Tissue Factor and CD40 Ligand

Markers for the Interplay of Coagulation and Inflammation in the Acute Coronary Syndrome

ANDERS MÄLARSTIG
Dissertation presented at Uppsala University to be publicly examined in Ebba Enghoff Sälen, Ingång 50, Uppsala, Tuesday, May 23, 2006 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract


BACKGROUND: Tissue factor (TF) is a 47 kDa transmembrane glycoprotein known as the main initiator of blood coagulation. CD40 ligand is another membrane molecule, which ligates to cell types associated with atherosclerotic plaques thereby mediating intraplaque inflammation and weakening of the fibrous cap. Acute coronary syndrome (ACS) is a multi-factorial disease in which TF and CD40 ligand have prominent roles. Single nucleotide poly-morphisms (SNPs) in the TF and CD40 ligand genes may influence the development, progression and outcome in ACS. AIM: The aim of this thesis was to investigate the genetic and molecular control of TF expression in healthy individuals and in patients with ACS. More-over, the aim was to investigate whether SNPs in the TF and CD40L genes respectively were associated with risk and outcome in ACS and/or with plasma concentrations of these pro-teins. RESULTS: A real-time PCR method that allowed sensitive and dynamic quantification of TF mRNA was established and used for the identification of a high and low response pheno-menon of TF mRNA. The TF high and low response correlated with the expression of toll-like receptor 4 (TLR-4) thus linking TF to innate immunity in a novel fashion. Investigation of several SNPs in the TF and CD40L genes led to the identification of the 5466 A>G in the TF gene and the -3459 A>G SNP in the CD40L gene. The 5466 G allele was associated with cardiovascular death in patients with ACS and increased TF procoagulant activity in human monocytes, which explained the clinical association. The -3459 G allele regulated the production of soluble CD40L but was not related with patient outcome. Soluble CD40L levels above median were associated with the risk of MI in patients with ACS. A prolonged treatment with dalteparin was more efficient in patients presenting with high levels of sCD40L, which further supports sCD40L as a marker of a prothrombotic state. CONCLUSIONS: The results of this thesis adds to our current knowledge of factors influencing TF expression and activity by demonstrating the effects of TF gene variants, cell signalling molecules, CD40 ligand protein and gene variation. All of these effects have the potential to modify the risk of development, progression and outcome in the acute coronary syndrome and exemplify the interplay between coagulation and inflammation, in which both TF and CD40 ligand are active.

Keywords: tissue factor, CD40 ligand, gene expression, single nucleotide polymorphism, acute coronary syndrome, myocardial infarction, coagulation, inflammation

Anders Målarstig, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© Anders Målarstig 2006

ISSN 1651-6206
ISBN 91-554-6558-7
urn:nbn:se:uu:diva-6841 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-6841)
To my family and to science
This thesis is based on the following papers


II Mälarstig A, Siegbahn A. The Intersubject variability of Tissue Factor mRNA Production in Human Monocytes – Relation with Toll-like receptor 4. *Manuscript*


IV Mälarstig A, Lindahl B, Wallentin L, Siegbahn A. Soluble CD40L Levels are Regulated by the -3 459 A>G Polymorphism and Predict Myocardial Infarction and the Efficacy of Anti-Thrombotic Treatment in Non-ST Elevation Acute Coronary Syndrome. *Arteriosclerosis, Thrombosis and Vascular Biology, in press*

*Article from THROMBOSIS RESEARCH, 112(3):175-183, copyright 2003 Elsevier Ltd. Reprinted with permission.*

*Article from Arteriosclerosis, Thrombosis and Vascular biology 2005;25(12):2667-72, copyright 2005 Lippincott Williams & Wilkins. Reprinted with permission*
DNA extraction ................................................................. 32
SNP genotyping .............................................................. 32
Minisequencing .............................................................. 32
SNP stream ................................................................. 33
Signal detection and data analysis .............................. 33

Results & analysis .......................................................... 34

Project 1 – A quantitative real-time PCR method for tissue factor mRNA .......................................................... 34
  Rationale ........................................................................ 34
  Aims .............................................................................. 34
  Results .......................................................................... 34

Other TF mRNA methods ........................................... 36

Project 2 - The Intersubject variability of Tissue Factor mRNA Production in Human Monocytes .......................................................... 36
  Rationale ........................................................................ 36
  Aims .............................................................................. 37
  Results .......................................................................... 37

Project 3 - Genetic variations in the TF gene are associated with clinical outcome in acute coronary syndrome and expression levels in human monocytes .......................................................... 40
  Rationale ........................................................................ 40
  Aims .............................................................................. 40
  Results .......................................................................... 41

The Fragmin and Fast Revascularization during Instability in Coronary Artery Disease II trial .......................................................... 41

Project 4 - Soluble CD40L Levels are Regulated by the -3459 A>G Polymorphism and Predict Myocardial Infarction and the Efficacy of Anti-Thrombotic Treatment in Non-ST Elevation ACS .......................................................... 45
  Rationale ........................................................................ 45
  Aims .............................................................................. 45
  Results .......................................................................... 45

Conclusions ........................................................................ 49

Summary of tissue factor and CD40 ligand interplay in coagulation and inflammation .......................................................... 51

Sammanfattning på svenska ............................................... 53

Acknowledgments .................................................................. 55

Appendix A – references .................................................. 57

Appendix B – references for table 2 .................................... 67
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
</tr>
<tr>
<td>asHTF</td>
<td>alternatively spliced human tissue factor</td>
</tr>
<tr>
<td>AT</td>
<td>antihtrombin</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass graft</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CK-MB</td>
<td>creatine kinase MB</td>
</tr>
<tr>
<td>CRP</td>
<td>c-reactive protein</td>
</tr>
<tr>
<td>ECG</td>
<td>elektrocardiography</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>F3</td>
<td>the tissue factor gene</td>
</tr>
<tr>
<td>FRISC-II</td>
<td>fragmin and fast revascularization during instability in coronary artery disease II</td>
</tr>
<tr>
<td>FVII, FX</td>
<td>coagulation factor seven, factor ten etc.</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL-1 beta</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amoebocyte Lysate</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LMWH</td>
<td>low molecular weight heparin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PCI</td>
<td>percutaneous coronary intervention</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>sCD40L</td>
<td>soluble CD40 ligand</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor, thromboplastin</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alfa</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular adhesion molecule-1</td>
</tr>
</tbody>
</table>
Introduction

-in which the pleiotropic effects of tissue factor are briefly described and followed by an overview of its important role in the acute coronary syndrome.

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein, present in various human tissues and cell types. Cells in the blood do not normally produce TF, whereas the expression is abundant in the brain, lung, placenta and in cells surrounding the vasculature. Production of TF in blood cells e.g. monocytes can be triggered as a consequence of e.g. internal bacterial infection, cancer, atherosclerotic plaque rupture or trauma.

When TF bearing cells come in contact with factor VIIa, a serine protease plasma protein, blood coagulation is started and an intracellular signalling cascade into the TF bearing cell is initiated. In acute clinical situations, the activity and quantity of TF, as well as the balance between TF and its inhibitors exert downstream effects that influence the extent of blood clot formation. The TF/FVIIa interaction also influences the subsequent degree of inflammation, cell activation and migration.

TF is recognized as a key player in the acute coronary syndrome, which is a multifactorial disease preceded by atherosclerosis, inflammation and thrombosis. The role of TF in coronary disease has been recognised in both plaque destabilisation, which in part is mediated via non-hemostatic TF functions, and as the main coagulation initiator in atherothrombosis. Hallmarks for the intimate relationship between TF and ACS is the abundant expression of TF in atherosclerotic plaques and the presence of circulating TF bearing microparticles in ACS patients.

The expression of TF can be up- or downregulated by a number of different processes in vivo or by artificial stimuli in cell cultures. One example is CD40 ligand, which is expressed on T-lymphocytes and activated platelets and induces TF by interacting with its counterpart CD40 on monocytes and macrophages. TF expression can also be modified by the immunoregulatory cytokine interleukin-10, which is predominantly produced by Th2 type T-lymphocytes. Regulation of TF expression through CD40 ligand and interleukin-10 are considered important mechanisms in the acute coronary syndrome.
Protein expression is regulated on the DNA, RNA and protein level, according to the central dogma of molecular biology. This thesis report studies on the regulation of the TF gene with a focus on DNA level control. Thus, single nucleotide polymorphisms in the TF gene as well as receptors and transcription factors regulating TF gene expression were studied.

The aim with this thesis was to increase the understanding of how TF and CD40 ligand are involved in the acute coronary syndrome by analysing interindividual differences in gene expressions, gene variations and plasma protein concentrations.

The variability in coagulatory and inflammatory response between different people may explain why some patients with the acute coronary syndrome suffer severe cardiac events and some do not. In this respect, the interindividual variability in TF expression, regardless of underlying cause, is a strong candidate.

The results of this thesis add to our current knowledge of factors influencing TF expression and activity by demonstrating the effects of TF gene variants, cell signalling molecules, and the platelet and T-lymphocyte associated CD40 ligand and a variation in its gene. All of these effects have the potential to modify the risk of development, progression and outcome of the acute coronary syndrome. Finally, these effects exemplify the interplay between coagulation and inflammation, in which both TF and CD40 ligand are active.
Background

Tissue Factor

-in which a detailed description on tissue factor’s gene, structure, normal functions and signalling effects is given.

The F3 gene
In 1985, the F3 gene, which encodes human TF, was mapped to chromosome 1 (p21-p22) in studies using somatic cell hybrids with a sensitive chromogenic assay. This observation was replicated some years later by using a cDNA probe for the tissue factor gene and in situ hybridization to metaphase chromosomes. In 1989, the full sequence of the 12.4 kBP long F3 gene including 6 exons was published.

F3 gene similarities in other species
TF has also been investigated in other species than human. The TF gene is located on chromosomes different from human in mouse, rat, cattle and dog. The amino acid sequence of TF in mouse and rat show 55 percent similarities with human whereas TF in cattle and dog is 70 percent similar. Evidence that TF based coagulation developed early in the evolution has been gathered by investigating TF sequence similarities in jawless fish, teleosts amphibia, reptiles and birds. However, the sequence similarities with human TF were relatively low and it seems that only the most crucial residues of the extracellular domain of TF were conserved.

Tissue factor transcription
Transcription of the F3 gene yields a 2.3 kBP TF mRNA, which contain a GC rich promoter region (72 %), a coding region of 788 nucleotides and a 3´ untranslated region that is 1151 nucleotides long. Full-length TF mRNA is polyadenylated after transcription, which may be regulated by a potential polyadenylation signal at the near end of the 3´ untranslated region (UTR).
In addition to full-length TF mRNA, two alternative mRNA transcripts have been described. The first transcript fails to remove the first intron of the gene and is not translated to protein. The other alternative transcript, namely asHTF, has exon 4 directly spliced to exon 6. This differential splice pattern causes the insertion of a stop codon, which creates a frame-shift in the open reading frame and the protein is translated with a unique C-terminal end. Thereby, asHTF has no functioning transmembrane or cytoplasmic domain and is therefore present in the blood circulation as a soluble protein.

**Regulation of TF transcription**
Similar to members of the cytokine family, the expression of TF is very dynamic and regulated on several different biological levels. On the DNA level putative mechanisms of control include transcription factor binding and epigenetic control. Several transcription factors have been identified in the 5' untranslated region of the TF gene. It is considered that the LPS response element predominantly controls the inducible TF expression through two AP-1 sites and one NFkappaB site whereas the constitutive expression of TF seems to be controlled through three overlapping Egr-1/Sp-1 sites. The presence of other enhancer / silencer elements in proximity of the characterised regions of the TF promoter has been indicated, but is at present relatively unknown.

**Tissue Factor translation**
The primary TF mRNA transcript is translated into a 295 amino acid precur- sor protein. The precursor is truncated at the C-terminal which generates the 263 amino acid mature TF (47 kDa). Three distinct domains of mature TF can be defined: extracellular (residues 1-219), transmembrane (residues 220-242), and cytoplasmic (residues 243-263). The extracellular domain is necessary for the binding of FVII and the protease activity of the TF-FVIIa complex.

Proteolytic cleavage of mature TF is not required for activity, since TF is a single-chain molecule. However, some other posttranslational modifications are known for TF: In the extracellular domain, the formation of two disulfide bonds between cystein pairs and the glycosylation of three threonine residues appears to be important for receptor function and TF stabilisation to the membrane. In the cytoplasmic domain, a cystein residue at position 245 may be acylated by palmitic and stearate acids. However, the procoagulant activity of TF has been shown to be similar in acylated and deacylated forms of the protein.
Tissue Factor expression

In tissues
That tissues with a high expression of TF causes blood to clot has been known since 1835, when De Blaineville investigated the effects of suspended brain tissue injections in animals. It was, however, not until 1981 that TF was purified and the expression of TF could be studied using antibody dependent immunological methods.

Today, the analysis of expressed sequence tags (EST) in hundreds of human tissues has revealed that TF is constitutively expressed in the brain, lung, placenta, pancreas and tongue in descending order (UniGene). Moreover, a high TF expression is usually observed in tumour cells. The pattern of TF expression in vivo led to the conclusion that TF constitutes a haemostatic envelope, which may protect against excessive bleeding.

In monocytes/macrophages
In contrast to cells with a constitutive TF expression, monocytes and macrophages do not normally produce TF but can be induced to do so upon various stimuli.

The monocyte is the largest of all leukocytes (18-25 uM) and has a “kidney bean” shaped nucleus. Monocytes develop along the myeloid lineage from multipotent stem cells in the bone marrow. They stay in the circulation for about one day and then migrate into tissues for further differentiation into macrophages. The monocyte/macrophage is a vital component of the innate and acquired immune systems. These cells engulf foreign materials by a process known as phagocytosis and then display fragments of the antigen on their MHC class-II molecules, which initiates cellular and humoral immune responses by B cells and T cells.

Today, it seems clear that stimulated monocytes are the prime source of TF in circulating blood whereas granulocytes and lymphocytes lack significant expression. Numerous co-factors that augment or attenuate the stimulated expression of TF have also been identified. These co-factors are produced by granulocytes, lymphocytes or platelets and therefore, the experimental system in which TF expression is investigated, has a profound effect on the magnitude. The monocyte expression of TF is usually investigated in whole-blood, isolated mononuclear cells or in highly purified monocyte preparations.

In vivo, the most potent inducer of TF is lipopolysacharide (LPS), which is a cell-wall component in gram-negative bacteria. LPS seems also as the only non-cellular agent, which has the ability to induce TF expression in ex-vivo whole-blood. The LPS induced TF production by LPS can be augmented by using PMA as a co-stimulatory agent.
In isolated mononuclear cells, platelet derived growth factor \(^\text{18}\) and P-selectin \(^\text{19}\) are known inducers of TF expression. These are factors associated with activated platelets. The chemokine monocyte chemoattractant protein has also been shown to induce TF in mononuclear cells.\(^\text{18}\)

CD40 ligand is expressed on activated T-lymphocytes and platelets and induces TF expression in monocytes via the CD40 receptor.\(^\text{20,21}\) C-reactive protein as well as tumour necrosis factor has also been shown to induce TF expression.\(^\text{22-24}\) However, it is unclear whether these stimulatory agents exert direct effects on monocyte TF production or if mechanisms that rely on the presence of other cell types are responsible for the observed effects.\(^\text{25,26}\)

Another potent inducer of TF expression in monocytes is interferon gamma (IFN-\(\gamma\)). This inflammatory mediator is often abundantly produced by T-cells within atherosclerotic lesions, where it not only induces TF expression in monocytes but also inhibits collagen synthesis thus contributing to plaque destabilisation through two different mechanisms.

**In soluble, circulatory forms**

In addition to the membrane bound form, TF has also been detected in plasma.\(^\text{27}\) There seems to be two different sources of this circulatory, non-cell bound TF. One of the forms is the result of a proteolytic cleavage of the TF extracellular domain, which then becomes an inactive TF form, since TF looses its procoagulant activity when it is no longer bound to a phospholipid surface. The other form has been detected on small vesicles of phospholipid cell debris. These vesicles, namely microparticles, are in general prothrombotic.\(^\text{28}\) If they are also positive for TF, their thrombogenic potential is even larger by exerting significant TF procoagulant activity.\(^\text{29-31}\)

**Interindividual variability**

When monocytes are stimulated with LPS, the amount of TF that is produced is dependent on the individual from which the monocytes were isolated. Moreover, the TF expression seems to be highly reproducible for any given individual over a period of time. This phenomenon has been designated the TF high low response and has been shown for both antigen production and TF procoagulant activity.\(^\text{18,32,33}\)

In clinical situations, being a high or low TF responder may play an important role in the course of diseases such as sepsis, cancer or ACS. Several studies have shown the intimate relationship between increased coagulation and inflammation activation and a poor prognosis in these conditions.\(^\text{34-36}\) It is therefore reasonable to speculate that high TF responders with these diseases experience a stronger activation of inflammation and coagulation. The opposite pattern may be seen in individuals who are low responders, with lower TF production and subsequent cell activation as a consequence. The identification of factors that determine whether an individual is a high or
low TF responder has the potential to improve diagnosis, prognosis, treatment and drug development in diseases related with TF expression.

**Tissue factor – factor VIIa signalling**

The binding of TF to FVIIa not only initiates the coagulation process but also a signalling cascade into the TF bearing cell. Intracellular signalling through TF/FVIIa affects other mechanisms within the cell either by direct effects or by triggering secondary signalling pathways. The mechanisms include calcium oscillation, the phosphorylation of Src-like kinases and the activation of PAR-2.38-41, 42

Today, data on the involvement of TF/FVIIa signalling in atherogenesis and plaque stability are sparse. However, TF/FVIIa signalling was responsible for a 100-fold increased sensitivity of platelet PDGF-BB induced chemotaxis in human fibroblasts and vascular SMC. 43 Interestingly, PDGF has been implicated in vessel wall response (neointima formation) to mechanical injury by mediating the migration and proliferation of smooth muscle cells and fibroblasts.

The cytoplasmic domain of TF is not required for FVII(a) binding and is thus not necessary for triggering blood coagulation. However, by investigating genetically modified cells that produces TF without the cytoplasmic domain, it has been shown that the cytoplasmic tail is essential for cell migration.42-44

Cellular mechanisms mediated by the TF/FVIIa intracellular signalling may explain the association between TF and the wide spectrum of biological functions that seemingly goes beyond blood coagulation such as inflammation activation, atherogenesis, cancer metastasis, cell adhesion and migration, angiogenesis and transplantation rejection of pancreatic islets.

**Measuring tissue factor expression and procoagulant activity**

Several methods are available for the determination of TF antigen levels. TF in plasma and serum is usually measured using the ELISA technique. Membrane bound and intracellular TF can be measured with flow-cytometry or western blot. The laboratory results using either of these techniques may be difficult to interpret, since one must take into consideration the different TF forms that are simultaneously measured. The antigen level does not necessarily correlate with the procoagulant activity of TF, which is dependent on several factors other than the availability of TF antigen. Among those factors, the availability of phosphatidylserine on monocytes and the presence of platelets or leukocytes may be particularly important.

TF activity can be measured e.g. by assessing the amount of factor Xa or thrombin that a particular sample can generate in well defined system of other coagulation factors with known concentrations. A chromogenous sub-
strate for factor Xa or thrombin that can be analysed spectrophotometrically is also needed. The unknown samples are related to a standard curve of lipo-dised TF protein dilutions.

The acute coronary syndrome

-in which the development, progression and outcome of ACS is discussed in the perspective of tissue factor and CD40 ligand

Acute coronary syndrome is one of the most common causes of emergency care and death in the Western world. The incidence of ACS also increases in countries with a strong economic development such as India or China. In Sweden, improved health care has decreased the incidence of myocardial infarction (MI) and cardiovascular deaths by 23% and 40% respectively during the last decade. In contrast, the incidence of diabetes and hypertension, known risk factors for ACS, shows a steady increase.

Etiology

The acute coronary syndrome (ACS) is used to describe a spectrum of clinical presentations that share an underlying pathophysiology, including unstable angina, ST-elevation MI and non-ST elevation MI. These disease conditions are predominantly caused by atherosclerosis, coronary plaque rupture and subsequent thrombosis, which may lead to cardiac ischemia by occluding the arterial blood flow. The development and progression of atherosclerosis as well as the mechanisms leading to coronary plaque rupture and thrombosis are influenced by numerous factors. Lifestyle factors such as social life, stress, diet, exercise and smoking play a role in addition to medical factors such as age, diabetes, hypertension and infectious diseases. Moreover, genetic factors such as gender or genetic variation in genes controlling lipid metabolism, endocrine systems, signal transduction, inflammation, haemostasis and innate immunity are important factors. Thus, patients with ACS are a heterogeneous population with different underlying causes for their disease.

Development and progression of atherosclerotic lesions

The development of atherosclerosis begins at early ages with the accumulation of fatty streaks within larger arteries. The fatty streaks contain macrophages, in which a significant uptake of oxidised lipoproteins through the macrophage scavenger receptor has taken place (foam-cells). The atheroscle-
rotic lesions progress with the migration of smooth muscle cells from the vessel media into the intima. Already at this stage, CD4+ T-lymphocytes may be present, which together with foam-cells produce proinflammatory cytokines and give rise to a low-graded inflammatory state.\textsuperscript{45, 46} The smooth muscle cells produce extracellular matrix proteins such as collagen type I and III, which in later stages of lesion formation completely separates the lesion from the vessel lumen. The collagen layer of matrix proteins is called the fibrous cap. Encapsulated within the fibrous cap are macrophages, mast cells, T-cells and low-density lipoproteins. These cells produce e.g. chemokines, which enhances the recruitment of leukocytes into the plaque.

**Stability of atherosclerotic plaques**

Some atherosclerotic plaques remain stable for a long period of time. It has been observed, that these plaques are characterized by a thick fibrous cap and a low content of LDL and leukocyte infiltration in the core of the plaque.\textsuperscript{47} Other plaques cause significant stenoses of coronary arteries but have a low content of proinflammatory cells and therefore do not constitute a high risk plaque.\textsuperscript{48} In contrast, plaques with a thin fibrous cap and a high content of LDL or oxidised forms of LDL and inflammatory cells are prone to plaque rupture, which can result in a life-threatening clinical event. The processes, by which plaques destabilise, have been topics of great attention recent years.

**Tissue factor**

Plaque destabilization by TF is likely mediated through several different mechanisms. The expression of TF in cells within the necrotic core of the plaque may cause intraplaque haemorrhage and thus decrease the plaque stability. Another mechanism starts with the production of interferon $\gamma$, TNF-$\alpha$ and interleukin-1 (IL-1) by cells within the plaque. These cytokines promote macrophage apoptosis, which leads to the shedding of thrombogenic microparticles. The microparticles expose both phosphatidylserine and TF, which makes them highly thrombogenic.\textsuperscript{28, 49} Finally, TF-FVIIa signalling may promote cell migration and play a role in the development of vasa vasorum neovascularisation in coronary arteries.\textsuperscript{42, 44}

**CD40 ligand**

Recent years progresses have identified the CD40-CD40 ligand (CD40L) interaction as a key process affecting the stability of atherosclerotic plaques.\textsuperscript{50} CD40 is present on a variety of immune cells within atherosclerotic lesions including endothelial cells, smooth muscle cells and macrophages, whereas CD40L is largely expressed on CD4+ T-cells and activated platelets.\textsuperscript{51 52 53 54} Interaction between CD40 and CD40L has been shown to induce the production of metalloproteinases (MMP).\textsuperscript{21} Evidence suggest that
MMPs, and in particular MMP-3, MMP-1 and MMP-9, degrade components in the extracellular matrix and thereby weakens the fibrous cap and makes it more prone to rupture. \(^{55}\) Blocking experiments in hyperlipidaemic mice have shown that the size of coronary plaques as well as inflammatory cell content can be decreased by using an inhibitory antibody against CD40L. \(^{56}\) In addition, evidence suggests that tissue factor is up regulated through CD40 ligation, which represents another mechanism promoting plaque destabilization. \(^{57}\)

Plaque rupture, thrombotic mechanisms

Two mechanisms responsible for coronary plaque rupture have been acknowledged. One is related to physical forces and often occurs at the shoulder region of the plaque whereas the other mechanism involves cellular processes that degrade the fibrous cap from within.

Tissue factor

Upon a coronary plaque rupture, the thrombogenic gruel within the plaque is exposed to circulating blood, shown in figure 1. As a consequence, the TF-FVIIa complex initiates blood coagulation by FVIIa binding of TF, which is exposed on cells in the vessel wall, on macrophages from the plaque or on shedded microparticles. Platelets, which are partially activated by the plaque rupture, are recruited and adhere to the site of injury. The TF-FVIIa complex further activates the coagulation factors IX to IXa and X to Xa and a trace amount of thrombin is generated. Factor IXa and Xa may propagate into circulating blood. However, when factor Xa dissociates from the TF expressing cell, it becomes rapidly inhibited by the protease inhibitors TFPI and AT. In contrast, factor IXa can be transported to an adjacent platelet or other cell as it is not inhibited by TFPI and only slowly so by AT. \(^{58}\)

The small amount of thrombin is a signal for further platelet activation and aggregation. Moreover, the thrombin activates factor V, factor VIII and factor XI on the platelet surface. \(^{59}\)

Activated factor IX, generated by both TF/FVIIa and by platelet-bound factor Xla, binds to factor VIIIa on the platelet surface. Since factor Xa cannot be transported from the TF expressing cell, it is provided on the platelet surface by the FIXa/VIIIa complex. When factor Xa associates with platelet factor Va it is protected from the protease inhibitors. Upon this association, a burst of thrombin is generated, which is sufficient for the clotting of fibrinogen and formation of the fibrin meshwork. Thereby, a stable thrombus is formed. \(^{60}\)
Coronary plaque rupture exposes TF expressing cells and microparticles to the coagulation factors in the lumen of the vessel and thereby initiates atherothrombosis. The advanced, unstable, coronary plaque is characterized by a weak collagen layer with infiltrated smooth muscle cells, which encapsulates oxidised LDL, macrophages and T-lymphocytes. These cells express matrix degrading proteins, proinflammatory cytokines such as IL-6, IFN-\(\gamma\) and TNF-\(\alpha\) and CD40-CD40 ligand. Macrophages, smooth muscle cells and thrombogenic microparticles express TF, which then initiates the coagulation process together with factor VIIa, upon the rupture. Another rich TF source comes from fibroblasts, which are exposed upon a deep fissure that goes all the way down to the adventitia. The TF-FVIIa complex initiates blood coagulation. Platelets, which are partially activated by the plaque rupture, are recruited and adhere to the site of injury. The TF-FVIIa complex further activates the coagulation factors IX to IXa and X to Xa and a trace amount of thrombin is generated. The small amount of thrombin is a signal for further platelet activation and aggregation. The thrombin activates factor V, factor VIII and factor XI on the platelet surface. Activated factor IX binds to factor VIIIa on the platelet surface. When factor Xa associates with platelet factor Va it is protected from TFPI and AT. Upon this association, a burst of thrombin is generated, which is sufficient for the clotting of fibrinogen and formation of the fibrin meshwork. A thrombus is formed.
CD40 ligand

The CD40 - CD40L pathway has also been acknowledged as a process which drives and mediates thrombotic complications in ACS. A constitutive and inducible expression of CD40 has been observed in the endothelium, which is a major site for infiltration of macrophages into the plaque via adhesion molecules such as E-selectin, ICAM-1 and VCAM-1. In addition, studies have shown that CD40L is abundantly expressed in CD4+ T-lymphocytes, which have adhered to an endothelial cell layer. Thus, CD40-CD40L has a role in potentiating an inflammatory reaction of the endothelium, including the release of chemokines, tissue factor, and vascular endothelial growth factor. Platelets, which together with T-lymphocytes are the most important source of CD40L, adhere to the damaged endothelial cell layer upon a coronary plaque rupture, thus giving rise to a thrombotic event.

A prominent expression of CD40 and CD40L has also been observed in the vessel intima of advanced atherosclerotic plaques. There, CD40L positive T-lymphocytes together with CD40L on platelets derived from encapsulated internal haemorrhage co-localises with CD40 expressing infiltrated macrophages. The intima is thus a significant place for cellular interactions resulting in the local production of MMPs, proinflammatory cytokines and TF. Upon a plaque rupture this highly thrombogenic gruel is exposed to the circulating blood, a process which rapidly initiates blood coagulation.

CD40L is also present as a soluble, proteolytically cleaved form, which seems to be biologically active and able for interaction with CD40. Elevation of sCD40L has been linked to prothrombotic states in patients with diabetes, hypercholesterolemia and peripheral arterial disease and it is therefore assumed that high concentrations of sCD40L represent abundant CD40L expression on cells.

Consequences of ischemia

The metabolic changes associated with the sudden onset of ischemia caused by occlusion of a major coronary artery include (a) cessation of aerobic metabolism, (b) depletion of creatine phosphate, (c) onset of anaerobic glycolysis, and (d) accumulation of glycolytic products, such as lactate and alpha glycerol phosphate (alpha GP), and catabolites of the nucleotide pools in the tissue. These changes are associated with contractile failure and electrocardiographic alterations. The changes also result in the leakage of creatine kinases and cardiac troponins as a consequence of myocyte necrosis and apoptosis. It is established that high systemic concentrations of these biomarkers are well correlated with the outcome of the patient.
Inflammation activation

Other changes that occur after ischemic events include the systemic elevation of proinflammatory agents such as TNF-α, IL-1β, IL-6 and CRP. This observation may have several underlying reasons. First, the inflammatory system is triggered by the event of plaque rupture and following thrombosis. Second, the inflammation activity increases by the cardiac ischemia and subsequent tissue damage. Elevation of IL-6 or CRP can be observed several months after the index cardiac event.

In addition, it has been shown that also healthy individuals at risk for future cardiac events have an increased low-graded inflammation. One may hypothesize that subclinical inflammation causes a priming of blood leukocytes and thus leads to increased inflammation activation in the case of ischemic events.

Genetics of the acute coronary syndrome

The human genome

The human genome encompasses approximately 3.1 billion nucleotides, in which approximately 23,000 genes have been defined (www.ensembl.org/index.html). The DNA sequence is, in general, very similar in different individuals. However, there are also variations. These variations can be categorized into different types of variations such as insertions, deletions, microsatellites, short tandem repeats and single base substitutions. A nucleotide base substitution, namely single nucleotide polymorphism is the most common human genetic variation. Of the 3.1 billion base-pairs in the genome, it has been estimated that every 300 base-pair varies, which gives approximately 10 million SNPs in total.

A SNP is usually biallelic, which means that there are two possible variants of the allele. In contrast, microsatellites often have 4 variants or more. The SNP density varies across different regions of the genome and certain hot-spots with a high density of independent SNPs have been observed.

As shown in table 1, there are several possible mechanisms by which SNPs may alter the phenotypic expression. Regardless of where in the genomic sequence the SNP is positioned, the effects of any given SNP on gene expression, on the translated product and subsequent molecular interactions are difficult to predict beforehand.
Table 1. Alterations in gene function by single nucleotide polymorphisms

<table>
<thead>
<tr>
<th>Position</th>
<th>Function*</th>
</tr>
</thead>
<tbody>
<tr>
<td>gene exon</td>
<td>synonymous change of amino-acid</td>
</tr>
<tr>
<td></td>
<td>non-synonymous change of amino-acid</td>
</tr>
<tr>
<td>gene proximity</td>
<td>interference with cis-acting silencers/enhancers and other transcription factors</td>
</tr>
<tr>
<td>5´untranslated region</td>
<td>interference with mRNA stability</td>
</tr>
<tr>
<td>gene proximity</td>
<td>interference with mRNA polyadenylation</td>
</tr>
<tr>
<td>3´untranslated region</td>
<td>interference with mRNA splicing</td>
</tr>
<tr>
<td>gene intron</td>
<td>interference with splicing factors such as donor/acceptor sites</td>
</tr>
<tr>
<td>other genes</td>
<td>variations in trans-acting genes</td>
</tr>
</tbody>
</table>

* Single nucleotide polymorphism may also be silent and with no effect on transcription or protein expression.

A genetic syndrome with several candidates, but few convictions

The acute coronary syndrome is a complex disease with a strong genetic component. This is evidenced by several studies showing that a family history of ACS is a strong independent risk factor for this syndrome. In addition, large human twin-studies have indicated a high heritability of ACS. The interindividual variations of intermediate phenotypes, which include clinical and biochemical risk factors for ACS, seem mostly to have a genetic cause, as reviewed by Lusis et al. It is hypothesised that genetic and environmental factors such as smoking, diet and exercise act cooperatively in the development and progression of ACS.

Despite a long known relation between ACS and genetics, few genes (and even fewer SNPs within genes) linking this relation are identified. Family studies, which have been successful in the identification of monogenic disease, have been less successful for complex diseases such as ACS. Genetic association studies comparing the frequency of a known allelic variant in cases and controls have been notoriously difficult to reproduce. Popular explanations for the lack of reproducibility include poor study designs, small number of subjects, hidden population stratifications, too few genetic variants in the investigations and too liberal levels of statistical significance.
Polymorphisms associated with thrombosis and inflammation

Although genetic association studies, in general, have been difficult to replicate some variants with a strong relation to ACS and/or to intermediate phenotypes have been identified. Table 2 shows a selection of genetic variants in genes coding for coagulatory and fibrinolytic factors, platelet receptors and proteins in the inflammatory system.

Table 2. Single nucleotide polymorphisms in genes that have been associated with the acute coronary syndrome or with the production of proteins involved in ACS pathogenesis.

<table>
<thead>
<tr>
<th>gene</th>
<th>polymorphism</th>
<th>intermediate phenotype</th>
<th>association with arterial thrombosis?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>coagulation factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prothrombin</td>
<td>20210 G&gt;A</td>
<td>changed plasma levels through altered polyadenylation of mRNA</td>
<td>yes, moderate 2</td>
</tr>
<tr>
<td>factor V</td>
<td>Arg506Gln</td>
<td>activated protein C resistance</td>
<td>yes, moderate 2</td>
</tr>
<tr>
<td>factor VII</td>
<td>- 402 G&gt;A</td>
<td>changed basal plasma levels through altered transcription factor binding</td>
<td>inconsistent</td>
</tr>
<tr>
<td>fibrinogen β</td>
<td>- 455 G&gt;A</td>
<td>changed plasma levels</td>
<td>inconsistent, no 2</td>
</tr>
<tr>
<td>factor XIII</td>
<td>Val34Leu</td>
<td>increased FXIII activation by thrombin</td>
<td>inconsistent</td>
</tr>
<tr>
<td><strong>fibrinolytic factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>675 4G&gt;5G</td>
<td>changed plasma levels</td>
<td>inconsistent, no 2</td>
</tr>
<tr>
<td>TAFI</td>
<td>Ala147Thr</td>
<td>changed plasma levels</td>
<td>inconsistent</td>
</tr>
<tr>
<td>t-PA</td>
<td>- 7351 C&gt;T</td>
<td>increases t-PA release</td>
<td>possible</td>
</tr>
<tr>
<td><strong>platelet receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GpIlla</td>
<td>Leu33Pro</td>
<td>fibrinogen and vWF binding?</td>
<td>inconsistent, no 2</td>
</tr>
<tr>
<td>GpIbα</td>
<td>- 5 T&gt;C</td>
<td>vWF, thrombin binding?</td>
<td>inconsistent, no 2</td>
</tr>
<tr>
<td>GpIα</td>
<td>807 C&gt;T</td>
<td>collagen receptor density?</td>
<td>inconsistent, no 2</td>
</tr>
<tr>
<td><strong>inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>1444C&gt;T</td>
<td>change of plasma levels</td>
<td>inconsistent</td>
</tr>
<tr>
<td>TNF-alfa</td>
<td>-308G&gt;A</td>
<td>change of plasma levels</td>
<td>inconsistent</td>
</tr>
<tr>
<td>LTA</td>
<td>252 A&gt;G</td>
<td>change of transcriptional activity</td>
<td>possible</td>
</tr>
<tr>
<td>MHCIITA</td>
<td>- 168 A&gt;G</td>
<td>changed MHC molecule expression</td>
<td>yes, moderate 11</td>
</tr>
</tbody>
</table>

This overview was composed with the aid of David Lane’s review in Blood 2001 and Barbara Voetsch’s review in Arteriosclerosis, Thrombosis and Vascular biology in 2005. It was updated by Anders Målarstig in March 2006. Appendix B contains the references indicated in this table. A recent meta-study published in Lancet indicated no association with coronary disease for factor VII, PAI-1, Gp1a, GpIbα and GpIIIa, which is the reason for giving information in this table on both previous inconsistent associations and the more recent lack of associations for these genes.
Why analyse single nucleotide polymorphisms?

- The analyses of genetic factors may further our understanding of the biological processes involved in ACS.
- SNPs may, in theory, be of use both as diagnostic and prognostic tools. It is believed that SNP information in the individual patient will be more important as a prognostic tool, rather than a diagnostic in complex diseases such as ACS.
- On the basis of genetic status, primary and secondary preventions can be achieved by tailoring individual and genotype specific treatments.
- A fully penetrant SNP with a major effect on disease may provide a target for directed gene therapy.
- The mapping of genetic factors contributing to ACS may increase our knowledge of the role of environmental factors in this syndrome.
Aims

Tissue factor is known as the principal initiator of blood coagulation and is therefore considered a major thrombogenic factor in ACS. In addition, it is now established that TF/FVIIa binding induces intracellular signalling, which makes TF a true receptor. The role of TF in ACS (and in other diseases related to TF expression) is becoming more important when new functions of TF are revealed. Both hemostatic and non-hemostatic functions of TF may be altered by factors influencing TF expression and as a consequence; ACS development, progression and outcome.

In vitro studies have identified factors that control TF transcription, translation and procoagulant activity. However, the differential TF expressions between individuals, which constitute another level of control, had not been addressed. Therefore, it was desirable to learn more about factors which determine TF expression levels in vivo and in vitro and in both healthy individuals and in patients with ACS. To be more precise, we focused on genetic variation in the TF gene and signalling molecules in the TF induction pathway, which we considered were important and unexplored factors. Concerning genetic variants in the TF gene, it was not known whether these correlated with the risk of MI and death in ACS. For these purposes, a method that allowed sensitive quantification for a wide range of TF mRNA expression levels was needed. These criteria were not fulfilled by existing TF mRNA methods.

In addition, current evidence suggested a key role of the CD40-CD40L pathway in TF induction within atherosclerotic plaques. CD40 ligation had also been shown to induce MMP production with the consequence of plaque structure weakening. However, it was unknown what high plasma concentrations of CD40L reflected in the ACS patient and it was unknown to what extent CD40L plasma concentrations mirrored TF expression. Therefore, the current thesis project was complemented with the investigation of soluble CD40L and SNPs within its gene in relation to MI, death and coagulation activation in ACS. The more specific aims of this thesis project were:

- To establish a method for tissue factor mRNA that was sensitive and reliable for a wide range of TF mRNA levels and which could be used for both in vivo and in vitro samples
- To investigate whether the tissue factor high and low response is present at the transcriptional level and if this phenomenon is related to genetic variation in the tissue factor gene and/or to lipopolysaccharide signal transduction pathways.

- To examine the role of tissue factor polymorphisms in patients with the acute coronary syndrome and to analyse clinically associated genetic variants in human monocytes.

- To examine the role and impact of genetic variation in the CD40 ligand gene in patients with ACS and to investigate the relationship between soluble CD40 ligand plasma concentrations and patient outcome.
Laboratory methods

This section provides a brief overview of the respective laboratory methods used in this thesis work. For more details, please see the respective papers in appendix A.

Cultivation of cells

The experimental systems used in the different projects are outlined below. Of note, buffers and reagents used for cell cultivations were routinely checked for endotoxin contamination using the CoaTest LAL assay (Haemochrom Diagnostica). If contamination was detected, the reagent or buffer solution was wasted. Glassware were heated at 180 °C for 3 hours in order to denature possible endotoxins.

Isolation of mononuclear cells

Venous blood was drawn and collected in endotoxin free EndoTube ET tubes, mixed with phosphate buffered saline solution (PBS) and subjected to density gradient centrifugation using ficoll-paque (Amersham, GE health care). The cells were thereafter collected, washed and counted using a flow-cytometer. The cells were diluted to a concentration of half a million monocytes per ml and cultivated for 3 hours in the presence or absence of 1 µg/ml of LPS. This procedure was used for mononuclear cell isolations from the seven individuals participating in the first project of this thesis, from 54 individuals in the second and from 25 individuals in the third project.

Experiments in the whole-blood environment

Routinely, 6 ml of endotoxin free heparin blood were drawn and used in experiments investigating the basal, incubated and LPS induced expression of TF mRNA. Two ml of blood was transferred to each of three polypropylene tubes for direct RNA isolation or subsequent incubation for 2 hours with or without prior LPS stimulation using a final concentration of 20 ng/ml LPS. This procedure was used on blood from two healthy individuals and five patients before and after PCI in the first project and on blood from nine healthy individuals in the second project of this thesis.
Cultivation of human cell lines

To establish the TF real-time PCR method in the first project of this thesis we investigated the TF expression in a panel of eleven different human cell lines. The cell lines investigated were human prostate cancer cell lines (LNCaP, PC3 and Du145), breast cancer cell lines (MCF-7 and MDA-MB-231), myeloid cells (U937, MonoMac-6, NB-4 and THP-1) and fibroblasts 1064sk and 1523. The cells were maintained in RPMI 1640 or D-MEM supplemented with 10 % fetal bovine serum, 1 % glutamine, 100 U/ml penicillin and 50 μg/ml of streptomycin.

Purification of monocytes

In the second project of the thesis, highly purified monocytes were obtained from eight individuals. The procedure started with isolation of mononuclear cells whereupon the cells were further purified using an affinity column that captured non-monocytes (Miltenyi Biotech). Depending on the monocyte / leukocyte ratio of the individual donor, the final cell suspension contained between 85 – 95 % monocytes.

RNA isolations and cDNA synthesis

Two methods were used for the isolation of total RNA – two-phase extraction using the phenol reagent Trizol (Invitrogen) and the Qiagen blood RNA kit. RT-PCR was performed by priming the RNA with Oligo dT primer and then reverse transciptase using the moloney murine leukemia virus enzyme (Invitrogen).

Real-time PCR

In the first project the ABI 7700 real-time PCR instrument was used and in project 2 and 3 the, ABI 7000 instrument. Primers and probes for the measurement of singleplex TF, singleplex IL-6, multiplex TF and singleplex alternatively spliced TF were designed using the software Vector NTI 6.0 or manually. The primers for β-actin, β-2-microglobulin (B2MG), glyceraldehyde-dehydrogenase (GAPDH) and ribosomal protein 0 (RPLP0) were previously published. Measurements of TNF-α, IL-1β, IL-8, TLR4, CHUK (IκB) and p65 were carried out using predesigned “assays on demand” (Applied Biosystems).
Flow-cytometry

The flow-cytometer (Epics XL, Beckam Coulter) was used in project 1, 2 and 3 for the counting of monocytes and leukocytes and to examine cell-viability using propidium iodide. Flow-cytometry analysis was also used for the measurements of TF surface expression in human cell lines in project 1 and in mononuclear cells in project 2 (unpublished). Briefly, cells were incubated with a primary TF mouse-anti-human antibody (10H10) for 30 minutes, whereupon they were washed 2 times. Thereafter, the cells were incubated with a secondary FITC-conjugated goat-anti-mouse antibody, washed and suspended in PBS+bovine serum albumine (BSA) and analysed in the cytometer.

Methods for TF antigen and procoagulant activity

Several methods of TF antigen and procoagulant activity measurement have been used in this thesis work. In project 1, TF was measured in human cell lines by the use of a semi-quantitative Western-blot analysis, which normalised the TF stainings on the Western gel to those of β-actin.

In project 2, both TF surface and total antigen expressions were investigated with flow-cytometry analysis and ELISA (American Diagnostica), respectively (unpublished). Monocytes were lysed using a lysis buffer containing 1 % Triton-X 100, 150 mM NaCl, 50 mM TRIS buffer and 0.5 % of protease inhibitors PMSF and DTT. Cell debris was removed from the extract, whereupon it was frozen to -70 °C until analysis.

In project 3, plasma TF was investigated in 725 FRISC-II patients and in 376 healthy controls with the use of a commercial ELISA (American Diagnostica). TF procoagulant activity was investigated in mononuclear cells from 25 healthy donors using a previously published method. In brief, intact monocytes were added to a mixture of 0.6 mmol/L S-2222, 2 mmol/L CaCl₂, and coagulation factors from the factor concentrate Prothromplex-TIM4 (Baxter, Vienna, Austria) at a final concentration of 1 U/mL FVII and 1.2 U/mL FX. Absorbance at 405 nm after incubation for 60 minutes at 37°C was determined and related to a standard curve of relipidised recombinant TF.

CD40 ligand in plasma

Venous blood was drawn in citrated tubes (Vacutainer®, Becton-Dickinson) at admission to the coronary care unit of the FRISC-II hospitals. Plasma was separated by centrifugation, aliquoted and stored at –70°C until
analysis. The median time-lapse between admission and blood sampling was 39 hours (interquartile range: 27-55) hours. Plasma levels of sCD40L were measured in 2,359 patients using the ELISA technique (Bender MedSystems, Burlingame, CA). Intra- and interassay coefficients of variation for a high and low sample were 6.9 -15.0 % and 8.0 – 16 %, respectively.

DNA extraction

EDTA blood was used for the extraction of DNA from the FRISC-II patients and healthy controls. The DNA was extracted at the Karolinska Institute biobank DNA extraction facility using the Promega Wizard DNA kit with slight modifications of the supplier’s protocol. The concentration of extracted DNA was measured by UV absorption at 260 nm, with the 260/280 absorbance ratio as a quality check.

SNP genotyping

The collection of in total 33,500 genotypes in the current thesis work was performed at the genotyping core facility of Uppsala University – the Wallenberg Platform North under supervision of Professor Syvänen and Doctor Axelsson. The choice of method was dependent on the location and nucleotide base change of the SNP.

A larger number of SNPs within the TF or CD40L encoding genes were evaluated in a method evaluation phase using a subset of 200 individuals in the FRISC-II study. Some SNPs that were selected from the SNP databases were either monomorphic in the present population or failed technically. To avoid the exclusion of genotype information of SNPs we considered crucial to the project, the method for genotyping was changed or another SNP in linkage disequilibrium with the desired SNP was genotyped instead. After the method evaluation stage, 12 SNPs in the TF gene and 6 SNPs in the CD40LG gene were selected for genotyping in the patients and controls participating in project 3 and 4 of the current thesis. In addition, four common SNPs in the TF gene were genotyped for 42 individuals in project 2.

Minisequencing

Excess of PCR primers and dNTPs were removed by adding a 5 µl mixture containing 0.1 U/µl shrimp alkaline phosphatase (USB Corporation) and 0.1 U/µl Exonuclease I (Fermentas GmbH) in 20mM Tris-HCl pH 8.0 and 10mM MgCl2 to the PCR product. The plate was incubated at 37°C for 45 minutes, followed by 10 minutes at 92°C to inactivate the enzymes. Five µL of extension mixture containing 1,0 µM extension primer, and 0.08 U/µl
KleenThermase™ DNA Polymerase containing 10 mM K-phosphate buffer (pH 7.0), 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.01% Tween 20; 50% glycerol (v/v) and fluorescently labeled and unlabeled ddNTPs was added to each reaction. The four ddNTPs were included at 0.125 µM concentration with the two, for the SNP relevant labeled ddNTPs, at a 1:5 ratio to unlabeled ddNTP. The fluorescent dye rhodamine 110 (R110) was used to label ddCTP and ddGTP and 6-carboxytetramethylrhodamine (TAMRA) was used to label ddATP and ddUTP. Fluorescently labeled ddNTPs were purchased from PerkinElmer Life Sciences. The cyclic extension reaction was performed at 95°C for 2 minute followed by 40 cycles of 94°C for 10 seconds and 55°C for 30 seconds.

SNP stream

Five µl of the multiplex PCR products were treated with 3 µl of SNP-IT Clean-Up reagent, containing exonuclease I and shrimp alkaline phosphatase diluted 25 fold (USB Corporation), at 37°C for 60 minutes followed by inactivation of the enzymes at 92°C for 15 min. Seven µl of reaction-mixture containing one Tamra-labelled ddNTP, one Fluorescein-labelled ddNTP, thermostable DNA polymerase (SNPware Reagent Kit, Beckman Coulter), and the tagged minisequencing primers, were added to a final concentration of 0.04 µM in a total volume of 15 µl. The cyclic extension reactions were performed at 96°C for 1 min followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. Eight µl of hybridization buffer from the SNPware Reagent Kit was added to the extension product and 10 µl of this mixture was transferred to the SNPware Tag Array plate (Beckman Coulter) containing the immobilized complementary tag oligonucleotides. The capturing reaction was allowed to proceed for 2 hours at 42°C in a humidified container, followed by rinsing the plate with washing buffer supplied with the reagent kit.

Signal detection and data analysis

For Method I, the fluorescence signals in the 384 well plates were read using an Analyst AD™ instrument (Molecular Devices). The genotypes were assigned using the software AlleleCaller™ supplied with the instrument. For Method II, a prototype version of the GenomeLab™ SNPstream Genotyping System® (Beckman Coulter) was used. The incorporated ddNTPs were detected using a CCD camera with excitation lasers of wavelength 488 nm and 532 nm integrated in the system. The fluorescence signals were extracted using the SNPstream Image™ and the SNPstream GetGenos™ software of the system.
Results & analysis

Project 1 – A quantitative real-time PCR method for tissue factor mRNA

Rationale

The present dissertation project was started out by establishing a method for TF mRNA quantification, which would be critical for further studies investigating TF expression. The current methods e.g. Northern blot and RT-PCR did not fulfil our demands of a sensitive and dynamic method that could be used for several different cell types, experimental systems or in vivo samples. Also, we demanded a method which could be adequately standardised. A short linear range, the need of a large RNA amount and the time-consuming experimental steps limited the Northern blot, whereas the RT-PCR was dismissed because of elaborate optimisation and control of each individual experiment.

Aims

Our specific goals with this method were to be able to:
- Standardise the measurements of TF mRNA over a long period of time
- Find a housekeeping gene, for which the expression was not significantly altered by the LPS treatment of monocytes
- Compensate for differences in cell proportions between individuals, reasoning that TF is only produced by monocytes and the housekeeping gene in all cell types
- Compare cells with a very high mRNA expression to those with a very low
- Use the method in several experimental systems including primary mononuclear cells, whole-blood and human cell lines.

Results

The standardisation of TF mRNA measurements over a longer period of time was solved by always including an RNA standard in each batch of samples subjected to the cDNA synthesis (figure 2). The RNA standard was
prepared from a high TF expressing source, aliquoted into several tubes and stored at -70°C.

We examined whether the expressions of β-actin, B2MG, GAPDH were significantly changed in monocytes after LPS stimulation. The results showed that B2MG was the transcript least altered by the LPS treatment whereas the use of actin and GAPDH in these experiments seemed unreliable. In project 2 we normalised mRNA expressions to that of RPLP0, which was previously reported to be unaffected by LPS.83

Figure 2. Four dilutions of the quantificatation RNA standard prepared from LPS stimulated human monocytes were included in each real-time PCR experiment.

We found a way to compensate for the differences in leukocyte ratios in both whole-blood and in isolated mononuclear cells by counting the cells in the flow-cytometer and normalise TF/housekeeping gene expression to that ratio.

To examine the dynamic range and sensitivity of the TF real-time method we used a plasmid containing a TF cDNA fragment, which was diluted between 1 and 10⁸ times. The plasmid, functioning as a calibrator of the assay, was detected in up to 10⁷ and down to 10⁰ copies by the TF method. In order to evaluate this dynamic range in a true experimental condition, we also investigated the TF expression in a panel of human cell lines with a very wide range of TF expression levels. The method proved equally well as the TF plasmid in this panel. TF mRNA and antigen levels in the cell lines were well correlated, as evaluated by a Western blot.

TF mRNA was also investigated in whole-blood samples taken from patients with stable angina before and after PCI (table 3). We observed that TF mRNA levels on average increased by 4-fold after this mechanic provocation. Although, the increase in TF mRNA depended strongly on the individual patient.
Table 3. TF mRNA in patients before and after percutaneous coronary intervention

<table>
<thead>
<tr>
<th></th>
<th>pre-PCI TF mean</th>
<th>SD</th>
<th>post-PCI TF mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.2</td>
<td>0.05</td>
<td>2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.1</td>
<td>0.04</td>
<td>0.4</td>
<td>0.22</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.4</td>
<td>0.21</td>
<td>1.0</td>
<td>0.42</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.2</td>
<td>0.06</td>
<td>0.4</td>
<td>0.21</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.4</td>
<td>0.03</td>
<td>0.4</td>
<td>0.16</td>
</tr>
</tbody>
</table>

This real-time method for TF mRNA quantification was reliable in true experimental conditions and for in vivo samples. Even small changes in mRNA levels, such as those in the PCI patients, could be monitored with this method.

Other TF mRNA methods

Although not a part of project 1, an update of the TF methods developed in project 3 has its place under this paragraph. To facilitate the simultaneous quantification of TF and the endogenous control B2MG a multiplex real-time PCR method was developed. This method, in which primer concentrations of B2MG are limited, saved time, reagents and abolished pipetting errors since the two mRNA transcripts were measured in the same PCR plate well. Furthermore, a real-time PCR method that facilitated the quantification of alternatively spliced TF mRNA was developed. The Taqman® probe hybridised to the unique exon 4 – exon 6 boundary and thus specificity for asHTF was accomplished. These methods are published.84

Project 2 - The Intersubject variability of Tissue Factor mRNA Production in Human Monocytes

Rationale

As reviewed in the introduction of this thesis, a high and low responder phenomenon of TF antigen and procoagulant activity has been observed. Cellular and environmental factors associated with the phenomenon have been suggested. However, the studies seem to only partly explain the TF interindividual variabilities.85,86 Thus, the TF high and low response may also be regulated by genetic variation in the TF gene and / or by components in the LPS signalling pathway.

As numerous factors affecting TF antigen expression and procoagulant activity have been reported and we wanted to avoid potential confounding factors we decided to study TF high and low response on the transcriptional level only. In addition, previous studies have shown that the expression of
TF is mainly controlled at the transcriptional level but TF high and low response has not been investigated at this level of control.  

Aims

We aimed to investigate whether the TF high and low response phenomenon was present at the transcriptional level and if so; what mechanisms were responsible for it? As a primary aim, we examined genetic variation of the TF promoter and gene in relation to the phenomenon and as a secondary aim, molecules in the LPS signalling pathway were analysed.

Results

This hypothesis-driven study was initiated with the screening of TF mRNA levels in monocytes after stimulation with LPS in 42 healthy individuals. Five high and three low responders were defined by their TF expression levels at the time of screening. The high and low response status of these individuals was further evaluated in three subsequent experiments at 8, 10 and 22 months after the screening.

By this analysis, we obtained evidence that the TF high and low response is present at the transcriptional level and that it is highly reproducible for almost two years (figure 3).

Figure 3. The LPS induced TF mRNA expression in four independent experiments according to the TF response defined at screening

In addition to this controlled experiment, we also observed high and low TF mRNA responders in PCI patients in project 1 of this thesis. Interestingly, patients responding highly upon the PCI treatment also responded high upon ex-vivo LPS stimulation of whole-blood (figure 4).
In project 3, we observed a high reproducibility of the LPS induced TF mRNA levels in 25 healthy individuals, from which mononuclear cells were isolated and tested on two occasions with 6 months in between.

To elucidate whether the high and low TF mRNA response phenomenon was related to variations in the TF gene, we sequenced the LRE region and genotyped four common SNPs in the TF gene. Furthermore, we calculated the intraclass correlation (ICC) of TF mRNA and compared it to the ICC of TNF-α, IL-1β, IL-6 and IL-8 to see whether these mRNAs showed a higher or similar correlation as TF between the different experimental occasions. The ICC is a measure for whether the variability within the individual is smaller than the variability between individuals.

No new SNPs in the LRE region of the TF gene were discovered. In addition, none of the four SNPs in the TF gene were correlated with TF mRNA levels. However, the ICC of TF was significantly higher compared to that of TNF-α, IL-1β and IL-6 whereas the ICC of IL-8 were somewhere in between TF and the cytokines. These results indicate that although no variations in the TF gene with a relation to TF high and low response were identified in the present study, there may be other TF gene specific mechanisms responsible for the TF high and low response.

Project 3 of the current thesis work demonstrated a functional variant in the TF intron 2, 5466 A>G, that conferred an increased risk of cardiovascular death and increased TF procoagulant activity. The three donors who carried the 5466 G allele were excluded from further analyses in the current project, with the motivation that the 5466 G allele frequency did not match the prevalence of the high and low response phenomenon and that other gene variants, apart from the 5466 A>G, may be associated with TF high and low response. Thus, the five high and three low responders defined in the current project had another underlying cause for their TF high or low response than
differences in LRE, the four common polymorphisms or presence of the 5466 G allele.

In order to examine whether TF high and low response was related to differences in the LPS signalling pathway we analysed correlations between TF and TNF-α, IL-1β, IL-6 and IL-8 as well as TF and TLR-4, CHUK and p65 (c-rel) in the eight donors, whose LPS induced TF expression was defined at the screening stage. The results showed that the TF expression was relatively well correlated with that of IL-8, TNF and IL-1 whereas IL-6 was not correlated. Furthermore, TF showed a considerable correlation with TLR-4 expression. To follow up this interesting finding, we compared the expressions of TF and TLR-4 mRNAs in two other independent groups in mononuclear cells and whole-blood (figure 5), respectively. The strong correlation between TF and TLR-4 was thus replicated ($R^2=0.82$, $p=0.001$) and ($R^2=0.69$, $p=0.03$).

The present study did not identify variants in the TF gene responsible for the TF high and low response. However, a TF gene specific control was implicated by the ICC analysis, which may suggest that gene variants not covered by our gene analyses do have effects on TF expression. Studies on the regulation of the TF gene has shown that LPS signalling is primarily mediated via the transcription factors AP-1 and NFκB, which bind to the LRE region. However, it can not be excluded that other parts of the promoter also has a part in the LPS induced transcription of the TF gene.

Figure 5. Tissue factor and toll-like receptor 4 mRNA expression after LPS stimulation of whole-blood from 9 different donors

In addition, a novel link between inflammation and coagulation was provided by showing a significant co-expression between TF, initiator of coagulation and TLR-4, the primary LPS signalling receptor. Endotoxin has been shown to induce the expression of proinflammatory cytokines in endothelial
cells, vascular smooth muscle cells and monocytes/macrophages and is rec-
ognised both as a factor driving atherosclerosis and as a factor which may
trigger acute thrombotic events. These results in combination with project 
3 in the current thesis indicate that several different mechanisms may be 
responsible for the individual capability of TF mRNA production after LPS 
stimulation. Possibly, the mechanisms for a high TF response may differ 
from the mechanisms regulating a low response. The mapping of interplay 
between genetic control and signalling pathways has potential benefits in 
both coagulation and non-hemostatic TF-induced processes.

Project 3 - Genetic variations in the TF gene are 
associated with clinical outcome in acute 
coronary syndrome and expression levels in 
human monocytes.

Rationale

TF has been recognised as major thrombogenic factor in the pathogenesis 
of the acute coronary syndrome. However, the current knowledge of TF 
SNPs in this syndrome was restricted to one common variant in the TF pro-
moter. Therefore, our aim was to further clarify the role of TF SNPs in 
ACS by genotyping a panel of SNPs, which covered the majority of variants 
in the TF gene, in a large prospective study of patients with ACS and in 
healthy controls who were similar in age, gender and geographic residence 
as the patients.

Aims

A total number of 12 SNPs in the TF gene were selected to represent the 
11 most common TF haplotypes, as evaluated in the Seattle SNP database. 
Thus, we avoided redundant genotyping with the exception of three SNPs 
representing the previously studied -1812 C>T SNP, which has been associ-
ated with TF expression. The specific study aims were to investigate 
whether

- Plasma TF was higher in patients compared to healthy controls
- Plasma TF was higher in patients who suffered a cardiac event during 
  follow-up
- Any of the 12 SNPs in the study were associated with plasma TF, patient 
  / control status or clinical outcome in patients.

To address these questions, patients from four hospitals participating in 
the prospective multicentre study FRISC-II were selected for the study to-
gether with 376 healthy controls recruited in the same parts of Sweden as the ACS patients. Primary genetic associations were followed-up in the full FRISC-II patient group, which enrolled 3 143 patients with non-ST elevation ACS.

The Fragmin and Fast Revascularization during Instability in Coronary Artery Disease II trial

The Scandinavian multicenter trial FRISC-II compared the benefits of an early invasive procedure over an early non-invasive strategy and a 90 days prolonged treatment with a LMWH (dalteparin) versus placebo (figure 6).92,93

Figure 6. Flow-scheme of the FRISC-II trial.

Patients were eligible if they had symptoms of ischemia that could be verified by electrocardiography or increased biochemical markers and if the last period of chest-pain had occurred within 48 hours from assignment to open-label dalteparin (study randomization). All patients received basic treatment with aspirin and at least 5 days with subcutaneous open-label dalteparin. If not contraindicated, patients were randomized within 72 hours
from admission to invasive / non-invasive treatment and secondly to long-term dalteparin / placebo. The endpoint of MI was defined by the occurrence of two out of three criteria, which were typical chest-pain, diagnostic ECG recording (mainly new Q-wave) and raised CK, CK-B, CK-MB or CK-MBmass according to the criteria of the local hospital. All causes of mortality were recorded. Events during the first 6 months were recorded and evaluated by an independent End Point Committee whereas the information after this time were collected by investigator reports, outpatient visits or telephone contact with surviving patients.

Apparently healthy individuals who were similar in age and gender proportions to the patients were recruited from the Swedish population registry. The healthy subjects were resident in southern, middle or northern parts of Sweden. Only those without a clinical history of cardiovascular disease or cardiovascular risk factors and with a normal electrocardiography and normal routine blood chemistry were eligible. Written consent was obtained from all study participants. These studies, including the genetic substudy protocols, were approved by the regional ethics review board of Uppsala.

Of note, three different settings of the described FRISC-II patients and healthy controls were included in the current thesis. In project 3, a primary setting with 725 patients and 376 healthy controls who were similar in age, gender and geographical residence as patients were included. The primary findings in this group were followed up in the full FRISC-II study group, consisting of 3 143 FRISC-II patients with available DNA samples.

In project 4, only the patients who were randomised to an invasive or non-invasive treatment strategy were included in the study. This selection included 2 359 patients with available plasma samples of which 2 164 also had available DNA samples.

Results

The plasma concentrations of TF were significantly higher in patients with ACS compared to the healthy controls (180 pg/ml vs. 143 pg/ml, p<0.001). Plasma concentrations of TF did not, however, correlate with outcome in the patient group. One possible reason for this finding is that the antibody used in the commercial ELISA seems to recognise both active and inactive forms of TF. Possibly, the analysis of TF procoagulant activity would have yielded a different result.

Out of 12 SNPs genotyped in the study, only the 5466 A>G SNP showed a significant difference in genotype distribution in the patients compared to the controls (15 % vs. 10 %, p=0.03). After permutation correction for multiple tests, the difference was no longer significant. The SNPs included in the study is shown in figure 7.
The 5466 A>G SNP was also weakly associated with the risk of death and MI in the ACS patient group. This association was considerably stronger for patients with the common -1812 CC genotype indicating a haplotype effect of these SNPs.

Figure 7. A total number of 12 SNPs in the TF gene were genotyped in 725 patients with ACS and in 376 healthy controls, similar in age, gender and geographic residence as the patients.

To follow up these genetic associations, the 5466 A>G and -1812 C>T SNPs were genotyped in all patients who participated in the FRISC-II trial, with the specific aim to investigate an association between these SNPs and the risk of death and death/MI within 6- or 24-months. A G allele dominant genetic model was used in the analyses, since 5466 GG homozygotes were extremely rare.

The results showed that the 5466 AG+GG genotypes were strongly associated with the 24-month risk of cardiovascular death, 1.8 (1.1 - 3.2, odds ratio and 95 % confidence interval (C.I). Recent years research on complex diseases such as ACS have indicated that most genetic risk factors are associated with a relative risk in the 1.1. – 1.5 range, which suggests that the 5466 G allele confers an unusually strong effect on the phenotype. However, the 5466 A>G by itself was not associated with non-fatal MI or the composite endpoint death/MI.

We confirmed a previous study, which indicated that the -1812 C>T SNP was unassociated with ACS. The combination of the 5466 A>G and -1812 C>T alleles resulted in three different haplotypes – C-A, T-A and C-G (in descending order of frequency). The C-G haplotype was strongly associated with cardiovascular death within 6- and 24 months and the composite endpoint of death/MI within 6-months, 3.1 (1.7 - 5.8) and 1.6 (1.1 - 2.3) (odds ratio and 95 % C.I), respectively.

In order to investigate the mechanisms underlying the clinical findings, we analysed the mRNA expressions of full-length and asHTF as well as the procoagulant activity in LPS induced mononuclear cells from 14 healthy
donors with the 5466 AA and -1812 CC genotypes and in 11 healthy donors with the 5466 AG and -1812 CC genotypes. This was equivalent with a comparison between the C-A/C-A and C-A/C-G haplotype combinations.

The median of basal and LPS induced TF mRNA levels was significantly lower in donors who carried the 5466 AG genotype. Also alternatively spliced TF was lower in this group of donors. Six months later, new experiments were performed, which showed the same results (table 4). Contrasting to TF mRNA, the increase in TF procoagulant activity upon LPS stimulation was significantly higher in 5466 AG carriers indicating a high response phenomenon in these individuals.

A possible explanation for the low TF mRNA quantities observed in 5466 AG carriers is that monocytes from this group were more readily activated, with the consequence that TF activity and mRNA expression peaked earlier and even during leukocyte isolation. One may speculate that when challenged with LPS, monocytes from 5466 AG carriers were already “exhausted” because of priming effects and therefore the TF mRNA production was blunted.

Table 4. Association of the 5466 A>G SNP and TF mRNA and activity in human monocytes

<table>
<thead>
<tr>
<th></th>
<th>5466 AA</th>
<th>5466 AG+GG</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF mRNA, control</td>
<td>Mean 1.4 Median 1.3 (0.3-2.0)</td>
<td>Mean 0.3 Median 0.04 (0.03-0.37)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TF mRNA, LPS</td>
<td>Mean 8.8 Median 5.8 (2.7-11.0)</td>
<td>Mean 3.0 Median 0.6 (0.1-1.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>TF activity, control</td>
<td>Mean 15.4 Median 14.6 (7.4-21.1)</td>
<td>Mean 7.5 Median 5.5 (2.9-7.8)</td>
<td>0.04</td>
</tr>
<tr>
<td>TF activity, LPS</td>
<td>Mean 51.4 Median 62.8 (38.9-76.6)</td>
<td>Mean 54.1 Median 41.2 (68.9)</td>
<td>0.97</td>
</tr>
<tr>
<td>TF activity, fold increase</td>
<td>Mean 2.1 Median 1.6 (1.3-1.9)</td>
<td>Mean 3.8 Median 3.2 (2.7-5.8)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The exact mechanism by which the 5466 G allele influences mRNA production seems yet to be determined. Possible explanations include a direct effect on transcription factor binding, the alteration of an unidentified silencer/enhancer region or effects mediated by a functional SNP in tight LD with the 5466 A>G. The 5466 A>G SNP is positioned in intron 2 in the TF gene. Previously, it was believed that gene introns were only “junk” DNA with no functions. This belief has been revised and the examples of intronic elements with a profound effect on gene function have steadily grown. One of the most important mechanisms by which intronic variation influence gene function is the splicing process. It has been estimated that 238 000 potential 3’ splice sites are located within introns of protein-coding genes, and each one can become an exon.

In this project we identified the 5466 A>G SNP, which is currently the only SNP in the TF gene with a clinical relevance. The 5466 A>G SNP fur-
thermore regulated the expression of TF mRNA in monocytes and conferred a high response of LPS induced TF activity. These in vitro results may provide a theoretical basis of the clinical observations in our patients with ACS.

Project 4 - Soluble CD40L Levels are Regulated by the -3459 A>G Polymorphism and Predict Myocardial Infarction and the Efficacy of Anti-Thrombotic Treatment in Non-ST Elevation ACS

Rationale

The interaction between CD40 and CD40L has been acknowledged as a process which drives atherosclerosis towards thrombotic complications in ACS. Elevated levels of sCD40L have been observed in prothrombotic states such as diabetes, PAD and ACS. However, the contribution of genetic variations in the CD40L encoding gene to sCD40L plasma concentrations and outcome in ACS was unknown. Likewise, the relationship between high plasma concentrations of sCD40L and outcome in patients with ACS had not been studied in a large prospective study, neither in relation to treatment strategies. Finally, it was not clear to what extent sCD40L plasma concentrations reflected coagulation activation. Activation of the coagulation system was assessed by plasma concentrations of d-dimer and prothrombin fragment 1+2, which are markers of thrombin generation and fibrin turnover and accurately mirrors an active coagulation.

Aims

The specific goals with this project was to

- investigate whether plasma concentrations of sCD40L were genetically controlled by SNPs in the CD40LG gene.
- analyse the relationship between CD40LG SNPs and risk factors for ACS and outcome in the patient group
- investigate whether high plasma concentrations of sCD40L were related with risk factors for ACS and/or predictive for the risk of future death and MI
- all of the above according to an early invasive treatment strategy and to the treatment with 90 days of dalteparin after the acute event

Results

A total of six SNPs in the CD40LG gene were selected for genotyping without beforehand information on LD between those. We observed an asso-
Association between sCD40L and three of the SNPs genotyped in the project. After LD analysis, shown in figure 8, we could pinpoint the association to the -3459 A>G SNP whereas the other associated SNPs showed a relatively high LD with this variant. The novel -3459 A>G SNP, identified in this project, is at present the only known genetic variant that regulates the expression of CD40L.

**Figure 8.** Linkage disequilibrium between the genotyped SNPs in the CD40LG gene. Black indicates $R^2=1.0$ and white $R^2=0$.

Although the -3459 G allele was associated with 10-15% higher concentrations of sCD40L there was no association between this SNP and death or MI, or with risk factors, in the ACS patients. Plasma concentrations according to -3459 A>G genotype is shown in figure 9.

**Figure 9.** Plasma concentrations of soluble CD40 ligand were higher in patients with the -3459 G allele.
Restricted to patients who received long-term placebo, sCD40L plasma concentrations above median were strongly associated with an increased risk of MI within the 2-year follow up. In contrast, sCD40L levels were not associated with the risk of death. Significantly higher concentrations of sCD40L were also observed in patients admitted with diabetes, chest pain at rest and in female patients. A multivariable analysis, with the use of a logistic regression model, showed that sCD40L was independent of any other parameter in the model including age, gender, troponin-T elevation, CRP elevation etc.

Since the predictive value of sCD40L was high in the placebo treated group and non-existent in the dalteparin treated group, a difference in the treatment efficacy of long-term dalteparin between patients who presented with low and high sCD40L plasma concentrations was implicated. Efficacy analyses of the respective treatments in the FRISC-II study in relation to sCD40L above median and TnT above 0.03 ug/L showed the following:

- soluble CD40L levels above median indicated a relative risk reduction of 40 % by the prolonged dalteparin treatment whereas TnT above 0.03 ug/L had no such indication
- soluble CD40L levels above median was no indication for an increased efficacy of early invasive treatment whereas this relation was indicated for TnT above 0.03 ug/L

A combination of sCD40L above median and TnT above 0.03 was associated with an even larger risk of MI and death/MI compared to each biomarker alone. However, the biomarker combination was not an indication for increased treatment efficacies of either sCD40L or TnT elevation.

Table 5. Combinations of soluble CD40 ligand and troponin-T concentrations at admission in the FRISC-II trial

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No elevation of sCD40L or TnT</td>
<td>362</td>
<td>16 %</td>
</tr>
<tr>
<td>elevation of TnT but not sCD40L</td>
<td>803</td>
<td>35 %</td>
</tr>
<tr>
<td>elevation of sCD40L but not TnT</td>
<td>361</td>
<td>16 %</td>
</tr>
<tr>
<td>elevation of both markers</td>
<td>741</td>
<td>33 %</td>
</tr>
</tbody>
</table>

Another interesting observation was that plasma concentrations of prothrombin fragment 1+2 and d-dimer were higher in FRISC-II patients admitted with high sCD40L levels. Whether high sCD40L levels are reflective of intense platelet activation or ongoing coagulation has been a matter of debate. Our data supported both views since the coagulation markers were higher in patients with sCD40L above median although there were also patients with elevation of sCD40L but less coagulation activation, as shown in figure 10.

Elevated sCD40L levels at hospital admission have previously been associated with clinical outcomes in patients with ACS within a 6 or 10 month follow up period using retrospective data and composite primary end
The present study is therefore the first investigation to demonstrate the prognostic value of sCD40L in a prospective, large, study of patients with ACS. It is also the only study, in which sCD40L have been examined in relation to SNPs and to coagulation activation.

Figure 10. Quartiles of plasma concentrations of prothrombin fragment 1+2 in relation to soluble CD40 ligand levels in patients with acute coronary syndrome.

Our combined results designate sCD40L as marker of a prothrombotic state that seems to be driven by T-cell and endothelial cell mediated inflammation and monocyte/macrophage and platelet mediated coagulation.
Conclusions

- A real-time PCR method that detected down to 100 and up to 100 million copies of TF mRNA was established in isolated mononuclear cells, whole blood and in human cell lines. By normalising the expression of TF to that of B2MG the method compensated for differences in mRNA quantity and integrity even in experimental systems using lipopolysaccharide as stimulating agent. To be able to compare the expression between different individuals with different compositions of leukocytes the TF/B2MG quotes were further normalised to the monocyte/leukocyte ratio.

- A multiplex real-time PCR method for the simultaneous quantification of TF and B2MG was established.

- By designing a Taqman probe directed at the unique exon 4-6 boundary in the TF gene, a method for the determination of alternatively spliced TF mRNA was established.

- High and low responders of TF mRNA after lipopolysaccharide stimulation of human monocytes were demonstrated and found stable over almost two years. Of note, the high and low TF responders were defined at a primary screening stage and the definitions held true in three independent, subsequent, experiments.

- Sequence variations in the LRE region (-252 to +26) of the TF promoter or four common SNPs in the TF gene were not associated with TF high and low response. In contrast, analyses of intraclass correlations suggested that TF mRNA expression levels between two independent experiments were more replicable over time compared to the mRNAs of IL-8, TNF-α, IL-1β and IL-6. This finding indicates a TF specific genetic control of the high and low response phenomenon not detected by our TF gene sequence analyses.

- A significant correlation between the high and low response phenomenon and the expression of the LPS signalling receptor TLR-4 was recorded. This observation provides a novel link between coagulation and inflammation.
• Plasma TF, measured at admission in patients with ACS, was not associated with the future risk of coronary events but was significantly higher in patients compared with a healthy control group.

• Carriers of the novel 5466 G allele were more frequent in patients with ACS compared with healthy controls in the uncorrected association analysis.

• The 5466 AG or GG genotypes were associated with a higher risk of cardiovascular death in patients with ACS.

• Haplotype construction according to the -1812 C>T and 5466 A>G SNPs revealed the C-G haplotype, which was associated with a three-fold higher risk of cardiovascular death and a 1.6 fold increased risk of death/MI in the ACS patients.

• The association between the 5466 A>G SNP and the cardiovascular risk was explained by a high TF procoagulant activity in LPS stimulated monocytes from a group of healthy donors.

• The novel -3459 A>G SNP in the CD40LG gene was associated with plasma concentrations of sCD40L.

• The -3459 A>G SNP was not associated with the risk of MI or death amongst all patients in the FRISC-II trial.

• Plasma sCD40L concentrations above median were associated with a higher risk of future MI, but not with death, in patients with ACS.

• Patients admitted with sCD40L concentrations above median showed a risk reduction of future MI by a prolonged treatment with LMWH whereas those with sCD40L below median had no benefit of this treatment.

• Patients who were admitted with sCD40L concentrations above median also had increased coagulation activation, as evaluated with prothrombin fragment 1+2 and d-dimer.

• Soluble CD40 ligand is marker reflecting platelet and coagulation activation in patients with ACS.
Summary of tissue factor and CD40 ligand interplay in coagulation and inflammation

Tissue factor is known for its ability to interact with factor VII and thus initiate blood coagulation. This function is important in the acute coronary syndrome and many other thrombotic disorders. Beyond its role in hemostasis TF is also a true receptor with the ability to influence downstream effects of TF/FVIIa binding such as activation of par-2, intracellular signal transduction and cell migration. These functions, together with the hemostatic function, are important in the destabilisation process of atherosclerotic plaques. By enhancing leukocyte infiltration into the plaque, inflammation, neovascularisation and internal hemorrhage TF regulates factors that increase the risk of coronary plaque rupture.

This concept was kept in mind, when we identified the high TF responding 5466 G allele in the TF gene as a risk factor for cardiovascular risk and outcome. The novel 5466 A>G, positioned in intron 2 of the TF gene, had a strong effect on TF mRNA and procoagulant activity in human monocytes, which potentially influences both the hemostatic function of TF as well as intracellular signal transduction by the TF/FVIIa complex. TF is the only coagulation factor for which a congenital deficiency has not previously been reported. We can now demonstrate the first TF polymorphism with a clinical relevance in ACS and suggest the in vivo functionality of this genetic variant.

This, however, did not completely solve the intriguing high and low TF responder phenomenon; the 5466 A>G SNP explained only a part of the large and replicable TF mRNA differences between individuals that we had identified in mononuclear cells from healthy donors. The examination of other common SNPs in the TF gene as well as sequence variation in the LRE promoter region of TF gave no further conclusions. In contrast, the toll-like receptor 4, which have been identified as the primary LPS signalling receptor, was more highly expressed in TF high responders compared to low responders thus linking innate immune response and coagulation in a novel fashion. In this context, the on-going discussion on how infection and subclinical endotoxemia may drive the atherosclerotic processes and perhaps even trigger thrombotic events is of great interest.

Another membrane bound molecule that was studied in the current thesis is CD40 ligand, also active in both inflammation and thrombosis. CD40
ligand promotes inflammation during atherogenesis by ligation to endothelial cells, smooth muscle cells, and macrophages, which in turn mediates functions such as the expression of cytokines, chemokines, growth factors, matrix metalloproteinases, and tissue factor. Thereby, the -3459 A>G SNP that was identified in the current thesis may play a role in the development of ACS, even if it was not associated with outcome in the FRISC-II patients.

We suggest that soluble CD40 ligand also has a key role in thrombosis, which we base on our observations that soluble CD40 ligand was associated with the risk of MI and that antithrombotic treatment effectively reduced the risk of new cardiovascular events in patients with high levels of this molecule. As a marker of thrombosis, a high plasma concentration of soluble CD40 ligand likely reflects platelet activation and adhesion as well as increased coagulation.

The findings demonstrated in the current dissertation may thus not only further the understanding of the biological roles of TF and CD40 ligand in the acute coronary syndrome, but may also provide new tools for molecular and genetic risk stratifications as well as antithrombotic treatments in this disease.
Sammanfattning på svenska

Vid en skada på ett blodkärl eller vid sjukdomar så som cancer, blodförgiftning och hjärtinfarkt börjar membranproteinet tissue factor (TF) produceras. När TF, som är bunden till celler, kommer i kontakt med plasmaproteinet factor VII startar blodets levring. Dessutom påverkas andra faktorer inne i den cell som har TF på ytan vilket leder till ökad cellvandring och inflammation. Celler i den yttre blodkärlsväggen har alltid en hög produktion av TF medan monocyter som cirkulerar runt i blodbanan har en hög produktion endast vid en skada eller vid infektion.

Instabil kranskärlssjukdom är ett förstadium till hjärtinfarkt som i huvudsak beror på ansamlingar av fettpartiklar och celler i hjärtat blodkärl. De s.k. placken skyddas av fibrös vävnad och är inte farliga så länge de inte brister. En hög produktion av TF hos de celler som är inneslutna i placket är en orsak till bristning av den fibrösa vävnaden. Vid en sådan bristning kommer celler med TF på ytan i kontakt med cirkulerande blod och levringen av blodet sätts igång. En annan typ av celler som kallas trombocyter täcker bristningen och bidrar också till blodlevring. En sådan proppbildning hotar att stänga av syretillförseln till hjärtat och leder till permanenta skador på hjärtat om den inte behandlas. Ett annat protein som heter CD40 ligand utöndras av celler inuti placket och av trombocyter. CD40 ligand binder till CD40 på andra celler och genom detta ökar benägenheten för ett plack att brista, för större blodproppsbildning och för ökad produktion av TF och inflammationsproteiner.

Människans hela DNA är uppdelat på 46 kromosomer och ungefär 23 000 gener. DNA sekvensen i våra gener är relativt lika mellan olika individer men i hela DNA:t förekommer ett stort antal variationer, varav 10 miljoner (av 3.1 miljarder) utgörs av polymorfer. Polymorferna kan påverka produktionen av protein på ett antal olika sätt och effekten av påverkan beror helt på i vilken gen variationen finns. Man tror att instabil kranskärlssjukdom till stor del är ärtlig men endast ett fåtal polymorfer som påverkar risken för sjukdomen har hittats.

I mitt avhandlingsarbete har jag utvecklat en metod för att mäta TF mRNA, vilket är ett förstadium till TF protein. Metoden visade sig vara tillförlitlig för både cellförsök med monocyter som provokerats till TF produktion med bakterierester och i patienter som genomgått ballongvidgning.

Jag har också undersökt hur TF mRNA varierar mellan olika friska individer i monocyter renade ur blod och vad som är orsaken till att olika indivi-
der får olika mycket TF på sina celler. Vi identifierade en polymorfi i TF genen som delvis förklarade variationen mellan individer, men vi förstod att det fanns andra faktorer som också spelade en roll. Ytterligare en av dessa identifierades; vi fann en koppling mellan hög produktion av det cellbundna proteinet toll-like receptor 4, som tillsammans med andra protein binder bakterierester till sig för att ”tala om” för cellen att den ska aktiveras. Infektioner och låga koncentrationer av bakterier eller rester av dessa i blodet anses öka risken för att få instabil karskärlssjukdom.


För att ta reda på mer om hur proteinet CD40 ligand är involverad i instabil karskärlssjukdom måtte vi detta och polymorfer i dess gen i FRISC-II studien. En ny polymorfi som ger ökad produktion av CD40 ligand, -3459 A>G, upptäcktes. Förekomsten av polymorfin föreföll däremot inte öka risken för död och hjärtinfarkt. Vår studie visade också att patienter som kom in till sjukhuset med höga koncentrationer av CD40 ligand hade en sämre prognos än övriga patienter. Patientgruppen som fick lägsmolekylärt heparin istället för placebo under 90 dagar efter inclusion i studien hade däremot ingen sämre prognos trots hög CD40 ligand. Därmed drog vi slutsatsen att en förlängd behandling med lägsmolekylärt heparin föreföll vara särskilt effektiv hos patienter med instabil karskärlssjukdom som har höga halter i blodet av CD40 ligand. Sammantaget visade studien att en hög halt av CD40 ligand återspeglar en ökad levringsbenägenhet av blodet.

Resultaten av dessa studier har gett ökad kunskap om genetisk kontroll av TF och CD40 ligand produktion och betydelsen av dessa i instabil karskärlssjukdom. Förutom att ge ökad kunskap om biologin bakom instabil karskärlssjukdom kan resultaten ge nya infallsvinklar för att kategorisera patienter som uppsöker vård p.g.a. misstänkt karskärlssjukdom och för nya behandlingsformer.
Acknowledgments

I would like to sincerely thank everyone who’s been involved in the studies leading to this doctoral dissertation, with special notice to:

**Agneta Siegbahn**, my supervisor, for teaching me the meaning of true science, for enthusiasm and wisdom, for boosting my self-confidence with words of praise following our joint achievements and finally, for successful co-authoring of our papers.

**Lars Wallentin**, my co-supervisor, for letting me be a part of the cardiology research group and for valuable advice and interesting discussions.

**Taavo Tenno**, my co-supervisor, for teaching me the difference between a bad and good experiment, for showing patience, for interesting and intellectual discussions and for friendship.

**Mikael Åberg**, for friendship, fun and fruity discussions.

**Birgitta Fahlström, Christina Christersson, Matilda Johnell, Anna Fläring, Helena Vretman, Teet Velling**, members of our research group for company and congresses, friendship, help, interesting discussions and words of encouragement. Birgitta and Helena have done a wonderful job of quantifying plasma protein and mRNA for my studies.

**Jenny Eriksson, Kristin Blom, Mirja Augustsson, Elin Nilsson, Göran Ronqvist and members of the IMV journal club**, for friendship and discussion during journal club meetings, lunch, coffee and ice-cream hours.

**Charlotte Woshnagg, Ulla-Britta Jansson, Lena Gröndahl, Lena Moberg, Riita Mållberg, Peter Ridefelt, Mia Lampinen, and others**, for donating blood to my studies.

**Lars Berglund, Johan Lindbäck and Lisa Wernroth**, for providing excellent statistical advice and evaluation. With Lars I have been involved in several interesting discussions of statistics and statistical genetics, which has helped me a lot in the course of my studies.
Ann-Christine Syvänen and Tomas Axelsson, for providing the Wallenberg Platform North courses in statistical genetics, for excellent and never-failing support with SNP genotyping and for co-authoring.

Bertil Lindahl, Nina Johnston and Bo Lagerqvist, for co-authoring and valuable discussions.

Enrique Vega and Lars-Ove Lundberg, for patience, humour and IT support.

Barbro Bjurhäll, Lena Berglund and administrators at Department of Medical Sciences, for rapid assistance with bills and administration.

All of the colleagues at Clinical Chemistry, for making this division a nice place to work.

Lasse Johansson and the guitar group, for letting me forget all about science once every fortnight

Daniel Bång and the rest of my friends, for taking me back to the real world once in a while.

Gudrun, Björn, Ida and Ylva, my parents and sisters, for love, support, baby-sitting and for being there.

Albin and Leah, my children, for unconditional love, joy and for reminding me of the real values in life.

Last but not least, Malin, my beloved wife, for always loving me, for patience, support and encouragement. And for being in my life!

Finally, Bella, our dog, for constantly reminding me that life is a walk in the park.
Appendix A – references

Reference List


Appendix B – references for table 2


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)