ratio of firefly luminescence to *Renilla* luminescence for pTm constructs in response to TNF-α (1 ng/ml; 15 h)

Experiment	Construct	Treatment	Relative Ratio
1	pGL-3-Basic	Untreated (TNF-α)	0.05
1	pGL-3-Basic	Untreated (TNF-α)	0.06
1	pGL-3-Basic	Untreated (TNF-α)	0.05
1	pGL-3-Basic	Untreated (TNF-α)	0.05
1	pGL-3-Basic	1 ng/ml TNF-α	0.02
1	pGL-3-Basic	1 ng/ml TNF-α	0.03
1	pGL-3-Basic	1 ng/ml TNF-α	0.02
1	pGL-3-Basic	1 ng/ml TNF-α	0.02
1	pTm WT	Untreated (TNF-α)	3.77
1	pTm WT	Untreated (TNF-α)	2.94
1	pTm WT	Untreated (TNF-α)	3.13
1	pTm WT	Untreated (TNF-α)	3.36
1	pTm WT	1 ng/ml TNF-α	0.47
1	pTm WT	1 ng/ml TNF-α	0.65
1	pTm WT	1 ng/ml TNF-α	0.53
1	pTm WT	1 ng/ml TNF-α	0.52
1	pTm -1748G>C	Untreated (TNF-α)	2.66
1	pTm -1748G>C	Untreated (TNF-α)	3.40
1	pTm -1748G>C	Untreated (TNF-α)	2.56
1	pTm -1748G>C	Untreated (TNF-α)	3.20
1	pTm -1748G>C	1 ng/ml TNF-α	0.46
1	pTm -1748G>C	1 ng/ml TNF-α	0.48
1	pTm -1748G>C	1 ng/ml TNF-α	0.47
1	pTm -1748G>C	1 ng/ml TNF-α	0.52
1	pTm -1166G>A	Untreated (TNF-α)	4.27
1	pTm -1166G>A	Untreated (TNF-α)	4.31
1	pTm -1166G>A	Untreated (TNF-α)	3.90
1	pTm -1166G>A	Untreated (TNF-α)	4.53
1	pTm -1166G>A	1 ng/ml TNF-α	0.81
1	pTm -1166G>A	1 ng/ml TNF-α	0.59
1	pTm -1166G>A	1 ng/ml TNF-α	0.65
1	pTm -1166G>A	1 ng/ml TNF-α	0.67
1	pTm -1208/09TT>delTT	Untreated (TNF-α)	3.90
1	pTm -1208/09TT>delTT	Untreated (TNF-α)	4.19
1	pTm -1208/09TT>delTT	Untreated (TNF-α)	4.90
1	pTm -1208/09TT>delTT	Untreated (TNF-α)	4.42
1	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.30
1	pTm -1208/09TT>delTT	1 ng/ml TNF-α	-
1	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.49
1	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.39
2	pGL-3-Basic	Untreated (TNF-α)	0.04
2	pGL-3-Basic	Untreated (TNF-α)	0.04
2	pGL-3-Basic	Untreated (TNF-α)	0.05
2	pGL-3-Basic	Untreated (TNF-α)	0.04
2	pGL-3-Basic	1 ng/ml TNF-α	0.02
2	pGL-3-Basic	1 ng/ml TNF-α	0.02

2	pGL-3-Basic	1 ng/ml TNF-α	0.01
2	pGL-3-Basic	1 ng/ml TNF-α	0.02
2	pTm WT	Untreated (TNF-α)	2.96
2	pTm WT	Untreated (TNF-α)	2.93
2	pTm WT	Untreated (TNF-α)	2.92
2	pTm WT	Untreated (TNF-α)	2.90
2	pTm WT	1 ng/ml TNF-α	0.51
2	pTm WT	1 ng/ml TNF-α	0.55
2	pTm WT	1 ng/ml TNF-α	0.47
2	pTm WT	1 ng/ml TNF-α	0.46
2	pTm -1748G>C	Untreated (TNF-α)	3.68
2	pTm -1748G>C	Untreated (TNF-α)	2.88
2	pTm -1748G>C	Untreated (TNF-α)	3.24
2	pTm -1748G>C	Untreated (TNF-α)	-
2	pTm -1748G>C	1 ng/ml TNF-α	0.76
2	pTm -1748G>C	1 ng/ml TNF-α	0.43
2	pTm -1748G>C	1 ng/ml TNF-α	1.15
2	pTm -1748G>C	1 ng/ml TNF-α	-
2	pTm -1166G>A	Untreated (TNF-α)	3.07
2	pTm -1166G>A	Untreated (TNF-α)	3.86
2	pTm -1166G>A	Untreated (TNF-α)	3.15
2	pTm -1166G>A	Untreated (TNF-α)	4.73
2	pTm -1166G>A	1 ng/ml TNF-α	0.93
2	pTm -1166G>A	1 ng/ml TNF-α	0.70
2	pTm -1166G>A	1 ng/ml TNF-α	0.95
2	pTm -1166G>A	1 ng/ml TNF-α	0.69
2	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.05
2	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.08
2	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.11
2	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.13
2	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.54
2	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.30
2	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.48
2	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.32
3	pGL-3-Basic	Untreated (TNF-α)	0.04
3	pGL-3-Basic	Untreated (TNF-α)	0.05
3	pGL-3-Basic	Untreated (TNF-α)	0.05
3	pGL-3-Basic	Untreated (TNF-α)	0.07
3	pGL-3-Basic	1 ng/ml TNF-α	0.02
3	pGL-3-Basic	1 ng/ml TNF-α	0.02
3	pGL-3-Basic	1 ng/ml TNF-α	0.02
3	pGL-3-Basic	1 ng/ml TNF-α	0.02
3	pTm WT	Untreated (TNF-α)	2.98
3	pTm WT	Untreated (TNF-α)	3.01
3	pTm WT	Untreated (TNF-α)	3.17
3	pTm WT	Untreated (TNF-α)	3.09
3	pTm WT	1 ng/ml TNF-α	0.29
3	pTm WT	1 ng/ml TNF-α	0.51
3	pTm WT	1 ng/ml TNF-α	0.32
3	pTm WT	1 ng/ml TNF-α	0.36
3	pTm -1748G>C	Untreated (TNF-α)	5.46
3	pTm -1748G>C	Untreated (TNF-α)	4.72
3	pTm -1748G>C	Untreated (TNF-α)	6.22
3	pTm -1748G>C	Untreated (TNF-α)	5.88

3	pTm -1748G>C	1 ng/ml TNF-α	0.33
3	pTm -1748G>C	1 ng/ml TNF-α	0.61
3	pTm -1748G>C	1 ng/ml TNF-α	0.64
3	pTm -1748G>C	1 ng/ml TNF-α	0.44
3	pTm -1166G>A	Untreated (TNF-α)	3.63
3	pTm -1166G>A	Untreated (TNF-α)	4.50
3	pTm -1166G>A	Untreated (TNF-α)	4.00
3	pTm -1166G>A	Untreated (TNF-α)	-
3	pTm -1166G>A	1 ng/ml TNF-α	0.98
3	pTm -1166G>A	1 ng/ml TNF-α	1.08
3	pTm -1166G>A	1 ng/ml TNF-α	1.00
3	pTm -1166G>A	1 ng/ml TNF-α	1.01
3	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.60
3	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.75
3	pTm -1208/09TT>delTT	Untreated (TNF-α)	2.20
3	pTm -1208/09TT>delTT	Untreated (TNF-α)	2.72
3	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.29
3	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.27
3	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.27
3	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.29
4	pGL-3-Basic	Untreated (TNF-α)	0.02
4	pGL-3-Basic	Untreated (TNF-α)	0.04
4	pGL-3-Basic	Untreated (TNF-α)	0.03
4	pGL-3-Basic	Untreated (TNF-α)	0.04
4	pGL-3-Basic	1 ng/ml TNF-α	0.02
4	pGL-3-Basic	1 ng/ml TNF-α	0.02
4	pGL-3-Basic	1 ng/ml TNF-α	0.02
4	pGL-3-Basic	1 ng/ml TNF-α	0.01
4	pTm WT	Untreated (TNF-α)	1.55
4	pTm WT	Untreated (TNF-α)	1.88
4	pTm WT	Untreated (TNF-α)	-
4	pTm WT	Untreated (TNF-α)	1.88
4	pTm WT	1 ng/ml TNF-α	0.38
4	pTm WT	1 ng/ml TNF-α	0.41
4	pTm WT	1 ng/ml TNF-α	0.46
4	pTm WT	1 ng/ml TNF-α	0.32
4	pTm -1748G>C	Untreated (TNF-α)	1.33
4	pTm -1748G>C	Untreated (TNF-α)	1.31
4	pTm -1748G>C	Untreated (TNF-α)	1.47
4	pTm -1748G>C	Untreated (TNF-α)	1.45
4	pTm -1748G>C	1 ng/ml TNF-α	0.27
4	pTm -1748G>C	1 ng/ml TNF-α	0.31
4	pTm -1748G>C	1 ng/ml TNF-α	0.47
4	pTm -1748G>C	1 ng/ml TNF-α	0.37
4	pTm -1166G>A	Untreated (TNF-α)	2.28
4	pTm -1166G>A	Untreated (TNF-α)	2.10
4	pTm -1166G>A	Untreated (TNF-α)	1.15
4	pTm -1166G>A	Untreated (TNF-α)	1.75
4	pTm -1166G>A	1 ng/ml TNF-α	-
4	pTm -1166G>A	1 ng/ml TNF-α	0.75
4	pTm -1166G>A	1 ng/ml TNF-α	0.67
4	pTm -1166G>A	1 ng/ml TNF-α	0.54
4	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.56
4	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.76

4	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.60
4	pTm -1208/09TT>delTT	Untreated (TNF-α)	-
4	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.31
4	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.37
4	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.21
4	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.40
5	pGL-3-Basic	Untreated (TNF-α)	0.02
5	pGL-3-Basic	Untreated (TNF-α)	0.03
5	pGL-3-Basic	Untreated (TNF-α)	0.02
5	pGL-3-Basic	Untreated (TNF-α)	0.03
5	pGL-3-Basic	1 ng/ml TNF-α	0.02
5	pGL-3-Basic	1 ng/ml TNF-α	0.01
5	pGL-3-Basic	1 ng/ml TNF-α	0.01
5	pGL-3-Basic	1 ng/ml TNF-α	0.01
5	pTm WT	Untreated (TNF-α)	1.39
5	pTm WT	Untreated (TNF-α)	1.33
5	pTm WT	Untreated (TNF-α)	1.40
5	pTm WT	Untreated (TNF-α)	1.55
5	pTm WT	1 ng/ml TNF-α	0.23
5	pTm WT	1 ng/ml TNF-α	0.39
5	pTm WT	1 ng/ml TNF-α	0.20
5	pTm WT	1 ng/ml TNF-α	-
5	pTm -1748G>C	Untreated (TNF-α)	1.50
5	pTm -1748G>C	Untreated (TNF-α)	1.29
5	pTm -1748G>C	Untreated (TNF-α)	1.38
5	pTm -1748G>C	Untreated (TNF-α)	1.58
5	pTm -1748G>C	1 ng/ml TNF-α	-
5	pTm -1748G>C	1 ng/ml TNF-α	-
5	pTm -1748G>C	1 ng/ml TNF-α	-
5	pTm -1748G>C	1 ng/ml TNF-α	-
5	pTm -1166G>A	Untreated (TNF-α)	-
5	pTm -1166G>A	Untreated (TNF-α)	2.61
5	pTm -1166G>A	Untreated (TNF-α)	3.24
5	pTm -1166G>A	Untreated (TNF-α)	4.33
5	pTm -1166G>A	1 ng/ml TNF-α	-
5	pTm -1166G>A	1 ng/ml TNF-α	0.28
5	pTm -1166G>A	1 ng/ml TNF-α	0.30
5	pTm -1166G>A	1 ng/ml TNF-α	0.33
5	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.28
5	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.58
5	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.26
5	pTm -1208/09TT>delTT	Untreated (TNF-α)	1.47
5	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.14
5	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.10
5	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.15
5	pTm -1208/09TT>delTT	1 ng/ml TNF-α	0.28

Appendix 5

Ratio of firefly luminescence to *Renilla* luminescence for pTm constructs in response to IL-1β (0.5 ng/ml; 15 h)

Experiment	Construct	Treatment	Relative Ratio
1	pGL-3-Basic	Untreated (IL-1β)	0.02
1	pGL-3-Basic	Untreated (IL-1β)	0.02
1	pGL-3-Basic	Untreated (IL-1β)	0.02
1	pGL-3-Basic	Untreated (IL-1β)	0.03
1	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
1	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
1	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
1	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
1	pTm WT	Untreated (IL-1β)	2.96
1	pTm WT	Untreated (IL-1β)	2.67
1	pTm WT	Untreated (IL-1β)	2.74
1	pTm WT	Untreated (IL-1β)	2.80
1	pTm WT	0.5 ng/ml IL-1β	1.45
1	pTm WT	0.5 ng/ml IL-1β	1.19
1	pTm WT	0.5 ng/ml IL-1β	2.12
1	pTm WT	0.5 ng/ml IL-1β	2.23
1	pTm -1748G>C	Untreated (IL-1β)	2.78
1	pTm -1748G>C	Untreated (IL-1β)	2.76
1	pTm -1748G>C	Untreated (IL-1β)	2.11
1	pTm -1748G>C	Untreated (IL-1β)	2.27
1	pTm -1748G>C	0.5 ng/ml IL-1β	1.50
1	pTm -1748G>C	0.5 ng/ml IL-1β	1.31
1	pTm -1748G>C	0.5 ng/ml IL-1β	-
1	pTm -1748G>C	0.5 ng/ml IL-1β	1.40
1	pTm -1166G>A	Untreated (IL-1β)	-
1	pTm -1166G>A	Untreated (IL-1β)	2.84
1	pTm -1166G>A	Untreated (IL-1β)	2.61
1	pTm -1166G>A	Untreated (IL-1β)	2.79
1	pTm -1166G>A	0.5 ng/ml IL-1β	1.70
1	pTm -1166G>A	0.5 ng/ml IL-1β	1.85
1	pTm -1166G>A	0.5 ng/ml IL-1β	1.14
1	pTm -1166G>A	0.5 ng/ml IL-1β	1.10
1	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.78
1	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.73
1	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.73
1	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.82
1	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.90
1	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.78
1	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	-
1	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.83
2	pGL-3-Basic	Untreated (IL-1β)	0.02
2	pGL-3-Basic	Untreated (IL-1β)	0.02
2	pGL-3-Basic	Untreated (IL-1β)	0.02
2	pGL-3-Basic	Untreated (IL-1β)	0.02
2	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
2	pGL-3-Basic	0.5 ng/ml IL-1β	0.02

2	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
2	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
2	pTm WT	Untreated (IL-1β)	3.07
2	pTm WT	Untreated (IL-1β)	2.72
2	pTm WT	Untreated (IL-1β)	3.01
2	pTm WT	Untreated (IL-1β)	2.82
2	pTm WT	0.5 ng/ml IL-1β	-
2	pTm WT	0.5 ng/ml IL-1β	2.69
2	pTm WT	0.5 ng/ml IL-1β	2.73
2	pTm WT	0.5 ng/ml IL-1β	2.26
2	pTm -1748G>C	Untreated (IL-1β)	2.20
2	pTm -1748G>C	Untreated (IL-1β)	2.15
2	pTm -1748G>C	Untreated (IL-1β)	2.45
2	pTm -1748G>C	Untreated (IL-1β)	2.20
2	pTm -1748G>C	0.5 ng/ml IL-1β	1.80
2	pTm -1748G>C	0.5 ng/ml IL-1β	1.76
2	pTm -1748G>C	0.5 ng/ml IL-1β	1.23
2	pTm -1748G>C	0.5 ng/ml IL-1β	-
2	pTm -1166G>A	Untreated (IL-1β)	3.15
2	pTm -1166G>A	Untreated (IL-1β)	2.55
2	pTm -1166G>A	Untreated (IL-1β)	2.01
2	pTm -1166G>A	Untreated (IL-1β)	2.24
2	pTm -1166G>A	0.5 ng/ml IL-1β	1.71
2	pTm -1166G>A	0.5 ng/ml IL-1β	2.11
2	pTm -1166G>A	0.5 ng/ml IL-1β	1.13
2	pTm -1166G>A	0.5 ng/ml IL-1β	1.13
2	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.31
2	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.99
2	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.96
2	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.40
2	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.67
2	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.15
2	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.32
2	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.19
3	pGL-3-Basic	Untreated (IL-1β)	0.01
3	pGL-3-Basic	Untreated (IL-1β)	0.02
3	pGL-3-Basic	Untreated (IL-1β)	0.02
3	pGL-3-Basic	Untreated (IL-1β)	0.02
3	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
3	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
3	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
3	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
3	pTm WT	Untreated (IL-1β)	2.24
3	pTm WT	Untreated (IL-1β)	2.15
3	pTm WT	Untreated (IL-1β)	2.20
3	pTm WT	Untreated (IL-1β)	2.35
3	pTm WT	0.5 ng/ml IL-1β	1.23
3	pTm WT	0.5 ng/ml IL-1β	2.03
3	pTm WT	0.5 ng/ml IL-1β	1.79
3	pTm WT	0.5 ng/ml IL-1β	1.33
3	pTm -1748G>C	Untreated (IL-1β)	1.46
3	pTm -1748G>C	Untreated (IL-1β)	1.55
3	pTm -1748G>C	Untreated (IL-1β)	1.61
3	pTm -1748G>C	Untreated (IL-1β)	1.65

3	pTm -1748G>C	0.5 ng/ml IL-1β	1.45
3	pTm -1748G>C	0.5 ng/ml IL-1β	2.45
3	pTm -1748G>C	0.5 ng/ml IL-1β	2.14
3	pTm -1748G>C	0.5 ng/ml IL-1β	1.41
3	pTm -1166G>A	Untreated (IL-1β)	1.76
3	pTm -1166G>A	Untreated (IL-1β)	2.49
3	pTm -1166G>A	Untreated (IL-1β)	2.00
3	pTm -1166G>A	Untreated (IL-1β)	1.50
3	pTm -1166G>A	0.5 ng/ml IL-1β	1.27
3	pTm -1166G>A	0.5 ng/ml IL-1β	1.25
3	pTm -1166G>A	0.5 ng/ml IL-1β	1.81
3	pTm -1166G>A	0.5 ng/ml IL-1β	0.92
3	pTm -1208/09TT>delTT	Untreated (IL-1β)	2.12
3	pTm -1208/09TT>delTT	Untreated (IL-1β)	2.20
3	pTm -1208/09TT>delTT	Untreated (IL-1β)	2.30
3	pTm -1208/09TT>delTT	Untreated (IL-1β)	2.19
3	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	-
3	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.17
3	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.93
3	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.02
4	pGL-3-Basic	Untreated (IL-1β)	0.02
4	pGL-3-Basic	Untreated (IL-1β)	0.02
4	pGL-3-Basic	Untreated (IL-1β)	0.02
4	pGL-3-Basic	Untreated (IL-1β)	0.03
4	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
4	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
4	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
4	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
4	pTm WT	Untreated (IL-1β)	3.43
4	pTm WT	Untreated (IL-1β)	2.22
4	pTm WT	Untreated (IL-1β)	2.14
4	pTm WT	Untreated (IL-1β)	2.42
4	pTm WT	0.5 ng/ml IL-1β	1.22
4	pTm WT	0.5 ng/ml IL-1β	1.75
4	pTm WT	0.5 ng/ml IL-1β	1.62
4	pTm WT	0.5 ng/ml IL-1β	1.47
4	pTm -1748G>C	Untreated (IL-1β)	2.04
4	pTm -1748G>C	Untreated (IL-1β)	3.66
4	pTm -1748G>C	Untreated (IL-1β)	2.76
4	pTm -1748G>C	Untreated (IL-1β)	-
4	pTm -1748G>C	0.5 ng/ml IL-1β	1.42
4	pTm -1748G>C	0.5 ng/ml IL-1β	1.70
4	pTm -1748G>C	0.5 ng/ml IL-1β	1.62
4	pTm -1748G>C	0.5 ng/ml IL-1β	1.20
4	pTm -1166G>A	Untreated (IL-1β)	3.05
4	pTm -1166G>A	Untreated (IL-1β)	2.96
4	pTm -1166G>A	Untreated (IL-1β)	-
4	pTm -1166G>A	Untreated (IL-1β)	3.17
4	pTm -1166G>A	0.5 ng/ml IL-1β	1.97
4	pTm -1166G>A	0.5 ng/ml IL-1β	2.09
4	pTm -1166G>A	0.5 ng/ml IL-1β	2.68
4	pTm -1166G>A	0.5 ng/ml IL-1β	1.16
4	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.34
4	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.55

4	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.42
4	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.40
4	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.12
4	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.25
4	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.48
4	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	1.01
5	pGL-3-Basic	Untreated (IL-1β)	0.02
5	pGL-3-Basic	Untreated (IL-1β)	0.02
5	pGL-3-Basic	Untreated (IL-1β)	0.02
5	pGL-3-Basic	Untreated (IL-1β)	0.02
5	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
5	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
5	pGL-3-Basic	0.5 ng/ml IL-1β	0.02
5	pGL-3-Basic	0.5 ng/ml IL-1β	0.01
5	pTm WT	Untreated (IL-1β)	1.22
5	pTm WT	Untreated (IL-1β)	0.88
5	pTm WT	Untreated (IL-1β)	1.72
5	pTm WT	Untreated (IL-1β)	1.57
5	pTm WT	0.5 ng/ml IL-1β	2.10
5	pTm WT	0.5 ng/ml IL-1β	1.54
5	pTm WT	0.5 ng/ml IL-1β	1.54
5	pTm WT	0.5 ng/ml IL-1β	1.56
5	pTm -1748G>C	Untreated (IL-1β)	1.39
5	pTm -1748G>C	Untreated (IL-1β)	-
5	pTm -1748G>C	Untreated (IL-1β)	1.29
5	pTm -1748G>C	Untreated (IL-1β)	1.48
5	pTm -1748G>C	0.5 ng/ml IL-1β	0.72
5	pTm -1748G>C	0.5 ng/ml IL-1β	0.83
5	pTm -1748G>C	0.5 ng/ml IL-1β	-
5	pTm -1748G>C	0.5 ng/ml IL-1β	0.89
5	pTm -1166G>A	Untreated (IL-1β)	1.63
5	pTm -1166G>A	Untreated (IL-1β)	1.30
5	pTm -1166G>A	Untreated (IL-1β)	1.42
5	pTm -1166G>A	Untreated (IL-1β)	1.80
5	pTm -1166G>A	0.5 ng/ml IL-1β	2.01
5	pTm -1166G>A	0.5 ng/ml IL-1β	2.36
5	pTm -1166G>A	0.5 ng/ml IL-1β	2.61
5	pTm -1166G>A	0.5 ng/ml IL-1β	2.21
5	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.26
5	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.14
5	pTm -1208/09TT>delTT	Untreated (IL-1β)	1.19
5	pTm -1208/09TT>delTT	Untreated (IL-1β)	0.98
5	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.67
5	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	-
5	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.70
5	pTm -1208/09TT>delTT	0.5 ng/ml IL-1β	0.72

Appendix 6

Genotyping the TNF-α -308G>A polymorphism

TNF-α -308G>A genotyping was performed using a TaqMan assay according to the manufacturer's recommendations. Allelic discrimination using TaqMan was based on the design of an assay mix, which consist of: two locus-specific PCR primers that flank the SNP of interest and two TaqMan probes, specific for the wild-type allele and the mutant allele, which were added into one assay mix. Each of the two probes was labelled with a different fluorescent dye. The assay was obtained as an Assays-on-Demand from the manufacturer; Assay ID: C___7514879_10. Each genotyping plate contained negative controls. PCR was performed using 384-well plates. The PCR mixture used contained 5 ng of genomic DNA, 2.5 μl of ABsolute QPCR Rox mix (2x)(ABgene), 0.25 μl of 20x *assay-on-demand* mix (designed by manufacturer) and 2.25 μl of purified dH_2O. PCR was performed under the following conditions: 50°C for 2 minutes, 95°C for 15 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The PCR product was then read on TaqMan HT7900 (Applied Biosystems, CA, USA).

TaqMan SNP genotyping assays consist of a single tube containing two primers for amplifying the sequence of interest and 2 probes for detecting alleles. Each probe contains a reporter dye at the 5' end (VIC dye linked to the 5' end of allele 1 probe and FAM dye linked to the 5' end of allele 2 probe). Each probe contains a nonfluorescent quencher (NFQ) at the 3'end. During PCR amplification each TaqMan probe anneals specifically to a complementary sequence between the forward and reverse primers. When the probe is intact, the proximity of the reporter dye to the quencher suppresses the reporter

fluorescence. AmpliTaq Gold® DNA polymerase cleaves probes that have hybridized to the target. Cleavage results in separation of the reporter dye from the quencher, thus increasing fluorescence by the reporter. Increased fluorescence only occurs if the amplified target sequence is complementary to the probe, thus indicating which allele is present in the sample.

Reference List

Abe,H., Okajima,K., Okabe,H., Takatsuki,K., and Binder,B.R. (1994). Granulocyte proteases and hydrogen peroxide synergistically inactivate thrombomodulin of endothelial cells in vitro. J. Lab Clin. Med. *123*, 874-881.

Abeyama,K., Stern,D.M., Ito,Y., Kawahara,K., Yoshimoto,Y., Tanaka,M., Uchimura,T., Ida,N., Yamazaki,Y., Yamada,S., Yamamoto,Y., Yamamoto,H., Iino,S., Taniguchi,N., and Maruyama,I. (2005). The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. J. Clin. Invest *115*, 1267-1274.

Adler,M., Seto,M.H., Nitecki,D.E., Lin,J.H., Light,D.R., and Morser,J. (1995). The structure of a 19-residue fragment from the C-loop of the fourth epidermal growth factor-like domain of thrombomodulin. J. Biol. Chem. *270*, 23366-23372.

Afshar-Kharghan,V., Li,C.Q., Khoshnevis-Asl,M., and Lopez,J.A. (1999). Kozak sequence polymorphism of the glycoprotein (GP) Ibalpha gene is a major determinant of the plasma membrane levels of the platelet GP Ib-IX-V complex. Blood *94*, 186-191.

Aleksic,N., Folsom A.R., Cushman M., Heckbert S.R., Tsai M.Y., and Wu K.K (2002). Prospective study of the A455V polymorphism in the thrombomodulin gene, plasma thrombomodulin, and incidence of venous thromboembolism: the LITE study. Journal of Thrombosis and Haemostasis *1*, 88-94.

Anber,V., Griffin,B.A., McConnell,M., Packard,C.J., and Shepherd,J. (1996). Influence of plasma lipid and LDL-subfraction profile on the interaction between low density lipoprotein with human arterial wall proteoglycans. Atherosclerosis *124*, 261-271.

Archipoff,G., Beretz,A., Freyssinet,J.M., Klein-Soyer,C., Brisson,C., and Cazenave,J.P. (1991). Heterogeneous regulation of constitutive thrombomodulin or inducible tissue-factor activities on the surface of human saphenous-vein endothelial cells in culture following stimulation by interleukin-1, tumour necrosis factor, thrombin or phorbol ester. Biochem. J. *273*, 679-684.

Ardissino,D., Mannucci,P.M., Merlini,P.A., Duca,F., Fetiveau,R., Tagliabue,L., Tubaro,M., Galvani,M., Ottani,F., Ferrario,M., Corral,J., and Margaglione,M. (1999). Prothrombotic genetic risk factors in young survivors of myocardial infarction. Blood *94*, 46-51.

Ariens,R.A., Philippou,H., Nagaswami,C., Weisel,J.W., Lane,D.A., and Grant,P.J. (2000). The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. Blood *96*, 988-995.

Armstrong,V.W., Cremer,P., Eberle,E., Manke,A., Schulze,F., Wieland,H., Kreuzer,H., and Seidel,D. (1986). The association between serum Lp(a) concentrations and angiographically assessed coronary atherosclerosis. Dependence on serum LDL levels. Atherosclerosis *62*, 249-257.

Arnalich,F., Hernanz,A., Lopez-Maderuelo,D., Pena,J.M., Camacho,J., Madero,R., Vazquez,J.J., and Montiel,C. (2000). Enhanced acute-phase response and oxidative stress in older adults with type II diabetes. Horm. Metab Res. *32*, 407-412.

Aso,Y., Fujiwara,Y., Tayama,K., Takanashi,K., Inukai,T., and Takemura,Y. (2001). Relationship between plasma soluble thrombomodulin levels and insulin resistance syndrome in type 2 diabetes: a comparison with von Willebrand factor. Exp. Clin. Endocrinol. Diabetes *109*, 210-216.

Aso,Y., Fujiwara,Y., Tayama,K., Takebayashi,K., Inukai,T., and Takemura,Y. (2000). Relationship between soluble thrombomodulin in plasma and coagulation or fibrinolysis in type 2 diabetes. Clin. Chim. Acta *301*, 135-145.

Aso,Y., Inukai,T., and Takemura,Y. (1998). Mechanisms of elevation of serum and urinary concentrations of soluble thrombomodulin in diabetic patients: possible application as a marker for vascular endothelial injury. Metabolism *47*, 362-365.

Assmann,G., Schulte,H., and von Eckardstein,A. (1996). Hypertriglyceridemia and elevated lipoprotein(a) are risk factors for major coronary events in middle-aged men. Am. J. Cardiol. *77*, 1179-1184.

Auro,K., Komulainen,K., Alanne,M., Silander,K., Peltonen,L., Perola,M., and Salomaa,V. (2006). Thrombomodulin gene polymorphisms and haplotypes and the risk of cardiovascular events: a prospective follow-up study. Arterioscler. Thromb. Vasc. Biol. *26*, 942-947.

Austin,M.A. (2000). Triglyceride, small, dense low-density lipoprotein, and the atherogenic lipoprotein phenotype. Curr. Atheroscler. Rep. *2*, 200-207.

Baeuerle,P.A. (1998). IkappaB-NF-kappaB structures: at the interface of inflammation control. Cell *95*, 729-731.

Baglin,T.P., Carrell,R.W., Church,F.C., Esmon,C.T., and Huntington,J.A. (2002). Crystal structures of native and thrombin-complexed heparin cofactor II reveal a multistep allosteric mechanism. Proc. Natl. Acad. Sci. U. S. A *99*, 11079-11084.

Bajaj,M.S., Birktoft,J.J., Steer,S.A., and Bajaj,S.P. (2001). Structure and biology of tissue factor pathway inhibitor. Thromb. Haemost. *86*, 959-972.

Bajaj,M.S., Kuppuswamy,M.N., Saito,H., Spitzer,S.G., and Bajaj,S.P. (1990). Cultured normal human hepatocytes do not synthesize lipoprotein- associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis. Proc. Natl. Acad. Sci. U. S. A *87*, 8869-8873.

Bajzar,L., Manuel,R., and Nesheim,M.E. (1995). Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. J. Biol. Chem. *270*, 14477-14484.

Bajzar,L., Morser,J., and Nesheim,M. (1996a). TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. J. Biol. Chem. *271*, 16603-16608.

Bajzar,L., Nesheim,M., Morser,J., and Tracy,P.B. (1998). Both cellular and soluble forms of thrombomodulin inhibit fibrinolysis by potentiating the activation of thrombin-activable fibrinolysis inhibitor. J. Biol. Chem. *273*, 2792-2798.

Bajzar,L., Nesheim,M.E., and Tracy,P.B. (1996b). The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. Blood *88*, 2093-2100.

Balarajan,R. (1991). Ethnic differences in mortality from ischaemic heart disease and cerebrovascular disease in England and Wales. BMJ *302*, 560-564.

Balarajan,R. (1995). Ethnicity and variations in the nation's health. Health Trends *27*, 114-119.

Bar-Shavit,R., Kahn,A., Fenton,J.W., and Wilner,G.D. (1983a). Chemotactic response of monocytes to thrombin. J. Cell Biol. *96*, 282-285.

Bar-Shavit,R., Kahn,A., Wilner,G.D., and Fenton,J.W. (1983b). Monocyte chemotaxis: stimulation by specific exosite region in thrombin. Science *220*, 728-731.

Barber,R.D., Harmer,D.W., Coleman,R.A., and Clark,B.J. (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics *21*, 389-395.

Barrett,A.J. and Starkey,P.M. (1973). The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. Biochem. J. *133*, 709-724.

Bartha,K., Brisson,C., Archipoff,G., de la,S.C., Lanza,F., Cazenave,J.P., and Beretz,A. (1993). Thrombin regulates tissue factor and thrombomodulin mRNA levels and activities in human saphenous vein endothelial cells by distinct mechanisms. J. Biol. Chem. *268*, 421-429.

Becker,B.F., Heindl,B., Kupatt,C., and Zahler,S. (2000). Endothelial function and hemostasis. Z. Kardiol. *89*, 160-167.

Behague,I., Poirier,O., Nicaud,V., Evans,A., Arveiler,D., Luc,G., Cambou,J.P., Scarabin,P.Y., Bara,L., Green,F., and Cambien,F. (1996). Beta fibrinogen gene polymorphisms are associated with plasma fibrinogen and coronary artery disease in patients with myocardial infarction. The ECTIM Study. Etude Cas-Temoins sur l'Infarctus du Myocarde. Circulation *93*, 440-449.

Bennett,J.S. and Vilaire,G. (1979). Exposure of platelet fibrinogen receptors by ADP and epinephrine. J. Clin. Invest *64*, 1393-1401.

Beral,V., Banks,E., and Reeves,G. (2002). Evidence from randomised trials on the long-term effects of hormone replacement therapy. Lancet *360*, 942-944.

Beretz,A., Freyssinet,J.M., Gauchy,J., Schmitt,D.A., Klein-Soyer,C., Edgell,C.J., and Cazenave,J.P. (1989). Stability of the thrombin-thrombomodulin complex on the surface of endothelial cells from human saphenous vein or from the cell line EA.hy 926. Biochem. J. *259*, 35-40.

Bernardi,F., Marchetti,G., Pinotti,M., Arcieri,P., Baroncini,C., Papacchini,M., Zepponi,E., Ursicino,N., Chiarotti,F., and Mariani,G. (1996). Factor VII gene polymorphisms contribute about one third of the factor VII level variation in plasma. Arterioscler. Thromb. Vasc. Biol. *16*, 72-76.

Berridge,M.J., Lipp,P., and Bootman,M.D. (2000). The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. *1*, 11-21.

Bertina,R.M., Koeleman,B.P., Koster,T., Rosendaal,F.R., Dirven,R.J., de Ronde,H., van der Velden,P.A., and Reitsma,P.H. (1994). Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature *369*, 64-67.

Bevilacqua,M.P., Pober,J.S., Majeau,G.R., Cotran,R.S., and Gimbrone,M.A., Jr. (1984). Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J. Exp. Med. *160*, 618-623.

Birkedal-Hansen,H. (1995). Proteolytic remodeling of extracellular matrix. Curr. Opin. Cell Biol. *7*, 728-735.

Bizios,R., Lai,L., Fenton,J.W., and Malik,A.B. (1986). Thrombin-induced chemotaxis and aggregation of neutrophils. J. Cell Physiol *128*, 485-490.

Blann,A.D. (1992). The acute influence of smoking on the endothelium. Atherosclerosis *96*, 249-250.

Blann,A.D., Steele,C., and McCollum,C.N. (1997). The influence of smoking on soluble adhesion molecules and endothelial cell markers. Thromb. Res. *85*, 433-438.

Blinder,M.A., Marasa,J.C., Reynolds,C.H., Deaven,L.L., and Tollefsen,D.M. (1988). Heparin cofactor II: cDNA sequence, chromosome localization, restriction fragment length polymorphism, and expression in Escherichia coli. Biochemistry *27*, 752-759.

Bode,W., Mayr,I., Baumann,U., Huber,R., Stone,S.R., and Hofsteenge,J. (1989). The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J. *8*, 3467-3475.

Boehme,M.W., Deng,Y., Raeth,U., Bierhaus,A., Ziegler,R., Stremmel,W., and Nawroth,P.P. (1996). Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: in vivo and in vitro studies. Immunology *87*, 134-140.

Boekholdt,S.M., Bijsterveld,N.R., Moons,A.H., Levi,M., Buller,H.R., and Peters,R.J. (2001). Genetic variation in coagulation and fibrinolytic proteins and their relation with acute myocardial infarction: a systematic review. Circulation *104*, 3063-3068.

Bombeli,T., Mueller,M., and Haeberli,A. (1997). Anticoagulant properties of the vascular endothelium. Thromb. Haemost. *77*, 408-423.

Booth,N.A. (1999). Fibrinolysis and thrombosis. Baillieres Best. Pract. Res. Clin. Haematol. *12*, 423-433.

Borawski,J., Naumnik,B., Pawlak,K., and Mysliwiec,M. (2001). Soluble thrombomodulin is associated with viral hepatitis, blood pressure, and medications in haemodialysis patients. Nephrol. Dial. Transplant. *16*, 787-792.

Bourin,M.C., Lundgren-Akerlund,E., and Lindahl,U. (1990). Isolation and characterization of the glycosaminoglycan component of rabbit thrombomodulin proteoglycan. J. Biol. Chem. *265*, 15424-15431.

Bourin,M.C., Ohlin,A.K., Lane,D.A., Stenflo,J., and Lindahl,U. (1988). Relationship between anticoagulant activities and polyanionic properties of rabbit thrombomodulin. J. Biol. Chem. *263*, 8044-8052.

Braunwald,E. (1997). Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N. Engl. J. Med. *337*, 1360-1369.

Breslow,J.L. (1997). Cardiovascular disease burden increases, NIH funding decreases. Nat. Med. *3*, 600-601.

Bromann,P.A., Korkaya,H., and Courtneidge,S.A. (2004). The interplay between Src family kinases and receptor tyrosine kinases. Oncogene *23*, 7957-7968.

Broze,G.J., Jr. (1995). Tissue factor pathway inhibitor and the revised theory of coagulation. Annu. Rev. Med. *46*, 103-112.

Broze,G.J., Jr., Girard,T.J., and Novotny,W.F. (1990). Regulation of coagulation by a multivalent Kunitz-type inhibitor. Biochemistry *29*, 7539-7546.

Broze,G.J., Jr., Lange,G.W., Duffin,K.L., and MacPhail,L. (1994). Heterogeneity of plasma tissue factor pathway inhibitor. Blood Coagul. Fibrinolysis *5*, 551-559.

Broze,G.J., Jr., Warren,L.A., Novotny,W.F., Higuchi,D.A., Girard,J.J., and Miletich,J.P. (1988). The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. Blood *71*, 335-343.

Brummel,K.E., Butenas,S., and Mann,K.G. (1999). An integrated study of fibrinogen during blood coagulation. J. Biol. Chem. *274*, 22862-22870.

Bullon,P., Machuca,G., Martinez-Sahuquillo,A., Rios,J.V., Velasco,E., Rojas,J., and Lacalle,J.R. (1996). Evaluation of gingival and periodontal conditions following causal periodontal treatment in patients treated with nifedipine and diltiazem. J. Clin. Periodontol. *23*, 649-657.

Busch,C., Cancilla,P.A., DeBault,L.E., Goldsmith,J.C., and Owen,W.G. (1982). Use of endothelium cultured on microcarriers as a model for the microcirculation. Lab Invest *47*, 498-504.

Butenas,S. and Mann,K.G. (2002). Blood coagulation. Biochemistry (Mosc.) *67*, 3-12.

Calnek,D.S. and Grinnell,B.W. (1998). Thrombomodulin-dependent anticoagulant activity is regulated by vascular endothelial growth factor. Exp. Cell Res. *238*, 294-298.

Campbell,I. and Bork,P. (1993). Epidermal growth factor-like modules. Current Opinion in Structural Biology 385-392.

Campbell,W., Okada,N., and Okada,H. (2001). Carboxypeptidase R is an inactivator of complement-derived inflammatory peptides and an inhibitor of fibrinolysis. Immunol. Rev. *180*, 162-167.

Campbell,W.D., Lazoura,E., Okada,N., and Okada,H. (2002). Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. Microbiol. Immunol. *46*, 131-134.

Canavy,I., Henry,M., Morange,P.E., Tiret,L., Poirier,O., Ebagosti,A., Bory,M., and Juhan-Vague,I. (2000). Genetic polymorphisms and coronary artery disease in the south of France. Thromb. Haemost. *83*, 212-216.

Capecchi,M.R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell *22*, 479-488.

Carter,A.M., Ossei-Gerning,N., Wilson,I.J., and Grant,P.J. (1997). Association of the platelet Pl(A) polymorphism of glycoprotein IIb/IIIa and the fibrinogen Bbeta 448 polymorphism with myocardial infarction and extent of coronary artery disease. Circulation *96*, 1424-1431.

Carter,A.M., Sachchithananthan,M., Stasinopoulos,S., Maurer,F., and Medcalf,R.L. (2002). Prothrombin G20210A is a bifunctional gene polymorphism. Thromb. Haemost. *87*, 846-853.

Castelli,W.P., Garrison,R.J., Wilson,P.W., Abbott,R.D., Kalousdian,S., and Kannel,W.B. (1986b). Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. JAMA *256*, 2835-2838.

Castelli,W.P., Garrison,R.J., Wilson,P.W., Abbott,R.D., Kalousdian,S., and Kannel,W.B. (1986a). Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. JAMA *256*, 2835-2838.

Chakravarti,A., Buetow,K.H., Antonarakis,S.E., Waber,P.G., Boehm,C.D., and Kazazian,H.H. (1984). Nonuniform recombination within the human beta-globin gene cluster. Am. J. Hum. Genet. *36*, 1239-1258.

Chan,H.M. and La Thangue,N.B. (2001). p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J. Cell Sci. *114*, 2363-2373.

Chaturvedi,N. (2003). Ethnic differences in cardiovascular disease. Heart *89*, 681-686.

Chen,C. and Okayama,H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol. *7*, 2745-2752.

Chen,L.B. and Buchanan,J.M. (1975). Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc. Natl. Acad. Sci. U. S. A *72*, 131-135.

Christensen,U. (1985). C-terminal lysine residues of fibrinogen fragments essential for binding to plasminogen. FEBS Lett. *182*, 43-46.

Chung,D.W., Harris,J.E., and Davie,E.W. (1990). Nucleotide sequences of the three genes coding for human fibrinogen. Adv. Exp. Med. Biol. *281*, 39-48.

Cines,D.B., Pollak,E.S., Buck,C.A., Loscalzo,J., Zimmerman,G.A., McEver,R.P., Pober,J.S., Wick,T.M., Konkle,B.A., Schwartz,B.S., Barnathan,E.S., McCrae,K.R., Hug,B.A., Schmidt,A.M., and Stern,D.M. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood *91*, 3527-3561.

Clarke,J.H., Light,D.R., Blasko,E., Parkinson,J.F., Nagashima,M., McLean,K., Vilander,L., Andrews,W.H., Morser,J., and Glaser,C.B. (1993). The short loop between epidermal growth factor-like domains 4 and 5 is critical for human thrombomodulin function. J. Biol. Chem. *268*, 6309-6315.

Colhoun,H.M., McKeigue,P.M., and Davey,S.G. (2003). Problems of reporting genetic associations with complex outcomes. Lancet *361*, 865-872.

Collen,D. (1999). The plasminogen (fibrinolytic) system. Thromb. Haemost. *82*, 259-270.

Collen,D. and Lijnen,H.R. (1991). Basic and clinical aspects of fibrinolysis and thrombolysis. Blood *78*, 3114-3124.

Coller,B.S., Owen,J., Jesty,J., Horowitz,D., Reitman,M.J., Spear,J., Yeh,T., and Comp,P.C. (1987). Deficiency of plasma protein S, protein C, or antithrombin III and arterial thrombosis. Arteriosclerosis *7*, 456-462.

Collins,C.L., Fink,L.M., Hsu,S.M., Schaefer,R., and Ordonez,N. (1992). Thrombomodulin staining of mesothelioma cells. Hum. Pathol. *23*, 966.

Colotta,F., Sciacca,F.L., Sironi,M., Luini,W., Rabiet,M.J., and Mantovani,A. (1994). Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am. J. Pathol. *144*, 975-985.

Colucci,M., Paramo,J.A., and Collen,D. (1985). Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. J. Clin. Invest *75*, 818-824.

Conway,E.M., Boffa,M.C., Nowakowski,B., and Steiner-Mosonyi,M. (1992a). An ultrastructural study of thrombomodulin endocytosis: internalization occurs via clathrin-coated and non-coated pits. J. Cell Physiol *151*, 604-612.

Conway,E.M., Liu,L., Nowakowski,B., Steiner-Mosonyi,M., and Jackman,R.W. (1994). Heat shock of vascular endothelial cells induces an up-regulatory transcriptional response of the thrombomodulin gene that is delayed in onset and does not attenuate. J. Biol. Chem. *269*, 22804-22810.

Conway,E.M. and Nowakowski,B. (1993). Biologically active thrombomodulin is synthesized by adherent synovial fluid cells and is elevated in synovial fluid of patients with rheumatoid arthritis. Blood *81*, 726-733.

Conway,E.M., Nowakowski,B., and Steiner-Mosonyi,M. (1992b). Human neutrophils synthesize thrombomodulin that does not promote thrombin-dependent protein C activation. Blood *80*, 1254-1263.

Conway,E.M., Pollefeyt,S., Collen,D., and Steiner-Mosonyi,M. (1997). The amino terminal lectin-like domain of thrombomodulin is required for constitutive endocytosis. Blood *89*, 652-661.

Conway,E.M., Pollefeyt,S., Cornelissen,J., DeBaere,I., Steiner-Mosonyi,M., Weitz,J.I., Weiler-Guettler,H., Carmeliet,P., and Collen,D. (1999). Structure-function analyses of thrombomodulin by gene-targeting in mice: the cytoplasmic domain is not required for normal fetal development. Blood *93*, 3442-3450.

Conway,E.M., Van De,W.M., Pollefeyt,S., Jurk,K., Van Aken,H., De Vriese,A., Weitz,J.I., Weiler,H., Hellings,P.W., Schaeffer,P., Herbert,J.M., Collen,D., and Theilmeier,G. (2002). The Lectin-like Domain of Thrombomodulin Confers Protection from Neutrophil-mediated Tissue Damage by Suppressing Adhesion Molecule Expression via Nuclear Factor kappaB and Mitogen-activated Protein Kinase Pathways. J. Exp. Med. *196*, 565-577.

Cooper,J., Miller GJ, Bauer KA, Morrissey JH, Meade TW, Howarth DJ, Barzegar S, Mitchell JP, and Rosenberg RD (2000). Comparison of novel hemostatic factors and conventional risk factors for prediction of coronary heart disease. Circulation *102*, 2816-1822.

Cordell,H.J. and Clayton,D.G. (2005). Genetic association studies. Lancet *366*, 1121-1131.

Corral,J., Gonzalez-Conejero,R., Iniesta,J.A., Rivera,J., Martinez,C., and Vicente,V. (2000a). The FXIII Val34Leu polymorphism in venous and arterial thromboembolism. Haematologica *85*, 293-297.

Corral,J., Lozano,M.L., Gonzalez-Conejero,R., Martinez,C., Iniesta,J.A., Rivera,J., and Vicente,V. (2000b). A common polymorphism flanking the ATG initiator codon of GPIb alpha does not affect expression and is not a major risk factor for arterial thrombosis. Thromb. Haemost. *83*, 23-28.

Coughlin,S.R. (1999). How the protease thrombin talks to cells. Proc. Natl. Acad. Sci. U. S. A *96*, 11023-11027.

Coughlin,S.R., Vu,T.K., Hung,D.T., and Wheaton,V.I. (1992). Characterization of a functional thrombin receptor. Issues and opportunities. J. Clin. Invest *89*, 351-355.

Courey,A.J. and Tjian,R. (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell *55*, 887-898.

Craig,W.Y., Palomaki,G.E., and Haddow,J.E. (1989). Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. BMJ *298*, 784-788.

Croft,S.A., Hampton,K.K., Daly,M.E., Steeds,R.P., Channer,K.S., and Samani,N.J. (2000). Kozak sequence polymorphism in the platelet GPIbalpha gene is not associated with risk of myocardial infarction. Blood *95*, 2183-2184.

Cross,J.C., Werb,Z., and Fisher,S.J. (1994). Implantation and the placenta: key pieces of the development puzzle. Science *266*, 1508-1518.

Cullen,P. (2000). Evidence that triglycerides are an independent coronary heart disease risk factor. Am. J. Cardiol. *86*, 943-949.

Cunningham,B.C. and Wells,J.A. (1989). High-resolution epitope mapping of hGH-receptor interactions by alanine- scanning mutagenesis. Science *244*, 1081-1085.

Dahlback,B. (1991). Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. Thromb. Haemost. *66*, 49-61.

Dahlback,B. (1999). Activated protein C resistance and thrombosis: molecular mechanisms of hypercoagulable state due to FVR506Q mutation. Semin. Thromb. Hemost. *25*, 273-289.

Dahlback,B. (2000). Blood coagulation. Lancet *355*, 1627-1632.

Dahlback,B., Lundwall,A., and Stenflo,J. (1986). Localization of thrombin cleavage sites in the amino-terminal region of bovine protein S. J. Biol. Chem. *261*, 5111-5115.

Daley,G.Q. and Cargill,M. (2001). The heart SNPs a beat: polymorphisms in candidate genes for cardiovascular disease. Trends Cardiovasc. Med. *11*, 60-66.

Damus,P.S., Hicks,M., and Rosenberg,R.D. (1973). Anticoagulant action of heparin. Nature *246*, 355-357.

Daniell,H., Vivekananda,J., Nielsen,B.L., Ye,G.N., Tewari,K.K., and Sanford,J.C. (1990). Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc. Natl. Acad. Sci. U. S. A *87*, 88-92.

Dart,A.M. and Chin-Dusting,J.P. (1999). Lipids and the endothelium. Cardiovasc. Res. *43*, 308-322.

David-Dufilho,M., Brussel,E.M., Topal,G., Walch,L., Brunet,A., and Rendu,F. (2005). Endothelial Thrombomodulin Induces Ca2+ Signals and Nitric Oxide Synthesis through Epidermal Growth Factor Receptor Kinase and Calmodulin Kinase II. J. Biol. Chem. *280*, 35999-36006.

Davie,E.W. (1995). Biochemical and molecular aspects of the coagulation cascade. Thromb. Haemost. *74*, 1-6.

Davie,E.W., Fujikawa,K., and Kisiel,W. (1991). The coagulation cascade: initiation, maintenance, and regulation. Biochemistry *30*, 10363-10370.

Davie,E.W. and Ratnoff,O.D. (1964). Waterfall sequence for intrinsic blood clotting. Science *145*, 1310-1312.

Davies,M.J. (2000). The pathophysiology of acute coronary syndromes. Heart *83*, 361-366.

Dawson,S., Hamsten,A., Wiman,B., Henney,A., and Humphries,S. (1991). Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. Arterioscler. Thromb. *11*, 183-190.

Dawson,S.J., Wiman,B., Hamsten,A., Green,F., Humphries,S., and Henney,A.M. (1993). The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. J. Biol. Chem. *268*, 10739-10745.

de Wet,J.R., Wood,K.V., DeLuca,M., Helinski,D.R., and Subramani,S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell Biol. *7*, 725-737.

Dittman,W.A., Kumada,T., and Majerus,P.W. (1989). Transcription of thrombomodulin mRNA in mouse hemangioma cells is increased by cycloheximide and thrombin. Proc. Natl. Acad. Sci. U. S. A *86*, 7179-7182.

Dittman,W.A., Kumada,T., Sadler,J.E., and Majerus,P.W. (1988). The structure and function of mouse thrombomodulin. Phorbol myristate acetate stimulates degradation and synthesis of thrombomodulin without affecting mRNA levels in hemangioma cells. J. Biol. Chem. *263*, 15815-15822.

Dittman,W.A., Nelson,S.C., Greer,P.K., Horton,E.T., Palomba,M.L., and McCachren,S.S. (1994). Characterization of thrombomodulin expression in response to retinoic acid and identification of a retinoic acid response element in the human thrombomodulin gene. J. Biol. Chem. *269*, 16925-16932.

Doggen,C.J., Cats,V.M., Bertina,R.M., and Rosendaal,F.R. (1998a). Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. Circulation *97*, 1037-1041.

Doggen,C.J., Kunz,G., Rosendaal,F.R., Lane,D.A., Vos,H.L., Stubbs,P.J., Manger,C., V, and Ireland,H. (1998b). A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. Thromb. Haemost. *80*, 743-748.

Doll,R. and Hill,A.B. (1966). Mortality of British doctors in relation to smoking: observations on coronary thrombosis. Natl. Cancer Inst. Monogr *19*, 205-268.

Douglas,H., Michaelides,K., Gorog,D.A., Durante-Mangoni,E., Ahmed,N., Davies,G.J., and Tuddenham,E.G. (2002). Platelet membrane glycoprotein Ibalpha gene -5T/C Kozak sequence polymorphism as an independent risk factor for the occurrence of coronary thrombosis. Heart *87*, 70-74.

Dovio,A. and Angeli,A. (2001). Cytokines and type 2 diabetes mellitus. JAMA *286*, 2233.

Drickamer,K. (1988). Two distinct classes of carbohydrate-recognition domains in animal lectins. J. Biol. Chem. *263*, 9557-9560.

Dullaart,R.P., Hoogenberg,K., Dikkeschei,B.D., and van Tol,A. (1994). Higher plasma lipid transfer protein activities and unfavorable lipoprotein changes in cigarette-smoking men. Arterioscler. Thromb. *14*, 1581-1585.

Eaton,D.L., Malloy,B.E., Tsai,S.P., Henzel,W., and Drayna,D. (1991). Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. J. Biol. Chem. *266*, 21833-21838.

Edgell,C.J., McDonald,C.C., and Graham,J.B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. U. S. A *80*, 3734-3737.

Egan,J.O., Kalafatis,M., and Mann,K.G. (1997). The effect of Arg306-->Ala and Arg506-->Gln substitutions in the inactivation of recombinant human factor Va by activated protein C and protein S. Protein Sci. *6*, 2016-2027.

Ehrlich,H.J., Grinnell,B.W., Jaskunas,S.R., Esmon,C.T., Yan,S.B., and Bang,N.U. (1990). Recombinant human protein C derivatives: altered response to calcium resulting in enhanced activation by thrombin. EMBO J. *9*, 2367-2373.

Eijnden-Schrauwen,Y., Lakenberg,N., Emeis,J.J., and de Knijff,P. (1995). Alu-repeat polymorphism in the tissue-type plasminogen activator (tPA) gene does not affect basal endothelial tPA synthesis. Thromb. Haemost. *74*, 1202.

Eisele,B. and Lamy,M. (1998). Clinical experience with antithrombin III concentrates in critically ill patients with sepsis and multiple organ failure. Semin. Thromb. Hemost. *24*, 71-80.

Eriksson,P., Nilsson,L., Karpe,F., and Hamsten,A. (1998). Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia. Arterioscler. Thromb. Vasc. Biol. *18*, 20-26.

Ernst,E. and Resch,K.L. (1993). Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. Ann. Intern. Med. *118*, 956-963.

Escobedo,J.A., Barr,P.J., and Williams,L.T. (1988). Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell Biol. *8*, 5126-5131.

Esmon,C.T. (1989). The roles of protein C and thrombomodulin in the regulation of blood coagulation. J. Biol. Chem. *264*, 4743-4746.

Esmon,C.T. (1995). Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J. *9*, 946-955.

Esmon,C.T. (2000). Regulation of blood coagulation. Biochim. Biophys. Acta *1477*, 349-360.

Esmon,C.T. (2001). Protein C anticoagulant pathway and its role in controlling microvascular thrombosis and inflammation. Crit Care Med. *29*, S48-S51.

Esmon,C.T. (2002). New mechanisms for vascular control of inflammation mediated by natural anticoagulant proteins. J. Exp. Med. *196*, 561-564.

Esmon,C.T. (2005). Is APC activation of endothelial cell PAR1 important in severe sepsis?: No. J. Thromb. Haemost. *3*, 1910-1911.

Esmon,C.T., Esmon,N.L., and Harris,K.W. (1982a). Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. J. Biol. Chem. *257*, 7944-7947.

Esmon,C.T. and Owen,W.G. (1981). Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc. Natl. Acad. Sci. U. S. A *78*, 2249-2252.

Esmon,C.T. and Owen,W.G. (2004). The discovery of thrombomodulin. J. Thromb. Haemost. *2*, 209-213.

Esmon,C.T., Taylor,F.B., Jr., and Snow,T.R. (1991). Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. Thromb. Haemost. *66*, 160-165.

Esmon,N.L., Carroll,R.C., and Esmon,C.T. (1983). Thrombomodulin blocks the ability of thrombin to activate platelets. J. Biol. Chem. *258*, 12238-12242.

Esmon,N.L., Owen,W.G., and Esmon,C.T. (1982b). Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem. *257*, 859-864.

Espana,F., Gruber,A., Heeb,M.J., Hanson,S.R., Harker,L.A., and Griffin,J.H. (1991). In vivo and in vitro complexes of activated protein C with two inhibitors in baboons. Blood *77*, 1754-1760.

Espinosa,R., III, Sadler,J.E., and Le Beau,M.M. (1989). Regional localization of the human thrombomodulin gene to 20p12-cen. Genomics *5*, 649-650.

Even-Ram,S., Uziely,B., Cohen,P., Grisaru-Granovsky,S., Maoz,M., Ginzburg,Y., Reich,R., Vlodavsky,I., and Bar-Shavit,R. (1998). Thrombin receptor overexpression in malignant and physiological invasion processes. Nat. Med. *4*, 909-914.

Farrall,M. and Morris,A.P. (2005). Gearing up for genome-wide gene-association studies. Hum. Mol. Genet. *14 Suppl 2*, R157-R162.

Fay,P.J. (1999). Regulation of factor VIIIa in the intrinsic factor Xase. Thromb. Haemost. *82*, 193-200.

Fay,P.J., Smudzin,T.M., and Walker,F.J. (1991). Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. J. Biol. Chem. *266*, 20139-20145.

Fazel,A., Vincenot,A., Malassine,A., Soncin,F., Gaussem,P., Alsat,E., and Evain-Brion,D. (1998). Increase in expression and activity of thrombomodulin in term human syncytiotrophoblast microvilli. Placenta *19*, 261-268.

Feldman,S.R. and Pizzo,S.V. (1986). A three-dimensional model of a unique proteinase inhibitor: alpha 2-macroglobulin. Semin. Thromb. Hemost. *12*, 223-225.

Felgner,P.L., Gadek,T.R., Holm,M., Roman,R., Chan,H.W., Wenz,M., Northrop,J.P., Ringold,G.M., and Danielsen,M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U. S. A *84*, 7413-7417.

Felgner,P.L. and Ringold,G.M. (1989). Cationic liposome-mediated transfection. Nature *337*, 387-388.

Ferraresi,P., Marchetti,G., Legnani,C., Cavallari,E., Castoldi,E., Mascoli,F., Ardissino,D., Palareti,G., and Bernardi,F. (1997). The heterozygous 20210 G/A prothrombin genotype is associated with early venous thrombosis in inherited thrombophilias and is not increased in frequency in artery disease. Arterioscler. Thromb. Vasc. Biol. *17*, 2418-2422.

Fickl,H., Van Antwerpen,V.L., Richards,G.A., Van der Westhuyzen,D.R., Davies,N., Van der,W.R., Van der Merwe,C.A., and Anderson,R. (1996). Increased levels of autoantibodies to cardiolipin and oxidised low density lipoprotein are inversely associated with plasma vitamin C status in cigarette smokers. Atherosclerosis *124*, 75-81.

Fleury,V. and Angles-Cano,E. (1991). Characterization of the binding of plasminogen to fibrin surfaces: the role of carboxy-terminal lysines. Biochemistry *30*, 7630-7638.

Frank,M.B., Reiner,A.P., Schwartz,S.M., Kumar,P.N., Pearce,R.M., Arbogast,P.G., Longstreth,W.T., Jr., Rosendaal,F.R., Psaty,B.M., and Siscovick,D.S. (2001). The Kozak sequence polymorphism of platelet glycoprotein Ibalpha and risk of nonfatal myocardial infarction and nonfatal stroke in young women. Blood *97*, 875-879.

Franscini,N., Bachli,E.B., Blau,N., Leikauf,M.S., Schaffner,A., and Schoedon,G. (2004). Gene expression profiling of inflamed human endothelial cells and influence of activated protein C. Circulation *110*, 2903-2909.

Frebelius,S., Hedin,U., and Swedenborg,J. (1994). Thrombogenicity of the injured vessel wall--role of antithrombin and heparin. Thromb. Haemost. *71*, 147-153.

Fried,S.K., Bunkin,D.A., and Greenberg,A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J. Clin. Endocrinol. Metab *83*, 847-850.

Fuentes-Prior,P., Iwanaga,Y., Huber,R., Pagila,R., Rumennik,G., Seto,M., Morser,J., Light,D.R., and Bode,W. (2000). Structural basis for the anticoagulant activity of the thrombin- thrombomodulin complex. Nature *404*, 518-525.

Fukudome,K. and Esmon,C.T. (1994). Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J. Biol. Chem. *269*, 26486-26491.

Fukudome,K., Ye,X., Tsuneyoshi,N., Tokunaga,O., Sugawara,K., Mizokami,H., and Kimoto,M. (1998). Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor. J. Exp. Med. *187*, 1029-1035.

Furie,B. and Furie,B.C. (1992). Molecular and cellular biology of blood coagulation. N. Engl. J. Med. *326*, 800-806.

Gailani,D. and Broze,G.J., Jr. (1991). Factor XI activation in a revised model of blood coagulation. Science *253*, 909-912.

Garcia,M.J., McNamara,P.M., Gordon,T., and Kannel,W.B. (1974). Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. Diabetes *23*, 105-111.

Gehring,N.H., Frede,U., Neu-Yilik,G., Hundsdoerfer,P., Vetter,B., Hentze,M.W., and Kulozik,A.E. (2001). Increased efficiency of mRNA 3' end formation: a new genetic mechanism contributing to hereditary thrombophilia. Nat. Genet. *28*, 389-392.

Gerlitz,B. and Grinnell,B.W. (1996). Mutation of protease domain residues Lys37-39 in human protein C inhibits activation by the thrombomodulin-thrombin complex without affecting activation by free thrombin. J. Biol. Chem. *271*, 22285-22288.

Gerlitz,B., Hassell,T., Vlahos,C.J., Parkinson,J.F., Bang,N.U., and Grinnell,B.W. (1993). Identification of the predominant glycosaminoglycan-attachment site in soluble recombinant human thrombomodulin: potential regulation of functionality by glycosyltransferase competition for serine474. Biochem. J. *295*, 131-140.

Gerth,C., Roberts,W.W., and Ferry,J.D. (1974). Rheology of fibrin clots. II. Linear viscoelastic behavior in shear creep. Biophys. Chem. *2*, 208-217.

Ghosh,S., May,M.J., and Kopp,E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. *16*, 225-260.

Gibbs,C.S., Coutre,S.E., Tsiang,M., Li,W.X., Jain,A.K., Dunn,K.E., Law,V.S., Mao,C.T., Matsumura,S.Y., Mejza,S.J., and . (1995). Conversion of thrombin into an anticoagulant by protein engineering. Nature *378*, 413-416.

Gimbrone,M.A., Jr., Topper,J.N., Nagel,T., Anderson,K.R., and Garcia-Cardena,G. (2000). Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann. N. Y. Acad. Sci. *902*, 230-239.

Girard,T.J., Warren,L.A., Novotny,W.F., Likert,K.M., Brown,S.G., Miletich,J.P., and Broze,G.J., Jr. (1989). Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature *338*, 518-520.

Gires,O., Kieu,C., Fix,P., Schmitt,B., Munz,M., Wollenberg,B., and Zeidler,R. (2001). Tumor necrosis factor alpha negatively regulates the expression of the carcinoma-associated antigen epithelial cell adhesion molecule. Cancer *92*, 620-628.

Glaser,C.B., Morser,J., Clarke,J.H., Blasko,E., McLean,K., Kuhn,I., Chang,R.J., Lin,J.H., Vilander,L., Andrews,W.H., and . (1992). Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. J. Clin. Invest *90*, 2565-2573.

Glass,C.K. and Witztum,J.L. (2001). Atherosclerosis. the road ahead. Cell *104*, 503-516.

Goldbourt,U. and Neufeld,H.N. (1986). Genetic aspects of arteriosclerosis. Arteriosclerosis *6*, 357-377.

Golino,P., Cirillo,P., Calabro',P., Ragni,M., D'Andrea,D., Avvedimento,E.V., Vigorito,F., Corcione,N., Loffredo,F., and Chiariello,M. (2001). Expression of exogenous tissue factor pathway inhibitor in vivo suppresses thrombus formation in injured rabbit carotid arteries. J. Am. Coll. Cardiol. *38*, 569-576.

Gomez,K., McVey,J.H., and Tuddenham,E. (2005). Inhibition of coagulation by macromolecular complexes. Haematologica *90*, 1570-1576.

Gonzalez-Conejero,R., Lozano,M.L., Rivera,J., Corral,J., Iniesta,J.A., Moraleda,J.M., and Vicente,V. (1998). Polymorphisms of platelet membrane glycoprotein Ib associated with arterial thrombotic disease. Blood *92*, 2771-2776.

Gordon,E.M., Hellerstein,H.K., Ratnoff,O.D., Arafah,B.M., and Yamashita,T.S. (1987). Augmented Hageman factor and prolactin titers, enhanced cold activation of factor VII, and spontaneous shortening of prothrombin time in survivors of myocardial infarction. J. Lab Clin. Med. *109*, 409-413.

Gosling,M., Golledge,J., Turner,R.J., and Powell,J.T. (1999). Arterial flow conditions downregulate thrombomodulin on saphenous vein endothelium. Circulation *99*, 1047-1053.

Grant,P.J. (2003). The genetics of atherothrombotic disorders: a clinician's view. J. Thromb. Haemost. *1*, 1381-1390.

Green,F., Kelleher,C., Wilkes,H., Temple,A., Meade,T., and Humphries,S. (1991). A common genetic polymorphism associated with lower coagulation factor VII levels in healthy individuals. Arterioscler. Thromb. *11*, 540-546.

Greengard,J.S., Sun,X., Xu,X., Fernandez,J.A., Griffin,J.H., and Evatt,B. (1994). Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet *343*, 1361-1362.

Gregory,S.A., Morrissey,J.H., and Edgington,T.S. (1989). Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Mol. Cell Biol. *9*, 2752-2755.

Grey,S.T., Csizmadia,V., and Hancock,W.W. (1998). Differential effect of tumor necrosis factor-alpha on thrombomodulin gene expression by human monocytoid (THP-1) cell versus endothelial cells. Int. J. Hematol. *67*, 53-62.

Grey,S.T., Tsuchida,A., Hau,H., Orthner,C.L., Salem,H.H., and Hancock,W.W. (1994). Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-gamma, or phorbol ester. J. Immunol. *153*, 3664-3672.

Griffith,M.J. (1982). Kinetics of the heparin-enhanced antithrombin III/thrombin reaction. Evidence for a template model for the mechanism of action of heparin. J. Biol. Chem. *257*, 7360-7365.

Griffith,M.J., Noyes,C.M., Tyndall,J.A., and Church,F.C. (1985). Structural evidence for leucine at the reactive site of heparin cofactor II. Biochemistry *24*, 6777-6782.

Gu,J.M., Fukudome,K., and Esmon,C.T. (2000a). Characterization and regulation of the 5'-flanking region of the murine endothelial protein C receptor gene. J. Biol. Chem. *275*, 12481-12488.

Gu,J.M., Katsuura,Y., Ferrell,G.L., Grammas,P., and Esmon,C.T. (2000b). Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding in vivo. Blood *95*, 1687-1693.

Haffner,S.M. (2002). Lipoprotein disorders associated with type 2 diabetes mellitus and insulin resistance. Am. J. Cardiol. *90*, 55i-61i.

Haffner,S.M. (2003). Insulin resistance, inflammation, and the prediabetic state. Am. J. Cardiol. *92*, 18J-26J.

Haffner,S.M., Valdez,R.A., Hazuda,H.P., Mitchell,B.D., Morales,P.A., and Stern,M.P. (1992). Prospective analysis of the insulin-resistance syndrome (syndrome X). Diabetes *41*, 715-722.

Haim,M., Benderly,M., Brunner,D., Behar,S., Graff,E., Reicher-Reiss,H., and Goldbourt,U. (1999). Elevated serum triglyceride levels and long-term mortality in patients with coronary heart disease: the Bezafibrate Infarction Prevention (BIP) Registry. Circulation *100*, 475-482.

Hammar,N., Kaprio,J., Hagstrom,U., Alfredsson,L., Koskenvuo,M., and Hammar,T. (2002). Migration and mortality: a 20 year follow up of Finnish twin pairs with migrant co-twins in Sweden. J. Epidemiol. Community Health *56*, 362-366.

Hamsten,A., Wiman,B., de Faire,U., and Blomback,M. (1985). Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. N. Engl. J. Med. *313*, 1557-1563.

Han,X., Fiehler,R., and Broze,G.J., Jr. (1998). Isolation of a protein Z-dependent plasma protease inhibitor. Proc. Natl. Acad. Sci. U. S. A *95*, 9250-9255.

Han,X., Fiehler,R., and Broze,G.J., Jr. (2000). Characterization of the protein Z-dependent protease inhibitor. Blood *96*, 3049-3055.

Han,X., Huang,Z.F., Fiehler,R., and Broze,G.J., Jr. (1999). The protein Z-dependent protease inhibitor is a serpin. Biochemistry *38*, 11073-11078.

Hansson,L., Zanchetti,A., Carruthers,S.G., Dahlof,B., Elmfeldt,D., Julius,S., Menard,J., Rahn,K.H., Wedel,H., and Westerling,S. (1998). Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group. Lancet *351*, 1755-1762.

Hardman,A.E. (1996). Exercise in the prevention of atherosclerotic, metabolic and hypertensive diseases: a review. J. Sports Sci. *14*, 201-218.

Harmon,J.T. and Jamieson,G.A. (1986). Activation of platelets by alpha-thrombin is a receptor-mediated event. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone-thrombin, but not N alpha-tosyl-L-lysine chloromethyl ketone-thrombin, binds to the high affinity thrombin receptor. J. Biol. Chem. *261*, 15928-15933.

Harris,K.W. and Esmon,C.T. (1985). Protein S is required for bovine platelets to support activated protein C binding and activity. J. Biol. Chem. *260*, 2007-2010.

Hayashi,T., Honda,G., and Suzuki,K. (1992). An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thrombomodulin expression in human umbilical vein endothelial cells. Blood *79*, 2930-2936.

Hayashi,T., Zushi,M., Yamamoto,S., and Suzuki,K. (1990). Further localization of binding sites for thrombin and protein C in human thrombomodulin. J. Biol. Chem. *265*, 20156-20159.

He,L., Vicente,C.P., Westrick,R.J., Eitzman,D.T., and Tollefsen,D.M. (2002). Heparin cofactor II inhibits arterial thrombosis after endothelial injury. J. Clin. Invest *109*, 213-219.

Healy,A.M., Rayburn,H.B., Rosenberg,R.D., and Weiler,H. (1995). Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc. Natl. Acad. Sci. U. S. A *92*, 850-854.

Heeb,M.J. and Griffin,J.H. (1988). Physiologic inhibition of human activated protein C by alpha 1-antitrypsin. J. Biol. Chem. *263*, 11613-11616.

Heeb,M.J., Gruber,A., and Griffin,J.H. (1991). Identification of divalent metal ion-dependent inhibition of activated protein C by alpha 2-macroglobulin and alpha 2-antiplasmin in blood and comparisons to inhibition of factor Xa, thrombin, and plasmin. J. Biol. Chem. *266*, 17606-17612.

Heemskerk,J.W., Bevers,E.M., and Lindhout,T. (2002). Platelet activation and blood coagulation. Thromb. Haemost. *88*, 186-193.

Heinrich,J., Balleisen,L., Schulte,H., Assmann,G., and van de,L.J. (1994). Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. Arterioscler. Thromb. *14*, 54-59.

Henkel,T., Machleidt,T., Alkalay,I., Kronke,M., Ben Neriah,Y., and Baeuerle,P.A. (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature *365*, 182-185.

Henry,M., Aubert,H., Morange,P.E., Nanni,I., Alessi,M.C., Tiret,L., and Juhan-Vague,I. (2001). Identification of polymorphisms in the promoter and the 3' region of the TAFI gene: evidence that plasma TAFI antigen levels are strongly genetically controlled. Blood *97*, 2053-2058.

Herbaczynska-Cedro,K., Wartanowicz,M., Panczenko-Kresowska,B., Cedro,K., Klosiewicz-Wasek,B., and Wasek,W. (1994). Inhibitory effect of vitamins C and E on the oxygen free radical production in human polymorphonuclear leucocytes. Eur. J. Clin. Invest *24*, 316-319.

Hermans,J.M. and Stone,S.R. (1993). Interaction of activated protein C with serpins. Biochem. J. *295 (Pt 1)*, 239-245.

Herrmann,S.M., Poirier,O., Marques-Vidal,P., Evans,A., Arveiler,D., Luc,G., Emmerich,J., and Cambien,F. (1997). The Leu33/Pro polymorphism (PlA1/PlA2) of the glycoprotein IIIa (GPIIIa) receptor is not related to myocardial infarction in the ECTIM Study. Etude Cas-Temoins de l'Infarctus du Myocarde. Thromb. Haemost. *77*, 1179-1181.

Hickey,M.J., Williams,S.A., and Roth,G.J. (1989). Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures. Proc. Natl. Acad. Sci. U. S. A *86*, 6773-6777.

Himmelmann,A., Hedner,T., Hansson,L., O'Donnell,C.J., and Levy,D. (1998). Isolated systolic hypertension: an important cardiovascular risk factor. Blood Press *7*, 197-207.

Hirose,K., Okajima,K., Taoka,Y., Uchiba,M., Tagami,H., Nakano,K., Utoh,J., Okabe,H., and Kitamura,N. (2000). Activated protein C reduces the ischemia/reperfusion-induced spinal cord injury in rats by inhibiting neutrophil activation. Ann. Surg. *232*, 272-280.

Hirschfield,G.M. and Pepys,M.B. (2003). C-reactive protein and cardiovascular disease: new insights from an old molecule. QJM. *96*, 793-807.

Hochner-Celnikier,D., Manor,O., Gotzman,O., Lotan,H., and Chajek-Shaul,T. (2002). Gender gap in coronary artery disease: comparison of the extent, severity and risk factors in men and women aged 45-65 years. Cardiology *97*, 18-23.

Hojima,Y., Cochrane,C.G., Wiggins,R.C., Austen,K.F., and Stevens,R.L. (1984). In vitro activation of the contact (Hageman factor) system of plasma by heparin and chondroitin sulfate E. Blood *63*, 1453-1459.

Horie,S., Ishii,H., Matsumoto,F., Kusano,M., Kizaki,K., Matsuda,J., and Kazama,M. (2001). Acceleration of thrombomodulin gene transcription by retinoic acid: retinoic acid receptors and Sp1 regulate the promoter activity through interactions with two different sequences in the 5'-flanking region of human gene. J. Biol. Chem. *276*, 2440-2450.

Horie,S., Kizaki,K., Ishii,H., and Kazama,M. (1992). Retinoic acid stimulates expression of thrombomodulin, a cell surface anticoagulant glycoprotein, on human endothelial cells. Differences between up-regulation of thrombomodulin by retinoic acid and cyclic AMP. Biochem. J. *28*, 149-154.

Horvat,R. and Palade,G.E. (1993). Thrombomodulin and thrombin localization on the vascular endothelium; their internalization and transcytosis by plasmalemmal vesicles. Eur. J. Cell Biol. *61*, 299-313.

Hosaka,Y., Takahashi,Y., and Ishii,H. (1998). Thrombomodulin in human plasma contributes to inhibit fibrinolysis through acceleration of thrombin-dependent activation of plasma procarboxypeptidase B. Thromb. Haemost. *79*, 371-377.

Hotamisligil,G.S., Arner,P., Caro,J.F., Atkinson,R.L., and Spiegelman,B.M. (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J. Clin. Invest *95*, 2409-2415.

Hoylaerts,M., Rijken,D.C., Lijnen,H.R., and Collen,D. (1982). Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J. Biol. Chem. *257*, 2912-2919.

Hsu-Hage,B.H. and Wahlqvist,M.L. (1993). Cardiovascular risk in adult Melbourne Chinese. Aust. J. Public Health *17*, 306-313.

Huang,E.M. and Detwiler,T.C. (1987). Thrombin-induced phosphoinositide hydrolysis in platelets. Receptor occupancy and desensitization. Biochem. J. *242*, 11-18.

Huang,H.C., Shi,G.Y., Jiang,S.J., Shi,C.S., Wu,C.M., Yang,H.Y., and Wu,H.L. (2003). Thrombomodulin-mediated cell adhesion: involvement of its lectin-like domain. J. Biol. Chem. *278*, 46750-46759.

Huang,Z.F., Wun,T.C., and Broze,G.J., Jr. (1993). Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. J. Biol. Chem. *268*, 26950-26955.

Humphries,S.E., Cook,M., Dubowitz,M., Stirling,Y., and Meade,T.W. (1987). Role of genetic variation at the fibrinogen locus in determination of plasma fibrinogen concentrations. Lancet *1*, 1452-1455.

Humphries,S.E. and Donati,M.B. (2002). Analysis of gene-environment interaction in coronary artery disease. Ital. Heart J. *3*, 3-5.

Humphries,S.E., Green,F.R., Temple,A., Dawson,S., Henney,A., Kelleher,C.H., Wilkes,H., Meade,T.W., Wiman,B., and Hamsten,A. (1992). Genetic factors determining thrombosis and fibrinolysis. Ann. Epidemiol. *2*, 371-385.

Humphries,S.E., Hawe,E., Dhamrait,S., Miller,G.J., and Talmud,P.J. (2003). In search of genetic precision. Lancet *361*, 1908-1909.

Humphries,S.E., Talmud,P.J., Hawe,E., Bolla,M., Day,I.N., and Miller,G.J. (2001). Apolipoprotein E4 and coronary heart disease in middle-aged men who smoke: a prospective study. Lancet *358*, 115-119.

Hunault,M., Arbini,A.A., Lopaciuk,S., Carew,J.A., and Bauer,K.A. (1997). The Arg353Gln polymorphism reduces the level of coagulation factor VII. In vivo and in vitro studies. Arterioscler. Thromb. Vasc. Biol. *17*, 2825-2829.

Iacoviello,L., Burzotta,F., Di Castelnuovo,A., Zito,F., Marchioli,R., and Donati,M.B. (1998). The 4G/5G polymorphism of PAI-1 promoter gene and the risk of myocardial infarction: a meta-analysis. Thromb. Haemost. *80*, 1029-1030.

Iber,H., Chen,Q., Cheng,P.Y., and Morgan,E.T. (2000). Suppression of CYP2C11 gene transcription by interleukin-1 mediated by NF-kappaB binding at the transcription start site. Arch. Biochem. Biophys. *377*, 187-194.

Ichinose,A., Takeya,H., Espling,E., Iwanaga,S., Kisiel,W., and Davie,E.W. (1990). Amino acid sequence of human protein Z, a vitamin K-dependent plasma glycoprotein. Biochem. Biophys. Res. Commun. *172*, 1139-1144.

Inukai,T., Fujiwara,Y., Tayama,K., Aso,Y., and Takemura,Y. (1996). Clinical significance of measurements of urinary and serum thrombomodulins in patients with non-insulin-dependent diabetes mellitus. Diabetes Res. Clin. Pract. *33*, 99-104.

Ireland,H., Konstantoulas,C.J., Cooper,J.A., Hawe,E., Humphries,S.E., Mather,H., Goodall,A.H., Hogwood,J., Juhan-Vague,I., Yudkin,J.S., Di Minno,G., Margaglione,M., Hamsten,A., Miller,G.J., Bauer,K.A., Kim,Y.T., Stearns-Kurosawa,D.J., and Kurosawa,S. (2005). EPCR Ser219Gly: elevated sEPCR, prothrombin F1+2, risk for coronary heart disease, and increased sEPCR shedding in vitro. Atherosclerosis *183*, 283-292.

Ireland,H., Kunz,G., Kyriakoulis,K., Stubbs,P.J., and Lane,D.A. (1997). Thrombomodulin gene mutations associated with myocardial infarction. Circulation *96*, 15-18.

Irigoyen,J.P., Munoz-Canoves,P., Montero,L., Koziczak,M., and Nagamine,Y. (1999). The plasminogen activator system: biology and regulation. Cell Mol. Life Sci. *56*, 104-132.

Isermann,B., Hendrickson,S.B., Hutley,K., Wing,M., and Weiler,H. (2001). Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block. Development *128*, 827-838.

Isermann,B., Sood,R., Pawlinski,R., Zogg,M., Kalloway,S., Degen,J.L., Mackman,N., and Weiler,H. (2003). The thrombomodulin-protein C system is essential for the maintenance of pregnancy. Nat. Med. *9*, 331-337.

Ishida,F., Furihata,K., Ishida,K., Kodaira,H., Han,K.S., Liu,D.Z., Kitano,K., and Kiyosawa,K. (1996). The largest isoform of platelet membrane glycoprotein Ib alpha is commonly distributed in eastern Asian populations. Thromb. Haemost. *76*, 245-247.

Ishii,H., Horie,S., Kizaki,K., and Kazama,M. (1992). Retinoic acid counteracts both the downregulation of thrombomodulin and the induction of tissue factor in cultured human endothelial cells exposed to tumor necrosis factor. Blood *80*, 2556-2562.

Ishii,H., Kizaki,K., Horie,S., and Kazama,M. (1996). Oxidized low density lipoprotein reduces thrombomodulin transcription in cultured human endothelial cells through degradation of the lipoprotein in lysosomes. J. Biol. Chem. *271*, 8458-8465.

Ishii,H. and Majerus,P.W. (1985). Thrombomodulin is present in human plasma and urine. J. Clin. Invest *76*, 2178-2181.

Ishii,H., Nakano,M., Tsubouchi,J., Ishikawa,T., Uchiyama,H., Hiraishi,S., Tahara,C., Miyajima,Y., and Kazama,M. (1990). Establishment of enzyme immunoassay of human thrombomodulin in plasma and urine using monoclonal antibodies. Thromb. Haemost. *63*, 157-162.

Ishii,H., Tezuka,T., Ishikawa,H., Takada,K., Oida,K., and Horie,S. (2003). Oxidized phospholipids in oxidized low-density lipoprotein down- regulate thrombomodulin transcription in vascular endothelial cells through a decrease in the binding of RAR{beta}-RXR{alpha} heterodimers and Sp1 and Sp3 to their binding sequences in the TM promoter. Blood *101*, 4765-4774.

Ishii,H., Uchiyama,H., and Kazama,M. (1991). Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb. Haemost. *65*, 618-623.

Jackman,R.W., Beeler,D.L., Fritze,L., Soff,G., and Rosenberg,R.D. (1987). Human thrombomodulin gene is intron depleted: nucleic acid sequences of the cDNA and gene predict protein structure and suggest sites of regulatory control. Proc. Natl. Acad. Sci. U. S. A *84*, 6425-6429.

Jackman,R.W., Beeler,D.L., VanDeWater,L., and Rosenberg,R.D. (1986). Characterization of a thrombomodulin cDNA reveals structural similarity to the low density lipoprotein receptor. Proc. Natl. Acad. Sci. U. S. A *83*, 8834-8838.

Jayaraman,G., Srinivas,R., Duggan,C., Ferreira,E., Swaminathan,S., Somasundaram,K., Williams,J., Hauser,C., Kurkinen,M., Dhar,R., Weitzman,S., Buttice,G., and Thimmapaya,B. (1999). p300/cAMP-responsive element-binding protein interactions with ets-1 and ets-2 in the transcriptional activation of the human stromelysin promoter. J. Biol. Chem. *274*, 17342-17352.

Jern,C., Ladenvall,P., Wall,U., and Jern,S. (1999). Gene polymorphism of t-PA is associated with forearm vascular release rate of t-PA. Arterioscler. Thromb. Vasc. Biol. *19*, 454-459.

Jesty,J., Lorenz,A., Rodriguez,J., and Wun,T.C. (1996). Initiation of the tissue factor pathway of coagulation in the presence of heparin: control by antithrombin III and tissue factor pathway inhibitor. Blood *87*, 2301-2307.

Johnson,E.J., Prentice,C.R., and Parapia,L.A. (1990). Premature arterial disease associated with familial antithrombin III deficiency. Thromb. Haemost. *63*, 13-15.

Jousilahti,P., Vartiainen,E., Tuomilehto,J., and Puska,P. (1999). Sex, age, cardiovascular risk factors, and coronary heart disease: a prospective follow-up study of 14 786 middle-aged men and women in Finland. Circulation *99*, 1165-1172.

Joyce,D.E., Gelbert,L., Ciaccia,A., DeHoff,B., and Grinnell,B.W. (2001). Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J. Biol. Chem. *276*, 11199-11203.

Juhan-Vague,I., Alessi,M.C., and Vague,P. (1991). Increased plasma plasminogen activator inhibitor 1 levels. A possible link between insulin resistance and atherothrombosis. Diabetologia *34*, 457-462.

Juhan-Vague,I., Morange,P.E., Aubert,H., Henry,M., Aillaud,M.F., Alessi,M.C., Samnegard,A., Hawe,E., Yudkin,J., Margaglione,M., Di Minno,G., Hamsten,A., and Humphries,S.E. (2002). Plasma thrombin-activatable fibrinolysis inhibitor antigen concentration and genotype in relation to myocardial infarction in the north and south of Europe. Arterioscler. Thromb. Vasc. Biol. *22*, 867-873.

Juhan-Vague,I., Pyke,S.D., Alessi,M.C., Jespersen,J., Haverkate,F., and Thompson,S.G. (1996). Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. ECAT Study Group. European Concerted Action on Thrombosis and Disabilities. Circulation *94*, 2057-2063.

Juhan-Vague,I., Thompson,S.G., and Jespersen,J. (1993). Involvement of the hemostatic system in the insulin resistance syndrome. A study of 1500 patients with angina pectoris. The ECAT Angina Pectoris Study Group. Arterioscler. Thromb. *13*, 1865-1873.

Junker,R., Heinrich,J., Schulte,H., van de,L.J., and Assmann,G. (1997). Coagulation factor VII and the risk of coronary heart disease in healthy men. Arterioscler. Thromb. Vasc. Biol. *17*, 1539-1544.

Juul,K., Tybjaerg-Hansen,A., Steffensen,R., Kofoed,S., Jensen,G., and Nordestgaard,B.G. (2002). Factor V Leiden: The Copenhagen City Heart Study and 2 meta-analyses. Blood *100*, 3-10.

Kafonek,S.D. (1994). Postmenopausal hormone replacement therapy and cardiovascular risk reduction. A review. Drugs *47 Suppl 2*, 16-24.

Kanaji,T., Okamura,T., Osaki,K., Kuroiwa,M., Shimoda,K., Hamasaki,N., and Niho,Y. (1998). A common genetic polymorphism (46 C to T substitution) in the 5'-untranslated region of the coagulation factor XII gene is associated with low translation efficiency and decrease in plasma factor XII level. Blood *91*, 2010-2014.

Kannel,W.B. (1986a). Hypertension. Relationship with other risk factors. Drugs *31 Suppl 1*, 1-11.

Kannel,W.B. (1986b). Prevalence, incidence, and hazards of hypertension in the elderly. Am. Heart J. *112*, 1362-1363.

Kannel,W.B. (2000). The Framingham Study: ITS 50-year legacy and future promise. J. Atheroscler. Thromb. *6*, 60-66.

Kannel,W.B., Belanger,A., D'Agostino,R., and Israel,I. (1986). Physical activity and physical demand on the job and risk of cardiovascular disease and death: the Framingham Study. Am. Heart J. *112*, 820-825.

Kaplan,N.M. (1989). The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. Arch. Intern. Med. *149*, 1514-1520.

Kaski,S., Kekomaki,R., and Partanen,J. (1996). Systematic screening for genetic polymorphism in human platelet glycoprotein Ibalpha. Immunogenetics *44*, 170-176.

Kawai,S. and Nishizawa,M. (1984). New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell Biol. *4*, 1172-1174.

Kelleher,C.C., Mitropoulos,K.A., Imeson,J., Meade,T.W., Martin,J.C., Reeves,B.E., and Hughes,L.O. (1992). Hageman factor and risk of myocardial infarction in middle-aged men. Atherosclerosis *97*, 67-73.

Kemkes-Matthes,B. and Matthes,K.J. (1995). Protein Z, a new haemostatic factor, in liver diseases. Haemostasis *25*, 312-316.

Kern,P.A., Saghizadeh,M., Ong,J.M., Bosch,R.J., Deem,R., and Simsolo,R.B. (1995). The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J. Clin. Invest *95*, 2111-2119.

Khrenov,A.V., Ananyeva,N.M., Griffin,J.H., and Saenko,E.L. (2002). Coagulation pathways in atherothrombosis. Trends Cardiovasc. Med. *12*, 317-324.

Kim,A.Y., Walinsky,P.L., Kolodgie,F.D., Bian,C., Sperry,J.L., Deming,C.B., Peck,E.A., Shake,J.G., Ang,G.B., Sohn,R.H., Esmon,C.T., Virmani,R., Stuart,R.S., and Rade,J.J. (2002). Early loss of thrombomodulin expression impairs vein graft thromboresistance: implications for vein graft failure. Circ. Res. *90*, 205-212.

King,H., Aubert,R.E., and Herman,W.H. (1998). Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care *21*, 1414-1431.

Kinlay,S., Libby,P., and Ganz,P. (2001). Endothelial function and coronary artery disease. Curr. Opin. Lipidol. *12*, 383-389.

Kirtane,A.J., Leder,D.M., Waikar,S.S., Chertow,G.M., Ray,K.K., Pinto,D.S., Karmpaliotis,D., Burger,A.J., Murphy,S.A., Cannon,C.P., Braunwald,E., and Gibson,C.M. (2005). Serum blood urea nitrogen as an independent marker of subsequent mortality among patients with acute coronary syndromes and normal to mildly reduced glomerular filtration rates. J. Am. Coll. Cardiol. *45*, 1781-1786.

Kisiel,W. (1979). Human plasma protein C: isolation, characterization, and mechanism of activation by alpha-thrombin. J. Clin. Invest *64*, 761-769.

Kisiel,W., Canfield,W.M., Ericsson,L.H., and Davie,E.W. (1977). Anticoagulant properties of bovine plasma protein C following activation by thrombin. Biochemistry *16*, 5824-5831.

Kohler,H.P., Carter,A.M., Stickland,M.H., and Grant,P.J. (1998a). Levels of activated FXII in survivors of myocardial infarction--association with circulating risk factors and extent of coronary artery disease. Thromb. Haemost. *79*, 14-18.

Kohler,H.P., Futers,T.S., and Grant,P.J. (1999). FXII (46C-->T) polymorphism and in vivo generation of FXII activity--gene frequencies and relationship in patients with coronary artery disease. Thromb. Haemost. *81*, 745-747.

Kohler,H.P., Stickland,M.H., Ossei-Gerning,N., Carter,A., Mikkola,H., and Grant,P.J. (1998b). Association of a common polymorphism in the factor XIII gene with myocardial infarction. Thromb. Haemost. *79*, 8-13.

Kokame,K., Zheng,X., and Sadler,J.E. (1998). Activation of thrombin-activable fibrinolysis inhibitor requires epidermal growth factor-like domain 3 of thrombomodulin and is inhibited competitively by protein C. J. Biol. Chem. *273*, 12135-12139.

Koski,K., Laippala,P., and Kivela,S.L. (2000). Predictors of coronary heart diseases among children and adolescents in families with premature coronary heart diseases in central eastern Finland. Scand. J. Prim. Health Care *18*, 170-176.

Kouba,D.J., Chung,K.Y., Nishiyama,T., Vindevoghel,L., Kon,A., Klement,J.F., Uitto,J., and Mauviel,A. (1999). Nuclear factor-kappa B mediates TNF-alpha inhibitory effect on alpha 2(I) collagen (COL1A2) gene transcription in human dermal fibroblasts. J. Immunol. *162*, 4226-4234.

Koyama,T., Parkinson,J.F., Sie,P., Bang,N.U., Muller-Berghaus,G., and Preissner,K.T. (1991). Different glycoforms of human thrombomodulin. Their glycosaminoglycan-dependent modulatory effects on thrombin inactivation by heparin cofactor II and antithrombin III. Eur. J. Biochem. *198*, 563-570.

Kris-Etherton,P.M., Pearson,T.A., Wan,Y., Hargrove,R.L., Moriarty,K., Fishell,V., and Etherton,T.D. (1999). High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. Am. J. Clin. Nutr. *70*, 1009-1015.

Kroeger,K.M., Carville,K.S., and Abraham,L.J. (1997). The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. Mol. Immunol. *34*, 391-399.

Kroeger,K.M., Steer,J.H., Joyce,D.A., and Abraham,L.J. (2000). Effects of stimulus and cell type on the expression of the -308 tumour necrosis factor promoter polymorphism. Cytokine *12*, 110-119.

Kroll,M.H., Hellums,J.D., McIntire,L.V., Schafer,A.I., and Moake,J.L. (1996). Platelets and shear stress. Blood *88*, 1525-1541.

Kruglyak,L. (1999). Prospects for whole-genome linkage disequilibrium mapping of common disease genes. Nat. Genet. *22*, 139-144.

Kruys,V., Marinx,O., Shaw,G., Deschamps,J., and Huez,G. (1989). Translational blockade imposed by cytokine-derived UA-rich sequences. Science *245*, 852-855.

Kumada,T., Dittman,W.A., and Majerus,P.W. (1988). A role for thrombomodulin in the pathogenesis of thrombin-induced thromboembolism in mice. Blood *71*, 728-733.

Kunz,G., Ireland,H.A., Stubbs,P.J., Kahan,M., Coulton,G.C., and Lane,D.A. (2000). Identification and characterization of a thrombomodulin gene mutation coding for an elongated protein with reduced expression in a kindred with myocardial infarction. Blood *95*, 569-576.

Kunz,G., Ohlin,A.K., Adami,A., Zoller,B., Svensson,P., and Lane,D.A. (2002). Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. Blood *99*, 3646-3653.

Kurosawa,S., Stearns,D.J., Jackson,K.W., and Esmon,C.T. (1988). A 10-kDa cyanogen bromide fragment from the epidermal growth factor homology domain of rabbit thrombomodulin contains the primary thrombin binding site. J. Biol. Chem. *263*, 5993-5996.

Kurtz,T.W. and Spence,M.A. (1993). Genetics of essential hypertension. Am. J. Med. *94*, 77-84.

Kwok,S., Kellogg,D.E., McKinney,N., Spasic,D., Goda,L., Levenson,C., and Sninsky,J.J. (1990). Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. *18*, 999-1005.

Ladenvall,P., Johansson,L., Jansson,J.H., Jern,S., Nilsson,T.K., Tjarnlund,A., Jern,C., and Boman,K. (2002). Tissue-type plasminogen activator -7,351C/T enhancer polymorphism is associated with a first myocardial infarction. Thromb. Haemost. *87*, 105-109.

Ladenvall,P., Wall,U., Jern,S., and Jern,C. (2000). Identification of eight novel single-nucleotide polymorphisms at human tissue-type plasminogen activator (t-PA) locus: association with vascular t-PA release in vivo. Thromb. Haemost. *84*, 150-155.

Laffan,M.A. and Tuddenham,E.G. (1997). Inherited thrombophilias. QJM. *90*, 375-378.

Lamarche,B. (1998). Abdominal obesity and its metabolic complications: implications for the risk of ischaemic heart disease. Coron. Artery Dis. *9*, 473-481.

Lander,E.S. and Schork,N.J. (1994). Genetic dissection of complex traits. Science *265*, 2037-2048.

Lane,D.A. and Grant,P.J. (2000). Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. Blood *95*, 1517-1532.

Lane,D.A., Mannucci,P.M., Bauer,K.A., Bertina,R.M., Bochkov,N.P., Boulyjenkov,V., Chandy,M., Dahlback,B., Ginter,E.K., Miletich,J.P., Rosendaal,F.R., and Seligsohn,U. (1996a). Inherited thrombophilia: Part 1. Thromb. Haemost. *76*, 651-662.

Lane,D.A., Mannucci,P.M., Bauer,K.A., Bertina,R.M., Bochkov,N.P., Boulyjenkov,V., Chandy,M., Dahlback,B., Ginter,E.K., Miletich,J.P., Rosendaal,F.R., and Seligsohn,U. (1996b). Inherited thrombophilia: Part 2. Thromb. Haemost. *76*, 824-834.

Lane,D.A., Olds,R.J., and Thein,S.L. (1994). Antithrombin III: summary of first database update. Nucleic Acids Res. *22*, 3556-3559.

Laszik,Z., Mitro,A., Taylor,F.B., Jr., Ferrell,G., and Esmon,C.T. (1997). Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. Circulation *96*, 3633-3640.

Laszik,Z.G., Zhou,X.J., Ferrell,G.L., Silva,F.G., and Esmon,C.T. (2001). Down-regulation of endothelial expression of endothelial cell protein C receptor and thrombomodulin in coronary atherosclerosis. Am. J. Pathol. *159*, 797-802.

Le Bonniec,B.F. and Esmon,C.T. (1991). Glu-192----Gln substitution in thrombin mimics the catalytic switch induced by thrombomodulin. Proc. Natl. Acad. Sci. U. S. A *88*, 7371-7375.

Le Bonniec,B.F., MacGillivray,R.T., and Esmon,C.T. (1991). Thrombin Glu-39 restricts the P'3 specificity to nonacidic residues. J. Biol. Chem. *266*, 13796-13803.

Le Flem,L., Mennen,L., Aubry,M.L., Aiach,M., Scarabin,P.Y., Emmerich,J., and Alhenc-Gelas,M. (2001). Thrombomodulin promoter mutations, venous thrombosis, and varicose veins. Arterioscler. Thromb. Vasc. Biol. *21*, 445-451.

Le Flem,L., Picard,V., Emmerich,J., Gandrille,S., Fiessinger,J.N., Aiach,M., and Alhenc-Gelas,M. (1999). Mutations in promoter region of thrombomodulin and venous thromboembolic disease. Arterioscler. Thromb. Vasc. Biol. *19*, 1098-1104.

Lentz,S.R. (1997). Homocysteine and vascular dysfunction. Life Sci. *61*, 1205-1215.

Lentz,S.R. (2003). Thrombosis of vein grafts: wall tension restrains thrombomodulin expression. Circ. Res. *92*, 12-13.

Lentz,S.R. and Sadler,J.E. (1991). Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J. Clin. Invest *88*, 1906-1914.

Lentz,S.R., Tsiang,M., and Sadler,J.E. (1991). Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. Blood *77*, 542-550.

Levi,M. and ten Cate,H. (1999). Disseminated intravascular coagulation. N. Engl. J. Med. *341*, 586-592.

Li,Y.H., Chen,C.H., Yeh,P.S., Lin,H.J., Chang,B.I., Lin,J.C., Guo,H.R., Wu,H.L., Shi,G.Y., Lai,M.L., and Chen,J.H. (2001). Functional mutation in the promoter region of thrombomodulin gene in relation to carotid atherosclerosis. Atherosclerosis *154*, 713-719.

Li,Y.H., Chen,J.H., Tsai,W.C., Chao,T.H., Guo,H.R., Tsai,L.M., Wu,H.L., and Shi,G.Y. (2002). Synergistic effect of thrombomodulin promoter -33G/A polymorphism and smoking on the onset of acute myocardial infarction. Thromb. Haemost. *87*, 86-91.

Li,Y.H., Chen,J.H., Wu,H.L., Shi,G.Y., Huang,H.C., Chao,T.H., Tsai,W.C., Tsai,L.M., Guo,H.R., Wu,W.S., and Chen,Z.C. (2000). G-33A mutation in the promoter region of thrombomodulin gene and its association with coronary artery disease and plasma soluble thrombomodulin levels. Am. J. Cardiol. *85*, 8-12.

Li,Y.P. and Stashenko,P. (1993). Characterization of a tumor necrosis factor-responsive element which down-regulates the human osteocalcin gene. Mol. Cell Biol. *13*, 3714-3721.

Liaw,P.C., Austin,R.C., Fredenburgh,J.C., Stafford,A.R., and Weitz,J.I. (1999). Comparison of heparin- and dermatan sulfate-mediated catalysis of thrombin inactivation by heparin cofactor II. J. Biol. Chem. *274*, 27597-27604.

Libby,P. and Simon,D.I. (2001). Inflammation and thrombosis: the clot thickens. Circulation *103*, 1718-1720.

Light,D.R., Glaser,C.B., Betts,M., Blasko,E., Campbell,E., Clarke,J.H., McCaman,M., McLean,K., Nagashima,M., Parkinson,J.F., Rumennik,G., Young,T., and Morser,J. (1999). The interaction of thrombomodulin with Ca2+. Eur. J. Biochem. *262*, 522-533.

Lijnen,H.R. and Collen,D. (1999). Matrix metalloproteinase system deficiencies and matrix degradation. Thromb. Haemost. *82*, 837-845.

Lin,J.H., McLean,K., Morser,J., Young,T.A., Wydro,R.M., Andrews,W.H., and Light,D.R. (1994). Modulation of glycosaminoglycan addition in naturally expressed and recombinant human thrombomodulin. J. Biol. Chem. *269*, 25021-25030.

Liu,S., Lee,I.M., Ajani,U., Cole,S.R., Buring,J.E., and Manson,J.E. (2001). Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The Physicians' Health Study. Int. J. Epidemiol. *30*, 130-135.

Lopez,J.A. (1994). The platelet glycoprotein Ib-IX complex. Blood Coagul. Fibrinolysis *5*, 97-119.

Lopez,J.A., Chung,D.W., Fujikawa,K., Hagen,F.S., Davie,E.W., and Roth,G.J. (1988). The alpha and beta chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. Proc. Natl. Acad. Sci. U. S. A *85*, 2135-2139.

Lopez,J.A., Ludwig,E.H., and McCarthy,B.J. (1992). Polymorphism of human glycoprotein Ib alpha results from a variable number of tandem repeats of a 13-amino acid sequence in the mucin-like macroglycopeptide region. Structure/function implications. J. Biol. Chem. *267*, 10055-10061.

Lorand,L., Losowsky,M.S., and Miloszewski,K.J. (1980). Human factor XIII: fibrin-stabilizing factor. Prog. Hemost. Thromb. *5*, 245-290.

Lowe,G.D. (2004). Hormone replacement therapy and cardiovascular disease: increased risks of venous thromboembolism and stroke, and no protection from coronary heart disease. J. Intern. Med. *256*, 361-374.

Loyter,A., Scangos,G.A., and Ruddle,F.H. (1982). Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. Proc. Natl. Acad. Sci. U. S. A *79*, 422-426.

Ludeman,M.J., Kataoka,H., Srinivasan,Y., Esmon,N.L., Esmon,C.T., and Coughlin,S.R. (2005). PAR1 cleavage and signaling in response to activated protein C and thrombin. J. Biol. Chem. *280*, 13122-13128.

Ludwig,M., Wohn,K.D., Schleuning,W.D., and Olek,K. (1992). Allelic dimorphism in the human tissue-type plasminogen activator (TPA) gene as a result of an Alu insertion/deletion event. Hum. Genet. *88*, 388-392.

Luno,J., Ayus,J.C., and Locatelli,F. (2002). Introduction. Kidney Int. Suppl 1.

Lupu,C., Poulsen,E., Roquefeuil,S., Westmuckett,A.D., Kakkar,V.V., and Lupu,F. (1999). Cellular effects of heparin on the production and release of tissue factor pathway inhibitor in human endothelial cells in culture. Arterioscler. Thromb. Vasc. Biol. *19*, 2251-2262.

Luria,M.H., Knoke,J.D., Margolis,R.M., Hendricks,F.H., and Kuplic,J.B. (1976). Acute myocardial infarction: prognosis after recovery. Ann. Intern. Med. *85*, 561-565.

Lusis,A.J. (2000). Atherosclerosis. Nature *407*, 233-241.

Ma,S.F., Garcia,J.G., Reuning,U., Little,S.P., Bang,N.U., and Dixon,E.P. (1997). Thrombin induces thrombomodulin mRNA expression via the proteolytically activated thrombin receptor in cultured bovine smooth muscle cells. J. Lab Clin. Med. *129*, 611-619.

Macfarlane,R.G. (1964). An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. Nature *202*, 498-499.

Maimone,M.M. and Tollefsen,D.M. (1990). Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. J. Biol. Chem. *265*, 18263-18271.

Malek,A.M., Jackman,R., Rosenberg,R.D., and Izumo,S. (1994). Endothelial expression of thrombomodulin is reversibly regulated by fluid shear stress. Circ. Res. *74*, 852-860.

Mammen,E.F. (1995). Clinical relevance of antithrombin deficiencies. Semin. Hematol. *32*, 2-6.

Mammen,E.F. (1998). Antithrombin: its physiological importance and role in DIC. Semin. Thromb. Hemost. *24*, 19-25.

Mann,J.F., Gerstein,H.C., Pogue,J., Lonn,E., and Yusuf,S. (2002). Cardiovascular risk in patients with early renal insufficiency: implications for the use of ACE inhibitors. Am. J. Cardiovasc. Drugs *2*, 157-162.

Mann,K.G. (1999). Biochemistry and physiology of blood coagulation. Thromb. Haemost. *82*, 165-174.

Mann,K.G., Nesheim,M.E., Church,W.R., Haley,P., and Krishnaswamy,S. (1990). Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood *76*, 1-16.

Mann,K.G., van't Veer,C., Cawthern,K., and Butenas,S. (1998). The role of the tissue factor pathway in initiation of coagulation. Blood Coagul. Fibrinolysis *9 Suppl 1*, S3-S7.

Mansfield,M.W., Stickland,M.H., and Grant,P.J. (1997). PAI-1 concentrations in first-degree relatives of patients with non-insulin-dependent diabetes: metabolic and genetic associations. Thromb. Haemost. *77*, 357-361.

Marchetti,G., Patracchini,P., Papacchini,M., Ferrati,M., and Bernardi,F. (1993). A polymorphism in the 5' region of coagulation factor VII gene (F7) caused by an inserted decanucleotide. Hum. Genet. *90*, 575-576.

Marcum,J.A. and Rosenberg,R.D. (1984). Anticoagulantly active heparin-like molecules from vascular tissue. Biochemistry *23*, 1730-1737.

Marenberg,M.E., Risch,N., Berkman,L.F., Floderus,B., and de Faire,U. (1994). Genetic susceptibility to death from coronary heart disease in a study of twins. N. Engl. J. Med. *330*, 1041-1046.

Margaglione,M., Di Minno,G., Grandone,E., Vecchione,G., Celentano,E., Cappucci,G., Grilli,M., Simone,P., Panico,S., and Mancini,M. (1994). Abnormally high circulation levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in patients with a history of ischemic stroke. Arterioscler. Thromb. *14*, 1741-1745.

Marguerie,G.A., Plow,E.F., and Edgington,T.S. (1979). Human platelets possess an inducible and saturable receptor specific for fibrinogen. J. Biol. Chem. *254*, 5357-5363.

Marmot,M.G., Syme,S.L., Kagan,A., Kato,H., Cohen,J.B., and Belsky,J. (1975). Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: prevalence of coronary and hypertensive heart disease and associated risk factors. Am. J. Epidemiol. *102*, 514-525.

Maron,D.J., Fazio,S., and Linton,M.F. (2000). Current perspectives on statins. Circulation *101*, 207-213.

Maruyama,I., Bell,C.E., and Majerus,P.W. (1985a). Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. J. Cell Biol. *101*, 363-371.

Maruyama,I. and Majerus,P.W. (1985). The turnover of thrombin-thrombomodulin complex in cultured human umbilical vein endothelial cells and A549 lung cancer cells. Endocytosis and degradation of thrombin. J. Biol. Chem. *260*, 15432-15438.

Maruyama,I. and Majerus,P.W. (1987). Protein C inhibits endocytosis of thrombin-thrombomodulin complexes in A549 lung cancer cells and human umbilical vein endothelial cells. Blood *69*, 1481-1484.

Maruyama,I., Salem,H.H., Ishii,H., and Majerus,P.W. (1985b). Human thrombomodulin is not an efficient inhibitor of the procoagulant activity of thrombin. J. Clin. Invest *75*, 987-991.

Marx,P.F., Bouma,B.N., and Meijers,J.C. (2002). Role of zinc ions in activation and inactivation of thrombin- activatable fibrinolysis inhibitor. Biochemistry *41*, 1211-1216.

Mather,T., Oganessyan,V., Hof,P., Huber,R., Foundling,S., Esmon,C., and Bode,W. (1996). The 2.8 A crystal structure of Gla-domainless activated protein C. EMBO J. *15*, 6822-6831.

Mathews,I.I., Padmanabhan,K.P., Ganesh,V., Tulinsky,A., Ishii,M., Chen,J., Turck,C.W., Coughlin,S.R., and Fenton,J.W. (1994a). Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. Biochemistry *33*, 3266-3279.

Mathews,I.I., Padmanabhan,K.P., Tulinksy,A., and Sadler,J.E. (1994b). Structure of a nonadecapeptide of the fifth EGF domain of thrombomodulin complexed with thrombin. Biochemistry *33*, 13547-13552.

Matsumura,J.S., Kim,R., Shively,V.P., MacDonald,R.C., and Pearce,W.H. (1999). Characterization of vascular gene transfer using a novel cationic lipid. J. Surg. Res. *85*, 339-345.

Matsuzawa,Y., Semba,K., Kawamura-Tsuzuku,J., Sudo,T., Ishii,S., Toyoshima,K., and Yamamoto,T. (1991). Characterization of the promoter region of the c-yes proto-oncogene: the importance of the GC boxes on its promoter activity. Oncogene *6*, 1561-1567.

Matts,J.P., Karnegis,J.N., Campos,C.T., Fitch,L.L., Johnson,J.W., and Buchwald,H. (1993). Serum creatinine as an independent predictor of coronary heart disease mortality in normotensive survivors of myocardial infarction. POSCH Group. J. Fam. Pract. *36*, 497-503.

Mbopi-Keou,F.X., Gresenguet,G., Mayaud,P., Weiss,H.A., Gopal,R., Matta,M., Paul,J.L., Brown,D.W., Hayes,R.J., Mabey,D.C., and Belec,L. (2000). Interactions between herpes simplex virus type 2 and human immunodeficiency virus type 1 infection in African women: opportunities for intervention. J. Infect. Dis. *182*, 1090-1096.

McCachren,S.S., Diggs,J., Weinberg,J.B., and Dittman,W.A. (1991). Thrombomodulin expression by human blood monocytes and by human synovial tissue lining macrophages. Blood *78*, 3128-3132.

McGuire,E.A. and Tollefsen,D.M. (1987). Activation of heparin cofactor II by fibroblasts and vascular smooth muscle cells. J. Biol. Chem. *262*, 169-175.

Meade,T.W., Mellows,S., Brozovic,M., Miller,G.J., Chakrabarti,R.R., North,W.R., Haines,A.P., Stirling,Y., Imeson,J.D., and Thompson,S.G. (1986). Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. Lancet *2*, 533-537.

Meade,T.W., Ruddock,V., Stirling,Y., Chakrabarti,R., and Miller,G.J. (1993). Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. Lancet *342*, 1076-1079.

Mendall,M.A., Patel,P., Asante,M., Ballam,L., Morris,J., Strachan,D.P., Camm,A.J., and Northfield,T.C. (1997). Relation of serum cytokine concentrations to cardiovascular risk factors and coronary heart disease. Heart *78*, 273-277.

Mendez,M.F., Perryman,K.M., Miller,B.L., and Cummings,J.L. (1998). Behavioral differences between frontotemporal dementia and Alzheimer's disease: a comparison on the BEHAVE-AD rating scale. Int. Psychogeriatr. *10*, 155-162.

Mertens,A. and Holvoet,P. (2001). Oxidized LDL and HDL: antagonists in atherothrombosis. FASEB J. *15*, 2073-2084.

Metcalfe,D.D., Lewis,R.A., Silbert,J.E., Rosenberg,R.D., Wasserman,S.I., and Austen,K.F. (1979). Isolation and characterization of heparin from human lung. J. Clin. Invest *64*, 1537-1543.

Miletich,J.P. and Broze,G.J., Jr. (1987). Human plasma protein Z antigen: range in normal subjects and effect of warfarin therapy. Blood *69*, 1580-1586.

Miller,G.J., Bauer,K.A., Barzegar,S., Cooper,J.A., and Rosenberg,R.D. (1996). Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease. Thromb. Haemost. *75*, 767-771.

Miller,G.J., Bauer,K.A., Barzegar,S., Foley,A.J., Mitchell,J.P., Cooper,J.A., and Rosenberg,R.D. (1995). The effects of quality and timing of venepuncture on markers of blood coagulation in healthy middle-aged men. Thromb. Haemost. *73*, 82-86.

Miller,G.J., Esnouf,M.P., Burgess,A.I., Cooper,J.A., and Mitchell,J.P. (1997). Risk of coronary heart disease and activation of factor XII in middle-aged men. Arterioscler. Thromb. Vasc. Biol. *17*, 2103-2106.

Mohri,M., Suzuki,M., Sugimoto,E., Sata,M., Yamamoto,S., and Maruyama,I. (1998). Effects of recombinant human thrombomodulin (rhs-TM) on clot-induced coagulation in human plasma. Thromb. Haemost *80*, 925-929.

Monroe,D.M., Hoffman,M., and Roberts,H.R. (2002). Platelets and thrombin generation. Arterioscler. Thromb. Vasc. Biol. *22*, 1381-1389.

Moore,K.L., Andreoli,S.P., Esmon,N.L., Esmon,C.T., and Bang,N.U. (1987). Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. J. Clin. Invest *79*, 124-130.

Moore,K.L., Esmon,C.T., and Esmon,N.L. (1989). Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. Blood *73*, 159-165.

Morange,P.E., Juhan-Vague,I., Scarabin,P.Y., Alessi,M.C., Luc,G., Arveiler,D., Ferrieres,J., Amouyel,P., Evans,A., and Ducimetiere,P. (2003). Association between TAFI antigen and Ala147Thr polymorphism of the TAFI gene and the angina pectoris incidence. The PRIME Study (Prospective Epidemiological Study of MI). Thromb. Haemost. *89*, 554-560.

Morrison,D.C. and Cochrane,C.G. (1974). Direct evidence for Hageman factor (factor XII) activation by bacterial lipopolysaccharides (endotoxins). J. Exp. Med. *140*, 797-811.

Morrissey,J.H., Macik,B.G., Neuenschwander,P.F., and Comp,P.C. (1993). Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood *81*, 734-744.

Mosnier,L.O., Meijers,J.C., and Bouma,B.N. (2001). Regulation of fibrinolysis in plasma by TAFI and protein C is dependent on the concentration of thrombomodulin. Thromb. Haemost. *85*, 5-11.

Murano,G., Williams,L., Miller-Andersson,M., Aronson,D.L., and King,C. (1980). Some properties of antithrombin-III and its concentration in human plasma. Thromb. Res. *18*, 259-262.

Musci,G., Berliner,L.J., and Esmon,C.T. (1988). Evidence for multiple conformational changes in the active center of thrombin induced by complex formation with thrombomodulin: an analysis employing nitroxide spin-labels. Biochemistry *27*, 769-773.

Mutch,N.J., Moore,N.R., Wang,E., and Booth,N.A. (2003). Thrombus lysis by uPA, scuPA and tPA is regulated by plasma TAFI. J. Thromb. Haemost. *1*, 2000-2007.

Nadanaka,S., Kitagawa,H., and Sugahara,K. (1998). Demonstration of the immature glycosaminoglycan tetrasaccharide sequence GlcAbeta1-3Galbeta1-3Galbeta1-4Xyl on recombinant soluble human alpha-thrombomodulin. An oligosaccharide structure on a "part- time" proteoglycan. J. Biol. Chem. *273*, 33728-33734.

Nagashima,M., Lundh,E., Leonard,J.C., Morser,J., and Parkinson,J.F. (1993). Alanine-scanning mutagenesis of the epidermal growth factor-like domains of human thrombomodulin identifies critical residues for its cofactor activity. J. Biol. Chem. *268*, 2888-2892.

Nakada,M.T., Amin,K., Christofidou-Solomidou,M., O'Brien,C.D., Sun,J., Gurubhagavatula,I., Heavner,G.A., Taylor,A.H., Paddock,C., Sun,Q.H., Zehnder,J.L., Newman,P.J., Albelda,S.M., and DeLisser,H.M. (2000). Antibodies against the first Ig-like domain of human platelet endothelial cell adhesion molecule-1 (PECAM-1) that inhibit PECAM-1-dependent homophilic adhesion block in vivo neutrophil recruitment. J. Immunol. *164*, 452-462.

Nakamura,T., Kambayashi,J., Okuma,M., and Tandon,N.N. (1999). Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both alpha2beta1 integrin and GP VI. J. Biol. Chem. *274*, 11897-11903.

Nakazawa,F., Koyama,T., Saito,T., Shibakura,M., Yoshinaga,H., Chung,D.H., Kamiyama,R., and Hirosawa,S. (1999). Thrombomodulin with the Asp468Tyr mutation is expressed on the cell surface with normal cofactor activity for protein C activation. Br. J. Haematol. *106*, 416-420.

Nakazawa,F., Koyama,T., Shibamiya,A., and Hirosawa,S. (2002). Characterization of thrombomodulin gene mutations of the 5'-regulatory region. Atherosclerosis *164*, 385.

Nan,B., Lin,P., Lumsden,A.B., Yao,Q., and Chen,C. (2005). Effects of TNF-alpha and curcumin on the expression of thrombomodulin and endothelial protein C receptor in human endothelial cells. Thromb. Res. *115*, 417-426.

Naski,M.C., Lorand,L., and Shafer,J.A. (1991). Characterization of the kinetic pathway for fibrin promotion of alpha- thrombin-catalyzed activation of plasma factor XIII. Biochemistry *30*, 934-941.

Navab,M., Berliner,J.A., Watson,A.D., Hama,S.Y., Territo,M.C., Lusis,A.J., Shih,D.M., Van Lenten,B.J., Frank,J.S., Demer,L.L., Edwards,P.A., and Fogelman,A.M. (1996). The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler. Thromb. Vasc. Biol. *16*, 831-842.

Nawroth,P.P., Handley,D.A., Esmon,C.T., and Stern,D.M. (1986). Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. Proc. Natl. Acad. Sci. U. S. A *83*, 3460-3464.

Nemerson,Y. (1995). Tissue factor: then and now. Thromb. Haemost. *74*, 180-184.

Nesheim,M. (2003). Thrombin and fibrinolysis. Chest *124*, 33S-39S.

Neumann,E., Schaefer-Ridder,M., Wang,Y., and Hofschneider,P.H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J. *1*, 841-845.

Ngeh,J., Anand,V., and Gupta,S. (2002). Chlamydia pneumoniae and atherosclerosis -- what we know and what we don't. Clin. Microbiol. Infect. *8*, 2-13.

Nicholls,S.J., Cutri,B., Worthley,S.G., Kee,P., Rye,K.A., Bao,S., and Barter,P.J. (2005). Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis in rabbits. Arterioscler. Thromb. Vasc. Biol. *25*, 2416-2421.

Nordenhem,A. and Wiman,B. (1998). Tissue plasminogen activator (tPA) antigen in plasma: correlation with different tPA/inhibitor complexes. Scand. J. Clin. Lab Invest *58*, 475-483.

Norlund,L., Holm,J., Zoller,B., and Ohlin,A.K. (1997a). A common thrombomodulin amino acid dimorphism is associated with myocardial infarction. Thromb. Haemost. *77*, 248-251.

Norlund,L., Zoller,B., and Ohlin,A.K. (1997b). A novel thrombomodulin gene mutation in a patient suffering from sagittal sinus thrombosis. Thromb. Haemost. *78*, 1164-1166.

Normand,S.T., Glickman,M.E., Sharma,R.G., and McNeil,B.J. (1996). Using admission characteristics to predict short-term mortality from myocardial infarction in elderly patients. Results from the Cooperative Cardiovascular Project. JAMA *275*, 1322-1328.

Novotny,W.F., Brown,S.G., Miletich,J.P., Rader,D.J., and Broze,G.J., Jr. (1991). Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. Blood *78*, 387-393.

O'Dell,S.D., Gaunt,T.R., and Day,I.N. (2000). SNP genotyping by combination of 192-well MADGE, ARMS and computerized gel image analysis. Biotechniques *29*, 500-506.

Ohji,T., Urano,H., Shirahata,A., Yamagishi,M., Higashi,K., Gotoh,S., and Karasaki,Y. (1995). Transforming growth factor beta 1 and beta 2 induce down-modulation of thrombomodulin in human umbilical vein endothelial cells. Thromb. Haemost. *73*, 812-818.

Ohlin,A.K., Larsson,K., and Hansson,M. (2005). Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. J. Thromb. Haemost. *3*, 976-982.

Ohlin,A.K., Linse,S., and Stenflo,J. (1988). Calcium binding to the epidermal growth factor homology region of bovine protein C. J. Biol. Chem. *263*, 7411-7417.

Ohlin,A.K. and Marlar,R.A. (1995). The first mutation identified in the thrombomodulin gene in a 45-year- old man presenting with thromboembolic disease. Blood *85*, 330-336.

Ohlin,A.K., Morser,J., and Ohlin,H. (1996). Soluble thrombomodulin antigen in plasma is increased in patients with acute myocardial infarction treated with thrombolytic therapy. Thromb. Res. *82*, 313-322.

Ohlin,A.K. and Stenflo,J. (1987). Calcium-dependent interaction between the epidermal growth factor precursor-like region of human protein C and a monoclonal antibody. J. Biol. Chem. *262*, 13798-13804.

Ohsawa,M., Koyama,T., Yamamoto,K., Hirosawa,S., Kamei,S., and Kamiyama,R. (2000). 1alpha,25-dihydroxyvitamin D(3) and its potent synthetic analogs downregulate tissue factor and upregulate thrombomodulin expression in monocytic cells, counteracting the effects of tumor necrosis factor and oxidized LDL. Circulation *102*, 2867-2872.

Oida,K., Takai,H., Maeda,H., Takahashi,S., Tamai,T., Nakai,T., Miyabo,S., and Ishii,H. (1990). Plasma thrombomodulin concentration in diabetes mellitus. Diabetes Res. Clin. Pract. *10*, 193-196.

Oida,K., Tohda,G., Ishii,H., Horie,S., Kohno,M., Okada,E., Suzuki,J., Nakai,T., and Miyamori,I. (1997). Effect of oxidized low density lipoprotein on thrombomodulin expression by THP-1 cells. Thromb. Haemost. *78*, 1228-1233.

Olson,S.T., Bjork,I., and Shore,J.D. (1993). Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. Methods Enzymol. *222*, 525-559.

Opal,S.M., Kessler,C.M., Roemisch,J., and Knaub,S. (2002). Antithrombin, heparin, and heparan sulfate. Crit Care Med. *30*, S325-S331.

Orbe,J., Chorda,C., Montes,R., and Paramo,J.A. (1999). Changes in the fibrinolytic components of cultured human umbilical vein endothelial cells induced by endotoxin, tumor necrosis factor-alpha and interleukin-1alpha. Haematologica *84*, 306-311.

Orchard,T. (1998). Diabetes: a time for excitement-and concern. Hopeful signs exist that the ravages of diabetes can be tamed. BMJ *317*, 691-692.

Owen,W.G. and Esmon,C.T. (1981). Functional properties of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem. *256*, 5532-5535.

Packard,C., Caslake,M., and Shepherd,J. (2000). The role of small, dense low density lipoprotein (LDL): a new look. Int. J. Cardiol. *74 Suppl 1*, S17-S22.

Panahloo,A., Mohamed-Ali,V., Lane,A., Green,F., Humphries,S.E., and Yudkin,J.S. (1995). Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. Diabetes *44*, 37-42.

Parise,L.V. and Phillips,D.R. (1985). Reconstitution of the purified platelet fibrinogen receptor. Fibrinogen binding properties of the glycoprotein IIb-IIIa complex. J. Biol. Chem. *260*, 10698-10707.

Parker,K.A. and Tollefsen,D.M. (1985). The protease specificity of heparin cofactor II. Inhibition of thrombin generated during coagulation. J. Biol. Chem. *260*, 3501-3505.

Parkinson,J.F., Vlahos,C.J., Yan,S.C., and Bang,N.U. (1992). Recombinant human thrombomodulin. Regulation of cofactor activity and anticoagulant function by a glycosaminoglycan side chain. Biochem. J. *283 (Pt 1)*, 151-157.

Pascal,E. and Tjian,R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. *5*, 1646-1656.

Pazzagli,M., Devine,J.H., Peterson,D.O., and Baldwin,T.O. (1992). Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. Anal. Biochem. *204*, 315-323.

Perneger,T.V. (1998). What's wrong with Bonferroni adjustments. BMJ *316*, 1236-1238.

Petersen,T.E. (1988). The amino-terminal domain of thrombomodulin and pancreatic stone protein are homologous with lectins. FEBS Lett. *231*, 51-53.

Pierce,J.W., Lenardo,M., and Baltimore,D. (1988). Oligonucleotide that binds nuclear factor NF-kappa B acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. U. S. A *85*, 1482-1486.

Pinotti,M., Toso,R., Girelli,D., Bindini,D., Ferraresi,P., Papa,M.L., Corrocher,R., Marchetti,G., and Bernardi,F. (2000). Modulation of factor VII levels by intron 7 polymorphisms: population and in vitro studies. Blood *95*, 3423-3428.

Pohjola-Sintonen,S., Rissanen,A., Liskola,P., and Luomanmaki,K. (1998). Family history as a risk factor of coronary heart disease in patients under 60 years of age. Eur. Heart J. *19*, 235-239.

Polgar,J., Lerant,I., Muszbek,L., and Machovich,R. (1986). Thrombomodulin inhibits the activation of factor XIII by thrombin. Thromb. Res. *43*, 685-690.

Pomerantz,M.W. and Owen,W.G. (1978). A catalytic role for heparin. Evidence for a ternary complex of heparin cofactor thrombin and heparin. Biochim. Biophys. Acta *535*, 66-77.

Poort,S.R., Rosendaal,F.R., Reitsma,P.H., and Bertina,R.M. (1996). A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood *88*, 3698-3703.

Pradhan,A.D. and Ridker,P.M. (2002). Do atherosclerosis and type 2 diabetes share a common inflammatory basis? Eur. Heart J. *23*, 831-834.

Pratt,C.W. and Church,F.C. (1992). Heparin binding to protein C inhibitor. J. Biol. Chem. *267*, 8789-8794.

Pratt,C.W., Whinna,H.C., and Church,F.C. (1992). A comparison of three heparin-binding serine proteinase inhibitors. J. Biol. Chem. *267*, 8795-8801.

Pratt,C.W., Whinna,H.C., Meade,J.B., Treanor,R.E., and Church,F.C. (1989). Physicochemical aspects of heparin cofactor II. Ann. N. Y. Acad. Sci. *556*, 104-115.

Preissner,K.T., Koyama,T., Muller,D., Tschopp,J., and Muller-Berghaus,G. (1990). Domain structure of the endothelial cell receptor thrombomodulin as deduced from modulation of its anticoagulant functions. Evidence for a glycosaminoglycan-dependent secondary binding site for thrombin. J. Biol. Chem. *265*, 4915-4922.

Pritchard,J.K. and Cox,N.J. (2002). The allelic architecture of human disease genes: common disease-common variant...or not? Hum. Mol. Genet. *11*, 2417-2423.

Pugh,B.F. and Tjian,R. (1990). Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell *61*, 1187-1197.

Raij,L., DeMaster,E.G., and Jaimes,E.A. (2001). Cigarette smoke-induced endothelium dysfunction: role of superoxide anion. J. Hypertens. *19*, 891-897.

Rajavashisth,T.B., Andalibi,A., Territo,M.C., Berliner,J.A., Navab,M., Fogelman,A.M., and Lusis,A.J. (1990). Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature *344*, 254-257.

Rapaport,S.I. (1989). Inhibition of factor VIIa/tissue factor-induced blood coagulation: with particular emphasis upon a factor Xa-dependent inhibitory mechanism. Blood *73*, 359-365.

Rapaport,S.I. (1991). The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. Thromb. Haemost. *66*, 6-15.

Rapaport,S.I. and Rao,L.V. (1992). Initiation and regulation of tissue factor-dependent blood coagulation. Arterioscler. Thromb. *12*, 1111-1121.

Rasmussen,U.B., Vouret-Craviari,V., Jallat,S., Schlesinger,Y., Pages,G., Pavirani,A., Lecocq,J.P., Pouyssegur,J., and Obberghen-Schilling,E. (1991). cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca2+ mobilization. FEBS Lett. *288*, 123-128.

Ravi,R., Mookerjee,B., van Hensbergen,Y., Bedi,G.C., Giordano,A., El Deiry,W.S., Fuchs,E.J., and Bedi,A. (1998). p53-mediated repression of nuclear factor-kappaB RelA via the transcriptional integrator p300. Cancer Res. *58*, 4531-4536.

Real,J.T., Chaves,F.J., Martinez-Uso,I., Garcia-Garcia,A.B., Ascaso,J.F., and Carmena,R. (2001). Importance of HDL cholesterol levels and the total/ HDL cholesterol ratio as a risk factor for coronary heart disease in molecularly defined heterozygous familial hypercholesterolaemia. Eur. Heart J. *22*, 465-471.

Reiner,A.P., Siscovick,D.S., and Rosendaal,F.R. (2001). Hemostatic risk factors and arterial thrombotic disease. Thromb. Haemost. *85*, 584-595.

Reny,J.L., Remones,V., Fontana,P., Bieche,I., Desvard,F., Aubry,M.L., Gaussen,P., and Aiach,M. (2005). The thrombomodulin-1208/-1209delTT gene promoter polymorphism does not affect basal or LPS-dependent monocyte TM mRNA transcription in healthy volunteers. Thromb. Haemost. *94*, 686-687.

Resnick,N., Collins,T., Atkinson,W., Bonthron,D.T., Dewey,C.F., Jr., and Gimbron,M.A., Jr. (1993). Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. Proc. Natl. Acad. Sci. U. S. A *90*, 7908.

Resnick,N., Yahav,H., Schubert,S., Wolfovitz,E., and Shay,A. (2000). Signalling pathways in vascular endothelium activated by shear stress: relevance to atherosclerosis. Curr. Opin. Lipidol. *11*, 167-177.

Rezaie,A.R. and Esmon,C.T. (1994a). Calcium inhibition of the activation of protein C by thrombin. Role of the P3 and P3' residues. Eur. J. Biochem. *223*, 575-579.

Rezaie,A.R. and Esmon,C.T. (1994b). Proline at the P2 position in protein C is important for calcium- mediated regulation of protein C activation and secretion. Blood *83*, 2526-2531.

Rezaie,A.R., Esmon,N.L., and Esmon,C.T. (1992). The high affinity calcium-binding site involved in protein C activation is outside the first epidermal growth factor homology domain. J. Biol. Chem. *267*, 11701-11704.

Rezaie,A.R., Mather,T., Sussman,F., and Esmon,C.T. (1994). Mutation of Glu-80-->Lys results in a protein C mutant that no longer requires Ca2+ for rapid activation by the thrombin-thrombomodulin complex. J. Biol. Chem. *269*, 3151-3154.

Ridker,P.M., Baker,M.T., Hennekens,C.H., Stampfer,M.J., and Vaughan,D.E. (1997a). Alu-repeat polymorphism in the gene coding for tissue-type plasminogen activator (t-PA) and risks of myocardial infarction among middle-aged men. Arterioscler. Thromb. Vasc. Biol. *17*, 1687-1690.

Ridker,P.M. and Hennekens,C.H. (1991). Hemostatic risk factors for coronary heart disease. Circulation *83*, 1098-1100.

Ridker,P.M., Hennekens,C.H., Lindpaintner,K., Stampfer,M.J., Eisenberg,P.R., and Miletich,J.P. (1995). Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. N. Engl. J. Med. *332*, 912-917.

Ridker,P.M., Hennekens,C.H., and Miletich,J.P. (1999). G20210A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in a large cohort of US men. Circulation *99*, 999-1004.

Ridker,P.M., Hennekens,C.H., Schmitz,C., Stampfer,M.J., and Lindpaintner,K. (1997b). PIA1/A2 polymorphism of platelet glycoprotein IIIa and risks of myocardial infarction, stroke, and venous thrombosis. Lancet *349*, 385-388.

Ridker,P.M., Hennekens,C.H., Stampfer,M.J., Manson,J.E., and Vaughan,D.E. (1994). Prospective study of endogenous tissue plasminogen activator and risk of stroke. Lancet *343*, 940-943.

Riewald,M., Petrovan,R.J., Donner,A., Mueller,B.M., and Ruf,W. (2002). Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science *296*, 1880-1882.

Rinne,T., Mutschler,E., Wimmer-Greinecker,G., Moritz,A., and Olbrich,H.G. (2000). Vitamins C and E protect isolated cardiomyocytes against oxidative damage. Int. J. Cardiol. *75*, 275-281.

Risch,N.J. (2000). Searching for genetic determinants in the new millennium. Nature *405*, 847-856.

Robbie,L.A., Booth,N.A., Croll,A.M., and Bennett,B. (1993). The roles of alpha 2-antiplasmin and plasminogen activator inhibitor 1 (PAI-1) in the inhibition of clot lysis. Thromb. Haemost. *70*, 301-306.

Roberts,W.W., Kramer,O., Rosser,R.W., Nestler,F.H., and Ferry,J.D. (1974). Rheology of fibrin clots. I. Dynamic viscoelastic properties and fluid permeation. Biophys. Chem. *1*, 152-160.

Rosenberg,J.S., McKenna,P.W., and Rosenberg,R.D. (1975). Inhibition of human factor IXa by human antithrombin. J. Biol. Chem. *250*, 8883-8888.

Rosenberg,R.D. (1997). Thrombomodulin gene disruption and mutation in mice. Thromb. Haemost. *78*, 705-709.

Rosenberg,R.D. and Damus,P.S. (1973). The purification and mechanism of action of human antithrombin-heparin cofactor. J. Biol. Chem. *248*, 6490-6505.

Rosendaal,F.R., Siscovick,D.S., Schwartz,S.M., Psaty,B.M., Raghunathan,T.E., and Vos,H.L. (1997). A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. Blood *90*, 1747-1750.

Rosing,J., Hoekema,L., Nicolaes,G.A., Thomassen,M.C., Hemker,H.C., Varadi,K., Schwarz,H.P., and Tans,G. (1995). Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J. Biol. Chem. *270*, 27852-27858.

Ross,R. (1999). Atherosclerosis--an inflammatory disease. N. Engl. J. Med. *340*, 115-126.

Rothman,K.J. (1990). No adjustments are needed for multiple comparisons. Epidemiology *1*, 43-46.

Rothwarf,D.M. and Karin,M. (1999). The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci. STKE. *1999*, RE1-16.

Ruggeri,Z.M. (1997). Mechanisms initiating platelet thrombus formation. Thromb. Haemost. *78*, 611-616.

Sadler,J.E. (1997). Thrombomodulin structure and function. Thromb. Haemost. *78*, 392-395.

Saito H (1994). The contact phase of blood coagulation. In: Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. Haemostasis and Thrombosis. 3rd ed. London, UK: Churchill Livingstone. 289-307.

Sakata,Y., Loskutoff,D.J., Gladson,C.L., Hekman,C.M., and Griffin,J.H. (1986). Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. Blood *68*, 1218-1223.

Sakharov,D.V., Plow,E.F., and Rijken,D.C. (1997). On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. J. Biol. Chem. *272*, 14477-14482.

Sakharov,D.V. and Rijken,D.C. (1995). Superficial accumulation of plasminogen during plasma clot lysis. Circulation *92*, 1883-1890.

Salomaa,V., Matei,C., Aleksic,N., Sansores-Garcia,L., Folsom,A.R., Juneja,H., Chambless,L.E., and Wu,K.K. (1999). Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. Lancet *353*, 1729-1734.

Sampoli Benitez,B.A., Hunter,M.J., Meininger,D.P., and Komives,E.A. (1997). Structure of the fifth EGF-like domain of thrombomodulin: An EGF-like domain with a novel disulfide-bonding pattern. J. Mol. Biol. *273*, 913-926.

Sandset,P.M., Abildgaard,U., and Larsen,M.L. (1988). Heparin induces release of extrinsic coagulation pathway inhibitor (EPI). Thromb. Res. *50*, 803-813.

Sandset,P.M., Warn-Cramer,B.J., Maki,S.L., and Rapaport,S.I. (1991a). Immunodepletion of extrinsic pathway inhibitor sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction. Blood *78*, 1496-1502.

Sandset,P.M., Warn-Cramer,B.J., Rao,L.V., Maki,S.L., and Rapaport,S.I. (1991b). Depletion of extrinsic pathway inhibitor (EPI) sensitizes rabbits to disseminated intravascular coagulation induced with tissue factor: evidence supporting a physiologic role for EPI as a natural anticoagulant. Proc. Natl. Acad. Sci. U. S. A *88*, 708-712.

Sanger,F. and Coulson,A.R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. *94*, 441-448.

Schleef,R.R., Bevilacqua,M.P., Sawdey,M., Gimbrone,M.A., Jr., and Loskutoff,D.J. (1988). Cytokine activation of vascular endothelium. Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor. J. Biol. Chem. *263*, 5797-5803.

Schneider,M., Nagashima,M., Knappe,S., Zhao,L., Morser,J., and Nesheim,M. (2002). Amino acid residues in the P6-P'3 region of thrombin-activable fibrinolysis inhibitor (TAFI) do not determine the thrombomodulin dependence of TAFI activation. J. Biol. Chem. *277*, 9944-9951.

Schonfeld,G., Patsch,W., Rudel,L.L., Nelson,C., Epstein,M., and Olson,R.E. (1982). Effects of dietary cholesterol and fatty acids on plasma lipoproteins. J. Clin. Invest *69*, 1072-1080.

Schroder,V. and Kohler,H.P. (2000). Effect of factor XIII Val34Leu on alpha2-antiplasmin incorporation into fibrin. Thromb. Haemost. *84*, 1128-1130.

Scully,M.F., Toh,C.H., Hoogendoorn,H., Manuel,R.P., Nesheim,M.E., Solymoss,S., and Giles,A.R. (1993). Activation of protein C and its distribution between its inhibitors, protein C inhibitor, alpha 1-antitrypsin and alpha 2-macroglobulin, in patients with disseminated intravascular coagulation. Thromb. Haemost. *69*, 448-453.

Segal,R. and Berk,A.J. (1991). Promoter activity and distance constraints of one versus two Sp1 binding sites. J. Biol. Chem. *266*, 20406-20411.

Sehnal,E. and Slany,J. (2002). Fibrinogen--the key to familial CHD or just another shadow in Plato's allegory? Eur. Heart J. *23*, 1231-1233.

Seligsohn,U., Osterud,B., Brown,S.F., Griffin,J.H., and Rapaport,S.I. (1979). Activation of human factor VII in plasma and in purified systems: roles of activated factor IX, kallikrein, and activated factor XII. J. Clin. Invest *64*, 1056-1065.

Selwyn,A.P. (2003). Prothrombotic and antithrombotic pathways in acute coronary syndromes. Am. J. Cardiol. *91*, 3H-11H.

Sen,R. and Baltimore,D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705-716.

Shaw,G. and Kamen,R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659-667.

Shen,L. and Dahlback,B. (1994). Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J. Biol. Chem. *269*, 18735-18738.

Sheppard,K.A., Rose,D.W., Haque,Z.K., Kurokawa,R., McInerney,E., Westin,S., Thanos,D., Rosenfeld,M.G., Glass,C.K., and Collins,T. (1999). Transcriptional activation by NF-kappaB requires multiple coactivators. Mol. Cell Biol. *19*, 6367-6378.

Shimano,H., Takahashi,K., Kawakami,M., Gotoda,T., Harada,K., Shimada,M., Yazaki,Y., and Yamada,N. (1994). Elevated serum and urinary thrombomodulin levels in patients with non- insulin-dependent diabetes mellitus. Clin. Chim. Acta *225*, 89-96.

Shimokawa,T., Yamamoto,K., Kojima,T., and Saito,H. (2000). Down-regulation of murine tissue factor pathway inhibitor mRNA by endotoxin and tumor necrosis factor-alpha in vitro and in vivo. Thromb. Res. *100*, 211-221.

Shirai,T., Shiojiri,S., Ito,H., Yamamoto,S., Kusumoto,H., Deyashiki,Y., Maruyama,I., and Suzuki,K. (1988). Gene structure of human thrombomodulin, a cofactor for thrombin-catalyzed activation of protein C. J. Biochem. (Tokyo) *103*, 281-285.

Shulman,N.B., Ford,C.E., Hall,W.D., Blaufox,M.D., Simon,D., Langford,H.G., and Schneider,K.A. (1989). Prognostic value of serum creatinine and effect of treatment of hypertension on renal function. Results from the hypertension detection and follow-up program. The Hypertension Detection and Follow-up Program Cooperative Group. Hypertension *13*, I80-I93.

Silveira,A., Schatteman,K., Goossens,F., Moor,E., Scharpe,S., Stromqvist,M., Hendriks,D., and Hamsten,A. (2000). Plasma procarboxypeptidase U in men with symptomatic coronary artery disease. Thromb. Haemost. *84*, 364-368.

Simioni,P. (1999). The molecular genetics of familial venous thrombosis. Baillieres Best. Pract. Res. Clin. Haematol. *12*, 479-503.

Simmonds,R.E., Hermida,J., Rezende,S.M., and Lane,D.A. (2001). Haemostatic genetic risk factors in arterial thrombosis. Thromb. Haemost. *86*, 374-385.

Simsek,S., Bleeker,P.M., van der Schoot,C.E., and dem Borne,A.E. (1994). Association of a variable number of tandem repeats (VNTR) in glycoprotein Ib alpha and HPA-2 alloantigens. Thromb. Haemost. *72*, 757-761.

Sing,C.F., Stengard,J.H., and Kardia,S.L. (2003). Genes, environment, and cardiovascular disease. Arterioscler. Thromb. Vasc. Biol. *23*, 1190-1196.

Sixma,J.J., van Zanten,G.H., Banga,J.D., Nieuwenhuls,H.K., and de Groot,P.G. (1995). Platelet adhesion. Semin. Hematol. *32*, 89-98.

Smith,E.B., Keen,G.A., Grant,A., and Stirk,C. (1990). Fate of fibrinogen in human arterial intima. Arteriosclerosis *10*, 263-275.

Smith,F.B., Lee,A.J., Fowkes,F.G., Price,J.F., Rumley,A., and Lowe,G.D. (1997). Hemostatic factors as predictors of ischemic heart disease and stroke in the Edinburgh Artery Study. Arterioscler. Thromb. Vasc. Biol. *17*, 3321-3325.

Soff,G.A., Jackman,R.W., and Rosenberg,R.D. (1991). Expression of thrombomodulin by smooth muscle cells in culture: different effects of tumor necrosis factor and cyclic adenosine monophosphate on thrombomodulin expression by endothelial cells and smooth muscle cells in culture. Blood *77*, 515-518.

Sohn,R.H., Deming,C.B., Johns,D.C., Champion,H.C., Bian,C., Gardner,K., and Rade,J.J. (2005). Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood *105*, 3910-3917.

Speir,E., Yu,Z.X., Takeda,K., Ferrans,V.J., and Cannon,R.O., III (2000). Competition for p300 regulates transcription by estrogen receptors and nuclear factor-kappaB in human coronary smooth muscle cells. Circ. Res. *87*, 1006-1011.

Speiser,W., Kapiotis,S., Kopp,C.W., Simonitsch,I., Jilma,B., Jansen,B., Exner,M., and Chott,A. (2001). Effect of intradermal tumor necrosis factor-alpha-induced inflammation on coagulation factors in dermal vessel endothelium. An in vivo study of human skin biopsies. Thromb. Haemost. *85*, 362-367.

Sperry,J.L., Deming,C.B., Bian,C., Walinsky,P.L., Kass,D.A., Kolodgie,F.D., Virmani,R., Kim,A.Y., and Rade,J.J. (2003). Wall tension is a potent negative regulator of in vivo thrombomodulin expression. Circ. Res. *92*, 41-47.

Spranger,J., Kroke,A., Mohlig,M., Hoffmann,K., Bergmann,M.M., Ristow,M., Boeing,H., and Pfeiffer,A.F. (2003). Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes *52*, 812-817.

Spronk,H.M., van,d., V, and ten Cate,H. (2004). Blood coagulation and the risk of atherothrombosis: a complex relationship. Thromb. J. *2*, 12.

Stamler,J., Stamler,R., Neaton,J.D., Wentworth,D., Daviglus,M.L., Garside,D., Dyer,A.R., Liu,K., and Greenland,P. (1999). Low risk-factor profile and long-term cardiovascular and noncardiovascular mortality and life expectancy: findings for 5 large cohorts of young adult and middle-aged men and women. JAMA *282*, 2012-2018.

Stead,N., Kaplan,A.P., and Rosenberg,R.D. (1976). Inhibition of activated factor XII by antithrombin-heparin cofactor. J. Biol. Chem. *251*, 6481-6488.

Stearns,D.J., Kurosawa,S., and Esmon,C.T. (1989). Microthrombomodulin. Residues 310-486 from the epidermal growth factor precursor homology domain of thrombomodulin will accelerate protein C activation. J. Biol. Chem. *264*, 3352-3356.

Stearns,D.J., Kurosawa,S., Sims,P.J., Esmon,N.L., and Esmon,C.T. (1988). The interaction of a Ca2+-dependent monoclonal antibody with the protein C activation peptide region. Evidence for obligatory Ca2+ binding to both antigen and antibody. J. Biol. Chem. *263*, 826-832.

Stearns-Kurosawa,D.J., Kurosawa,S., Mollica,J.S., Ferrell,G.L., and Esmon,C.T. (1996). The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc. Natl. Acad. Sci. U. S. A *93*, 10212-10216.

Steinberg,D. (1997). Low density lipoprotein oxidation and its pathobiological significance. J. Biol. Chem. *272*, 20963-20966.

Stenflo,J., Ohlin,A.K., Owen,W.G., and Schneider,W.J. (1988). beta-Hydroxyaspartic acid or beta-hydroxyasparagine in bovine low density lipoprotein receptor and in bovine thrombomodulin. J. Biol. Chem. *263*, 21-24.

Stern,M.P. (1995). Diabetes and cardiovascular disease. The "common soil" hypothesis. Diabetes *44*, 369-374.

Stern,M.P. (1996). Do non-insulin-dependent diabetes mellitus and cardiovascular disease share common antecedents? Ann. Intern. Med. *124*, 110-116.

Stevens,J., Cai,J., Pamuk,E.R., Williamson,D.F., Thun,M.J., and Wood,J.L. (1998). The effect of age on the association between body-mass index and mortality. N. Engl. J. Med. *338*, 1-7.

Stewart,C.L., Schuetze,S., Vanek,M., and Wagner,E.F. (1987). Expression of retroviral vectors in transgenic mice obtained by embryo infection. EMBO J. *6*, 383-388.

Stork,P.J. and Schmitt,J.M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. *12*, 258-266.

Stubbs,M.T. and Bode,W. (1995). The clot thickens: clues provided by thrombin structure. Trends Biochem. Sci. *20*, 23-28.

Suh,Y. and Vijg,J. (2005). SNP discovery in associating genetic variation with human disease phenotypes. Mutat. Res. *573*, 41-53.

Suzuki,K., Kusumoto,H., Deyashiki,Y., Nishioka,J., Maruyama,I., Zushi,M., Kawahara,S., Honda,G., Yamamoto,S., and Horiguchi,S. (1987). Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. EMBO J. *6*, 1891-1897.

Suzuki,K., Nishioka,J., and Hashimoto,S. (1983). Protein C inhibitor. Purification from human plasma and characterization. J. Biol. Chem. *258*, 163-168.

Suzuki,K., Nishioka,J., Hayashi,T., and Kosaka,Y. (1988). Functionally active thrombomodulin is present in human platelets. J. Biochem. (Tokyo) *104*, 628-632.

Tabor,H.K., Risch,N.J., and Myers,R.M. (2002). Opinion: Candidate-gene approaches for studying complex genetic traits: practical considerations. Nat. Rev. Genet. *3*, 391-397.

Taddei,S., Virdis,A., Ghiadoni,L., Salvetti,G., and Salvetti,A. (2000). Endothelial dysfunction in hypertension. J. Nephrol. *13*, 205-210.

Takada,Y., Shinkai,F., Kondo,S., Yamamoto,S., Tsuboi,H., Korenaga,R., and Ando,J. (1994). Fluid shear stress increases the expression of thrombomodulin by cultured human endothelial cells. Biochem. Biophys. Res. Commun. *205*, 1345-1352.

Takahashi,Y., Hosaka,Y., Imada,K., Adachi,T., Niina,H., and Mochizuki,H. (1998). Species specificity of the anticoagulant activity of human urinary soluble thrombomodulin. Thromb. Res. *89*, 187-197.

Takano,S., Kimura,S., Ohdama,S., and Aoki,N. (1990). Plasma thrombomodulin in health and diseases. Blood *76*, 2024-2029.

Talmud,P.J. and Humphries,S.E. (2002). Gene:environment interaction in lipid metabolism and effect on coronary heart disease risk. Curr. Opin. Lipidol. *13*, 149-154.

Tan,A.K. and Eaton,D.L. (1995). Activation and characterization of procarboxypeptidase B from human plasma. Biochemistry *34*, 5811-5816.

Tanaga,K., Bujo,H., Inoue,M., Mikami,K., Kotani,K., Takahashi,K., Kanno,T., and Saito,Y. (2002). Increased circulating malondialdehyde-modified LDL levels in patients with coronary artery diseases and their association with peak sizes of LDL particles. Arterioscler. Thromb. Vasc. Biol. *22*, 662-666.

Tapper,H. and Herwald,H. (2000). Modulation of hemostatic mechanisms in bacterial infectious diseases. Blood *96*, 2329-2337.

Taskinen,M.R. (2002). Diabetic dyslipidemia. Atheroscler. Suppl *3*, 47-51.

Taylor,F.B., Jr., Peer,G.T., Lockhart,M.S., Ferrell,G., and Esmon,C.T. (2001). Endothelial cell protein C receptor plays an important role in protein C activation in vivo. Blood *97*, 1685-1688.

Tazawa,R., Hirosawa,S., Suzuki,K., Hirokawa,K., and Aoki,N. (1993). Functional characterization of the 5'-regulatory region of the human thrombomodulin gene. J. Biochem. (Tokyo) *113*, 600-606.

Thogersen,A.M., Jansson,J.H., Boman,K., Nilsson,T.K., Weinehall,L., Huhtasaari,F., and Hallmans,G. (1998). High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. Circulation *98*, 2241-2247.

Thompson,S.G., Kienast,J., Pyke,S.D., Haverkate,F., and van de Loo,J.C. (1995). Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N. Engl. J. Med. *332*, 635-641.

Thompson,W.D. and Smith,E.B. (1989). Atherosclerosis and the coagulation system. J. Pathol. *159*, 97-106.

Thorelli,E., Kaufman,R.J., and Dahlback,B. (1999). Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. Blood *93*, 2552-2558.

Thorisson,G.A., Smith,A.V., Krishnan,L., and Stein,L.D. (2005). The International HapMap Project Web site. Genome Res. *15*, 1592-1593.

Tollefsen,D.M. (1997). Heparin cofactor II. Adv. Exp. Med. Biol. *425*, 35-44.

Tollefsen,D.M., Majerus,D.W., and Blank,M.K. (1982). Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma. J. Biol. Chem. *257*, 2162-2169.

Tollefsen,D.M. and Pestka,C.A. (1985). Heparin cofactor II activity in patients with disseminated intravascular coagulation and hepatic failure. Blood *66*, 769-774.

Tollefsen,D.M., Pestka,C.A., and Monafo,W.J. (1983). Activation of heparin cofactor II by dermatan sulfate. J. Biol. Chem. *258*, 6713-6716.

Tracy,P.B., Eide,L.L., and Mann,K.G. (1985). Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. J. Biol. Chem. *260*, 2119-2124.

Tracy,R.P. (2001). Is visceral adiposity the "enemy within"? Arterioscler. Thromb. Vasc. Biol. *21*, 881-883.

Tregouet,D.A., Aubert,H., Henry,M., Morange,P., Visvikis,S., Juhan-Vague,I., and Tiret,L. (2001). Combined segregation-linkage analysis of plasma thrombin activatable fibrinolysis inhibitor (TAFI) antigen levels with TAFI gene polymorphisms. Hum. Genet. *109*, 191-197.

Tsiang,M., Lentz,S.R., and Sadler,J.E. (1992). Functional domains of membrane-bound human thrombomodulin. EGF-like domains four to six and the serine/threonine-rich domain are required for cofactor activity. J. Biol. Chem. *267*, 6164-6170.

Uchiba,M., Okajima,K., Murakami,K., Johno,M., Okabe,H., and Takatsuki,K. (1996). Recombinant thrombomodulin prevents endotoxin-induced lung injury in rats by inhibiting leukocyte activation. Am. J. Physiol *271*, L470-L475.

Uehara,S., Gotoh,K., and Handa,H. (2001). Separation and characterization of the molecular species of thrombomodulin in the plasma of diabetic patients. Thromb. Res. *104*, 325-332.

Ueno,A., Murakami,K., Yamanouchi,K., Watanabe,M., and Kondo,T. (1996). Thrombin stimulates production of interleukin-8 in human umbilical vein endothelial cells. Immunology *88*, 76-81.

Vaheri,A. and Pagano,J.S. (1965). Infectious poliovirus RNA: a sensitive method of assay. Virology *27*, 434-436.

van 't Hooft,F.M., Silveira,A., Tornvall,P., Iliadou,A., Ehrenborg,E., Eriksson,P., and Hamsten,A. (1999). Two common functional polymorphisms in the promoter region of the coagulation factor VII gene determining plasma factor VII activity and mass concentration. Blood *93*, 3432-3441.

Van Deerlin,V.M. and Tollefsen,D.M. (1991). The N-terminal acidic domain of heparin cofactor II mediates the inhibition of alpha-thrombin in the presence of glycosaminoglycans. J. Biol. Chem. *266*, 20223-20231.

van der Bom,J.G., de Knijff,P., Haverkate,F., Bots,M.L., Meijer,P., de Jong,P.T., Hofman,A., Kluft,C., and Grobbee,D.E. (1997). Tissue plasminogen activator and risk of myocardial infarction. The Rotterdam Study. Circulation *95*, 2623-2627.

van der Velden,P.A., Krommenhoek-Van Es,T., Allaart,C.F., Bertina,R.M., and Reitsma,P.H. (1991). A frequent thrombomodulin amino acid dimorphism is not associated with thrombophilia. Thromb. Haemost. *65*, 511-513.

van Hinsbergh,V.W. (2001). The endothelium: vascular control of haemostasis. Eur. J. Obstet. Gynecol. Reprod. Biol. *95*, 198-201.

Vandesompele,J., De Preter,K., Pattyn,F., Poppe,B., Van Roy,N., De Paepe,A., and Speleman,F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. *3*, Research0034.

Varadi,K., Rosing,J., Tans,G., Pabinger,I., Keil,B., and Schwarz,H.P. (1996). Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor VR506Q mutation. Thromb. Haemost. *76*, 208-214.

Vendrell,J., Fernandez-Real,J.M., Gutierrez,C., Zamora,A., Simon,I., Bardaji,A., Ricart,W., and Richart,C. (2003). A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. Atherosclerosis *167*, 257-264.

Villoutreix, B. O and Dahlback, B. (1998) Molecular Model for the C-type Lectin Domain of Human Thrombomodulin. Journal of Molecular Modeling , 310-322.

Vincenot,A., Gaussem,P., Pittet,J.L., Debost,S., and Aiach,M. (1995). Amino acids 225-235** of the protein C serine-protease domain are important for the interaction with the thrombin-thrombomodulin complex. FEBS Lett. *367*, 153-157.

Vo,N. and Goodman,R.H. (2001). CREB-binding protein and p300 in transcriptional regulation. J. Biol. Chem. *276*, 13505-13508.

von der Ahe,D., Nischan,C., Kunz,C., Otte,J., Knies,U., Oderwald,H., and Wasylyk,B. (1993). Ets transcription factor binding site is required for positive and TNF alpha-induced negative promoter regulation. Nucleic Acids Res. *21*, 5636-5643.

Voorberg,J., Roelse,J., Koopman,R., Buller,H., Berends,F., ten Cate,J.W., Mertens,K., and van Mourik,J.A. (1994). Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet *343*, 1535-1536.

Vu,T.K., Hung,D.T., Wheaton,V.I., and Coughlin,S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell *64*, 1057-1068.

Wagner,E.F., Vanek,M., and Vennstrom,B. (1985). Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter. EMBO J. *4*, 663-666.

Walker,F.J. (1980). Regulation of activated protein C by a new protein. A possible function for bovine protein S. J. Biol. Chem. *255*, 5521-5524.

Wang,W., Nagashima,M., Schneider,M., Morser,J., and Nesheim,M. (2000). Elements of the primary structure of thrombomodulin required for efficient thrombin-activable fibrinolysis inhibitor activation. J. Biol. Chem. *275*, 22942-22947.

Wartiovaara,U., Mikkola,H., Szoke,G., Haramura,G., Karpati,L., Balogh,I., Lassila,R., Muszbek,L., and Palotie,A. (2000). Effect of Val34Leu polymorphism on the activation of the coagulation factor XIII-A. Thromb. Haemost. *84*, 595-600.

Wartiovaara,U., Perola,M., Mikkola,H., Totterman,K., Savolainen,V., Penttila,A., Grant,P.J., Tikkanen,M.J., Vartiainen,E., Karhunen,P.J., Peltonen,L., and Palotie,A. (1999). Association of FXIII Val34Leu with decreased risk of myocardial infarction in Finnish males. Atherosclerosis *142*, 295-300.

Waugh,J.M., Yuksel,E., Li,J., Kuo,M.D., Kattash,M., Saxena,R., Geske,R., Thung,S.N., Shenaq,S.M., and Woo,S.L. (1999). Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. Circ. Res. *84*, 84-92.

Weiler,H. and Isermann,B.H. (2003). Thrombomodulin. J. Thromb. Haemost. *1*, 1515-1524.

Weiler-Guettler,H., Christie,P.D., Beeler,D.L., Healy,A.M., Hancock,W.W., Rayburn,H., Edelberg,J.M., and Rosenberg,R.D. (1998). A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J. Clin. Invest *101*, 1983-1991.

Weinberger,M.H. (1988). Hypertension: the sodium connection. Clin. Physiol Biochem. *6*, 130-135.

Weiner,D.E., Tighiouart,H., Stark,P.C., Amin,M.G., MacLeod,B., Griffith,J.L., Salem,D.N., Levey,A.S., and Sarnak,M.J. (2004). Kidney disease as a risk factor for recurrent cardiovascular disease and mortality. Am. J. Kidney Dis. *44*, 198-206.

Weisel,J.W., Nagaswami,C., Young,T.A., and Light,D.R. (1996). The shape of thrombomodulin and interactions with thrombin as determined by electron microscopy. J. Biol. Chem. *271*, 31485-31490.

Weiss,E.J., Bray,P.F., Tayback,M., Schulman,S.P., Kickler,T.S., Becker,L.C., Weiss,J.L., Gerstenblith,G., and Goldschmidt-Clermont,P.J. (1996). A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. N. Engl. J. Med. *334*, 1090-1094.

Wen,D.Z., Dittman,W.A., Ye,R.D., Deaven,L.L., Majerus,P.W., and Sadler,J.E. (1987). Human thrombomodulin: complete cDNA sequence and chromosome localization of the gene. Biochemistry *26*, 4350-4357.

Wilhelmsen,L., Svardsudd,K., Korsan-Bengtsen,K., Larsson,B., Welin,L., and Tibblin,G. (1984). Fibrinogen as a risk factor for stroke and myocardial infarction. N. Engl. J. Med. *311*, 501-505.

Williams,R.R., Hunt,S.C., Heiss,G., Province,M.A., Bensen,J.T., Higgins,M., Chamberlain,R.M., Ware,J., and Hopkins,P.N. (2001). Usefulness of cardiovascular family history data for population-based preventive medicine and medical research (the Health Family Tree Study and the NHLBI Family Heart Study). Am. J. Cardiol. *87*, 129-135.

Wong,T.K. and Neumann,E. (1982). Electric field mediated gene transfer. Biochem. Biophys. Res. Commun. *107*, 584-587.

Woodward,M., Lowe,G.D., Rumley,A., and Tunstall-Pedoe,H. (1998). Fibrinogen as a risk factor for coronary heart disease and mortality in middle-aged men and women. The Scottish Heart Health Study. Eur. Heart J. *19*, 55-62.

Wu,K.K., Aleksic,N., Ahn,C., Boerwinkle,E., Folsom,A.R., and Juneja,H. (2001). Thrombomodulin Ala455Val polymorphism and risk of coronary heart disease. Circulation *103*, 1386-1389.

Wun,T.C., Kretzmer,K.K., Girard,T.J., Miletich,J.P., and Broze,G.J., Jr. (1988). Cloning and characterization of a cDNA coding for the lipoprotein- associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J. Biol. Chem. *263*, 6001-6004.

Xu,J., Esmon,N.L., and Esmon,C.T. (1999). Reconstitution of the human endothelial cell protein C receptor with thrombomodulin in phosphatidylcholine vesicles enhances protein C activation. J. Biol. Chem. *274*, 6704-6710.

Yagi,M., Edelhoff,S., Disteche,C.M., and Roth,G.J. (1995). Human platelet glycoproteins V and IX: mapping of two leucine-rich glycoprotein genes to chromosome 3 and analysis of structures. Biochemistry *34*, 16132-16137.

Yang,L., Manithody,C., Walston,T.D., Cooper,S.T., and Rezaie,A.R. (2003). Thrombomodulin enhances the reactivity of thrombin with protein C inhibitor by providing both a binding site for the serpin and allosterically modulating the activity of thrombin. J. Biol. Chem. *278*, 37465-37470.

Yang,L. and Rezaie,A.R. (2003). The fourth epidermal growth factor-like domain of thrombomodulin interacts with the basic exosite of protein C. J. Biol. Chem. *278*, 10484-10490.

Ye,J., Esmon,C.T., and Johnson,A.E. (1993). The chondroitin sulfate moiety of thrombomodulin binds a second molecule of thrombin. J. Biol. Chem. *268*, 2373-2379.

Ye,J., Esmon,N.L., Esmon,C.T., and Johnson,A.E. (1991). The active site of thrombin is altered upon binding to thrombomodulin. Two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation. J. Biol. Chem. *266*, 23016-23021.

Ye,J., Liu,L.W., Esmon,C.T., and Johnson,A.E. (1992). The fifth and sixth growth factor-like domains of thrombomodulin bind to the anion-binding exosite of thrombin and alter its specificity. J. Biol. Chem. *267*, 11023-11028.

Yegneswaran,S., Wood,G.M., Esmon,C.T., and Johnson,A.E. (1997). Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J. Biol. Chem. *272*, 25013-25021.

Yu,H.I., Sheu,W.H., Lai,C.J., Lee,W.J., and Chen,Y.T. (2001). Endothelial dysfunction in type 2 diabetes mellitus subjects with peripheral artery disease. Int. J. Cardiol. *78*, 19-25.

Yu,K., Morioka,H., Fritze,L.M., Beeler,D.L., Jackman,R.W., and Rosenberg,R.D. (1992). Transcriptional regulation of the thrombomodulin gene. J. Biol. Chem. *267*, 23237-23247.

Yudkin JS, Juhan-Vague I, Hawe E, Humphries SE, di Minno G, Margaglione M, Tremoli E, Kooistra T, Morange PE, Lundham P, Mohammed-Ali V, and Hamsten A (2003). The Insulin Resistance Syndrome and Inflammatory Markers in Relation to Myocardial Infarction in North and South Europe. Metabolism.

Yudkin,J.S. (1999). Abnormalities of coagulation and fibrinolysis in insulin resistance. Evidence for a common antecedent? Diabetes Care *22 Suppl 3*, C25-C30.

Yudkin,J.S. (2000). Relationship of serum C3 complement with insulin resistance and coronary heart disease-cause, consequence or common antecedent? Eur. Heart J. *21*, 1036-1039.

Yudkin,J.S., Stehouwer,C.D., Emeis,J.J., and Coppack,S.W. (1999). C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler. Thromb. Vasc. Biol. *19*, 972-978.

Zabel,U., Schreck,R., and Baeuerle,P.A. (1991). DNA binding of purified transcription factor NF-kappa B. Affinity, specificity, Zn2+ dependence, and differential half-site recognition. J. Biol. Chem. *266*, 252-260.

Zhang,Y., Weiler-Guettler,H., Chen,J., Wilhelm,O., Deng,Y., Qiu,F., Nakagawa,K., Klevesath,M., Wilhelm,S., Bohrer,H., Nakagawa,M., Graeff,H., Martin,E., Stern,D.M., Rosenberg,R.D., Ziegler,R., and Nawroth,P.P. (1998). Thrombomodulin modulates growth of tumor cells independent of its anticoagulant activity. J. Clin. Invest *101*, 1301-1309.

Ziccardi,P., Nappo,F., Giugliano,G., Esposito,K., Marfella,R., Cioffi,M., D'Andrea,F., Molinari,A.M., and Giugliano,D. (2002). Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. Circulation *105*, 804-809.

Zito,F., Drummond,F., Bujac,S.R., Esnouf,M.P., Morrissey,J.H., Humphries,S.E., and Miller,G.J. (2000). Epidemiological and genetic associations of activated factor XII concentration with factor VII activity, fibrinopeptide A concentration, and risk of coronary heart disease in men. Circulation *102*, 2058-2062.