

From the Department of Medicine Solna, Clinical Pharmacology Unit  
Karolinska Institutet, Stockholm, Sweden

# **EFFECTS OF LIPID-LOWERING TREATMENT ON PLATELET FUNCTION AND HEMOSTATIC MECHANISMS IN DIABETIC PATIENTS:**

## **INFLUENCE OF CHRONIC KIDNEY DISEASE AND INFLAMMATORY PARAMETERS**

Tora Almquist



**Karolinska  
Institutet**

Stockholm 2014

All previously published papers were reproduced with permission from the publisher.  
The picture on front page is reprinted from Platelets (Third edition) 2013 with permission from Elsevier.  
Published by Karolinska Institutet.  
Printed by Åtta.45 Tryckeri AB  
© Tora Almquist, 2014  
ISBN 978-91-7549-703-7

# EFFECTS OF LIPID-LOWERING TREATMENT ON PLATELET FUNCTION AND HEMOSTATIC MECHANISMS IN DIABETIC PATIENTS:

## INFLUENCE OF CHRONIC KIDNEY DISEASE AND INFLAMMATORY PARAMETERS

### THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Tora Almquist**

*Principal Supervisor:*

Professor Paul Hjemdahl  
Karolinska Institutet  
Department of Medicine, Solna  
Clinical Pharmacology Unit

*Co-supervisor(s):*

Professor Stefan Jacobson  
Karolinska Institutet  
Department of Clinical Sciences  
Danderyd Hospital  
Division of Nephrology

Professor Per-Eric Lins  
Karolinska Institutet  
Department of Clinical Sciences  
Danderyd Hospital  
Division of Internal Medicine

*Opponent:*

Professor Bengt Fellström  
Uppsala University  
Department of Medical Sciences  
Division of Renal Medicine

*Examination Board:*

Docent Thomas Nyström  
Karolinska Institutet  
Department of Clinical Sciences and Education  
Södersjukhuset  
Division of Internal Medicine

Professor Cecilia Linde  
Karolinska Institutet  
Department of Medicine Solna  
Cardiology Unit

Docent Sofia Ramström  
University of Linköping  
Department of Clinical and  
Experimental Medicine



“Just don’t give up trying to do what you really want to do. Where there is love and inspiration, I don’t think you can go wrong.”

*Ella Fitzgerald*

To Magnus, Johan, Sofia and Gustaf



## ABSTRACT

Diabetes mellitus (DM) and chronic kidney disease (CKD) are both associated with increased cardiovascular morbidity and mortality, and the risk is even higher when they are concurrent. Both diseases are considered to be prothrombotic states with increased inflammatory activity and major disturbances in the hemostasis. Lipid-lowering treatment (LLT) may have beneficial effects on inflammation, platelet activation and atherothrombotic mechanisms.

We evaluated the prognostic implications of impaired renal function, measured as estimated creatinine clearance (eCrCl), in 808 patients with stable angina pectoris in a *post hoc* analysis of the Angina Prognosis Study In Stockholm (APSIS), which compared metoprolol and verapamil treatment in stable angina with a median follow-up of 40 months. A multivariate Cox analysis showed an independent prognostic importance of eCrCl for cardiovascular (CV) death and for CV death or myocardial infarction (MI). Patients with eCrCl <60 ml/min had a doubled risk of suffering CV death or MI, compared to patients with eCrCl ≥90 ml/min.

We investigated the effects of LLT with simvastatin alone or in combination with ezetimibe in 18 patients with an estimated GFR (eGFR) of 15-59 ml/min/1.73m<sup>2</sup> (DM-CKD) and 21 DM patients with eGFR >75 ml/min/1.73m<sup>2</sup> (DM-only) in a randomized, double blind, cross-over study. Parameters reflecting platelet activity, microparticles (MP) formation and inflammatory parameters were measured. At baseline, after a placebo run-in period, we found signs of increased inflammatory activity, increased platelet activation and hypercoagulability in DM-CKD compared to DM-only patients with increased formation of platelet-leukocyte aggregates (PLA), elevated levels of proinflammatory cytokines and soluble CD40L (sCD40L) in plasma, as well as elevated levels of MPs derived from platelets (PMPs), monocytes (MMPs) and endothelial cells. Simvastatin treatment alone reduced the expression of P-selectin, tissue factor (TF) and CD40L on PMPs, and TF on MMPs in both patient groups. Simvastatin also reduced levels of total procoagulant MPs, PMPs and MMPs as well as IFN $\gamma$  and MCP-1 in DM-CKD but not in DM-only patients. Furthermore, the combination of simvastatin+ezetimibe reduced PLA formation and sCD40L levels in DM patients with CKD compared to DM-only patients. Most differences between DM-CKD and DM-only patients were reduced or disappeared with LLT despite similar lipid levels in the two groups both before and during LLT.

In conclusion, impaired renal function carries independent prognostic information in patients with stable angina pectoris, in agreement with findings in other patient categories. Patients with CKD should be identified early, as there is need for improved CV risk reduction therapy in these high-risk patients. DM patients with CKD stages 3-4 (eGFR 15-59 mL/min/1.73m<sup>2</sup>) have signs of increased inflammatory activity and platelet activation, and hypercoagulability compared to DM-patients with normal eGFR. LLT counteracted the differences between DM-CKD and DM-only patients, with reduced inflammatory activation and a less procoagulant milieu especially in the presence of CKD. This may contribute to the beneficial effects of LLT on atherothrombotic complications in DM patients with concurrent CKD.

## LIST OF SCIENTIFIC PAPERS

- I. **Almquist T**, Forslund L, Rehnqvist N, Hjemdahl P  
Prognostic implications of renal dysfunction in patients with stable angina pectoris. *J Intern Med*. 2006 Dec; 260(6):537-44.
- II. **Almquist T**, Jacobson SH, Lins PE, Farndale RW, Hjemdahl P  
Effects of lipid-lowering treatment on platelet reactivity and platelet-leukocyte aggregation in diabetic patients without and with chronic kidney disease. *Nephrol Dial Transplant* 2012 Sep; 27(9):3540-6.
- III. **Almquist T**, Jacobson SH, Mobarrez F, Näsman P, Hjemdahl P  
Lipid-lowering treatment and inflammatory mediators in diabetes and chronic kidney disease. *Eur J Clin Invest* 2014 Mar; 44(3):276-84.
- IV. **Almquist T**, Mobarrez F, Jacobson SH, Wallen H, Hjemdahl P  
Effects of lipid-lowering treatment on circulating microparticles in patients with diabetes and chronic kidney disease. *Manuscript*.

# CONTENTS

1	Introduction.....	1
1.1	Background.....	1
1.2	Platelet function, platelet-leukocyte interactions and inflammation – importance in atherosclerosis and thrombosis .....	2
1.2.1	Platelet physiology .....	2
1.2.2	Platelet activation.....	3
1.2.3	The coagulation system .....	5
1.2.4	Platelet-leukocyte aggregates .....	6
1.2.5	The CD40-CD40L system.....	6
1.2.6	Microparticles .....	7
1.3	Hemostasis in Diabetes Mellitus.....	7
1.4	Hemostasis in Chronic Kidney Disease – bleeding and clotting.....	7
1.4.1	Platelets in CKD .....	8
1.4.2	The coagulation system in CKD .....	8
1.5	The endothelium in DM AND CKD.....	10
1.6	Dyslipidemia in DM and CKD .....	10
1.7	Inflammation in DM and CKD .....	10
1.8	Inflammation in atherosclerosis and thrombosis .....	11
1.9	Antiinflammatory and anticoagulant effects of lipid-lowering treatment.....	11
2	Aims of the project .....	13
3	Patients & methods .....	15
3.1	Study design and population .....	15
3.1.1	Paper I .....	15
3.1.2	Papers II-IV .....	15
3.2	Laboratory investigations .....	16
3.2.1	Blood sampling.....	16
3.2.2	Assessment of platelet function ( <i>Paper II</i> ) .....	16
3.2.3	Platelet-leukocyte aggregation ( <i>Paper II</i> ) .....	17
3.2.4	Microparticles analyses ( <i>Paper IV</i> ).....	17
3.2.5	Biochemical analyses .....	17
3.3	Statistical analyses.....	18
4	Results & disussion.....	19
4.1	Paper I.....	19
4.2	Paper II .....	22
4.3	Paper III .....	25
4.4	Paper IV.....	26
5	General Discussion .....	31
5.1	Renal function as a prognostic predictor .....	31
5.2	Signs of increased inflammation and hypercoagulability in DM-CKD patients .....	31
5.3	Effects of lipid-lowering treatment .....	33

5.4	The influence of CKD was counteracted by lipid-lowering treatment .....	34
5.5	Lipid-lowering treatment in CKD and clinical outcomes .....	35
5.6	Limitations .....	35
6	Future Perspectives.....	37
7	Summary and Conclusions.....	39
8	Svensk sammanfattning.....	40
9	Acknowledgements .....	42
10	References .....	45

## LIST OF ABBREVIATIONS

ADP	Adenosine-diphosphate
CKD	Chronic kidney disease
COX-1	Cyclooxygenase-1
CVD	Cardiovascular disease
DM	Diabetes mellitus
eCrCl	Estimated creatinine clearance
eGFR	Estimated glomerular filtration rate
EMPs	Endothelial microparticles
GP	Glycoprotein
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
INF $\gamma$	Interferon- $\gamma$
LLT	Lipid-lowering treatment
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMPs	Monocyte microparticles
MPs	Microparticles
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor -1
PLA	Platelet-leukocyte aggregates
PMPs	Platelet microparticles
PS	Phosphatidylserine
sCr	Serum creatinine
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNF $\alpha$	Tumor necrosis factor- $\alpha$
tPA	Tissue plasminogen activator
TxA <sub>2</sub>	Tromboxane A <sub>2</sub>
VCAM-1	Vascular cell adhesion molecule-1
VWF	Von Willebrand factor



# 1 INTRODUCTION

## 1.1 BACKGROUND

Diabetes mellitus (DM) is one of the most common causes of chronic kidney disease (CKD) mainly as a consequence of the global increase in type 2 DM and obesity (Tuttle 2014). Both DM and CKD are associated with increased cardiovascular morbidity and mortality and large cohort studies have shown that those with kidney disease predominantly account for the increased mortality in patients with both type 1 and type 2 DM (Groop 2009, Afkarian 2013).

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with CKD, and individuals with mild to moderate CKD are more likely to die of CVD than to develop end-stage renal disease (Levey 2012). Both CKD and albuminuria are independent risk factor for CV mortality both in the general population and in high-risk patients (history of hypertension, diabetes, or cardiovascular disease) with an exponentially increasing risk for CVD with declining renal function (Chronic Kidney Disease Prognosis 2010, van der Velde 2011).

The pathophysiological links between CKD and CVD have been subject to rapidly growing interest the last ten years. Patients with CKD have a high prevalence of “traditional” risk factors such as diabetes, hypertension and dyslipidaemia. In addition, “non-traditional” risk factors such as chronic inflammation, mineral disorders, anemia, increased oxidative stress and procoagulant mechanisms may contribute to the excess risk (Stenvinkel 2002, Sarnak 2003, Shlipak 2005). However, the complex pathophysiological links between CKD and CVD are still not fully understood and need to be further explored.

The Kidney Disease Improving Global Outcomes (KDIGO) statement has defined CKD as the presence of kidney damage (i.e., albuminuria) or a decreased glomerular filtration rate (GFR)  $<60\text{ml/min per }1.73\text{m}^2$  during 3 months or more, irrespective of the clinical diagnosis (Levey 2012). Since GFR plays an essential role in the pathophysiology of complications, CKD is divided into five stages based on GFR:

Stage	Description	GFR (ml/min/1.73m <sup>2</sup> )
1	Kidney damage with normal renal function	$>90$
2	Mildly decreased renal function	60-89
3	Moderately decreased renal function	30-59
4	Severely decreased renal function	15-29
5	Kidney failure	$<15$

GFR can be estimated (eGFR) by equations based on serum creatinine or cystatin-C (Levey 2012).

Albuminuria has an important role in the progression of CKD, and epidemiological studies show a graded relationship between albuminuria and mortality as well as kidney outcomes (Levey 2012). Since albuminuria is an independent risk factor for CVD morbidity and mortality, the classification of CKD has recently been modified by the addition of stages based on albuminuria (Levey 2012).

Lipid-lowering treatment (LLT) with statins reduces cardiovascular morbidity in DM patients without (Collins 2003, Colhoun 2004) or with (Collins 2003) cardiovascular disease, as well as in patients with non-dialysis dependent CKD (Baigent 2011, Palmer 2012). In patients with CKD and no history of MI or coronary revascularization, the SHARP study showed a 17 % reduction of major atherosclerotic events with simvastatin and ezetimibe co-treatment compared to placebo (Baigent 2011). Ezetimibe inhibits intestinal cholesterol absorption and is mainly used concomitantly with a statin to further reduce LDL cholesterol (Kalogirou 2010). Statins seem to have antiinflammatory and antithrombotic effects, which may influence markers of coagulation, inflammation and platelet activation independently of their lipid-lowering effects, i.e., so-called pleiotropic effects (Bonetti 2003). Ezetimibe may also have additional effects on inflammation and coagulation independent of the lipid-lowering effect, but this remains controversial (Kalogirou 2010).

Both DM and CKD are associated with inflammation and have complex disturbances in hemostatic function, leading to a prothrombotic state (Hess 2011, Lutz 2014). The present work concerns the influence of CKD on inflammatory and hemostatic mechanisms in patients with DM and the effects of LLT.

## **1.2 PLATELET FUNCTION, PLATELET-LEUKOCYTE INTERACTIONS AND INFLAMMATION – IMPORTANCE IN ATHEROSCLEROSIS AND THROMBOSIS**

Platelets have a central role in the pathophysiology of atherosclerosis and thrombosis, in interaction with coagulation, endothelial function and inflammation (Gawaz 2005).

### **1.2.1 Platelet physiology**

Platelets are small dynamic anuclear cell fragments formed from megakaryocytes and with a lifespan of approximately 7-10 days. Around 2/3 of the platelets circulate in the blood and the remaining 1/3 are stored in the spleen. In the absence of vessel injury or other stimuli platelets circulate in the blood in a resting discoid shape; they are normally inhibited from

activation by nitric oxide (NO) and prostaglandin I<sub>2</sub> (prostacyclin) released from healthy endothelial cells. The primary function of the platelets is to stop hemorrhage after damage to the vessel wall (Jurk 2005). Platelets contain three different secretory granules:  $\alpha$ -granules, dense granules and lysosomes, in which a large number of proteins with various biological functions are stored.  $\alpha$ -Granules are the most abundant ones and contain proteins such as P-selectin, von Willebrand factor (VWF),  $\beta$ -thromboglobulin, fibrinogen, GPIIb/IIIa, Factor V, Factor X, plasminogen activator inhibitor (PAI-1) and CD40 ligand (CD40L). The dense granules contain adenosine diphosphate (ADP), serotonin and Ca<sup>++</sup>, and lysosomes contain hydrolytic enzymes (Jurk 2005).

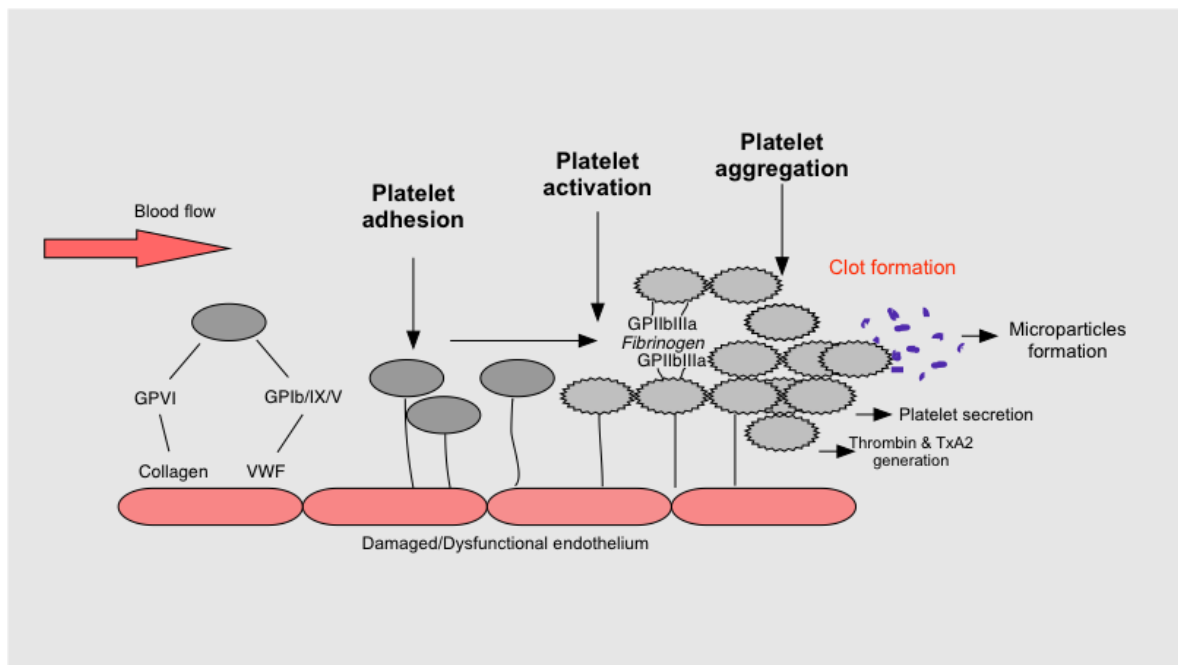
The platelet cytoplasmic membrane consists of a bilayer of polarized phospholipids containing arachidonic acid, which is released and converted to thromboxane A<sub>2</sub> (TxA<sub>2</sub>) via cyclooxygenase-1 (COX-1) during platelet activation. The external layer of the platelet contains numerous glycoproteins (e.g., GPIV, GPIb/V/IX and GPIIb/IIIa). These glycoproteins act as receptors for several ligands and are important for platelet adhesion and activation. Platelets also have other membrane receptors for a number of agonists.

### **1.2.2 Platelet activation**

Adhesion of resting platelets to a damaged vessel wall is the first step of primary hemostasis (Jurk 2005). The adhesion is mainly mediated by interactions between the GPIb/V/IX receptor complex on the platelet surface and VWF, and interactions between GPVI and collagen at the site of endothelial damage. The adhesion leads to a shape change of the platelet from a smooth disc to a small sphere with pseudopods, and then spreading of the platelets on the damaged vessel wall. VWF plays an important role in platelet adhesion as it binds to both collagen and two major platelet receptors (GPIb/V/IX and GPIIb/IIIa) (Davi 2007). VWF supports platelet adhesion predominately during high shear flow.

Upon shape-change and activation of the adhering platelets there is secretion of granule components and activation of the fibrinogen receptor (GPIIb/IIIa) leading to fibrinogen binding and platelet-platelet aggregation. Resting platelets are not able to bind fibrinogen. Local platelet activators, such as ADP, TxA<sub>2</sub>, serotonin and thrombin, help to recruit additional circulating platelet to the primary hemostatic plug. The release of ADP and TxA<sub>2</sub> from activated platelets provides an important positive feed-back to reinforce platelet shape change, activation and aggregation, and secretion of granule contents. Thrombin is the most potent endogenous platelet activator and acts through stimulation of specific thrombin receptors (PAR-1 and PAR-4) on the platelet surface (Davi 2007). Thrombin activates platelets at lower concentrations than those required to activate the coagulation cascade.

The primary platelet plug is rather unstable and needs reinforcing, which is the goal of the secondary hemostasis. Secondary hemostasis involves activation of the coagulation cascade and thrombin-mediated conversion of fibrinogen to fibrin, leading to formation of a fibrin network and consolidation of the blood clot. Fig 1 shows a schematic presentation of platelet activation in the atherosclerotic plaque.



**Figure 1.** Schematic presentation of platelet activation in an atherosclerotic vessel.

Activated platelets have a procoagulant surface, mainly because of increased expression of the anionic phospholipid phosphatidylserine (PS) on the external layer of the platelet membrane, allowing assembly and activation of calcium-binding coagulation factors, as a result of its negative charge. This activation of the coagulation cascade leads to a burst of thrombin generation thus creating a more stable clot (Jurk 2005). Furthermore, platelet binding to monocytes leads to increased tissue factor (TF) expression and thrombin generation. Thus, there are important links between platelets, leukocytes and blood coagulation.

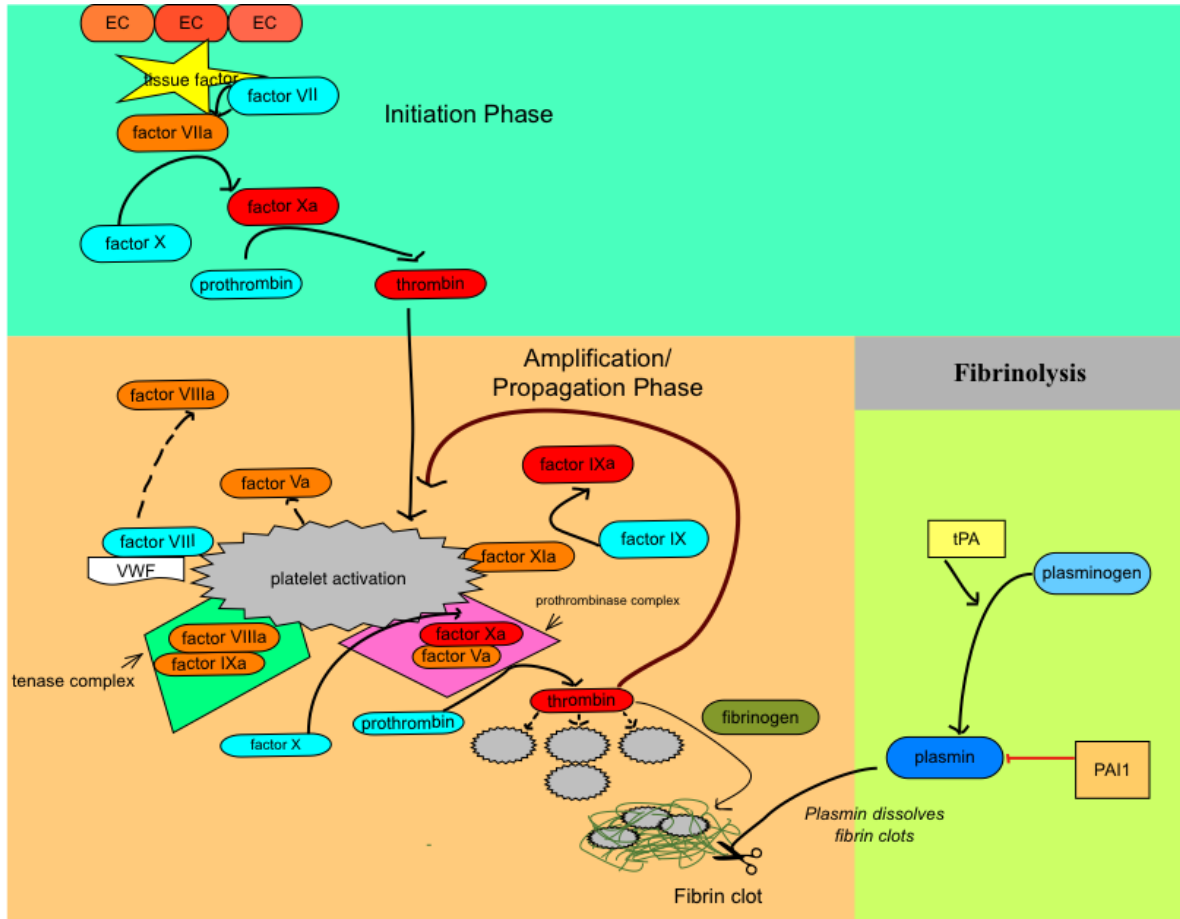


**Figure 2.** Discoid resting platelet (A) and early activated platelet (B).

Reprinted from Platelets (Third edition) 2013 with permission from Elsevier.

### 1.2.3 The coagulation system

The cell-based model of hemostasis (Fig 3) suggests that coagulation consists of three stages, which occur on different cell surfaces: 1) Initiation, which occurs on a TF bearing cell, 2) Amplification, in which platelets and cofactors are activated; and 3) Propagation, in which large amounts of thrombin are generated on the platelet surface (Hoffman 2001).



**Figure 3.** The cell-based model of the hemostasis.

TF (FIII) is essential for blood coagulation (Mackman 2009). At the time of vessel injury, TF is released into plasma by cells in the vessel wall and activates the clotting cascade. Atherosclerotic plaques contain large amounts of TF, which is exposed to the circulation upon plaque rupture. TF may also be expressed by circulating monocytes, endothelial cells and circulating microparticles (MPs) in the presence of inflammation, and TF also contributes to inflammation by various mechanisms such as increased release of proinflammatory cytokines (Mackman 2009). During the initiation phase, when TF comes in contact with plasma it binds to FVII and forms the TF-FVIIa complex which activates FX to FXa and FIX to FIXa. On the cell surface, mainly on platelets, FXa binds to FVa and forms the prothrombinase complex, which converts prothrombin to small amounts of active thrombin (FIIa) by cleavage. Since thrombin is the most potent platelet activator, the small amounts of thrombin that are generated lead to intensified platelet adhesion and activation at the site of injury. The amplification phase occurs on the platelet surface as it becomes activated. During activation platelets release FV in a partially activated form. FV is then fully activated by

thrombin or FXa. Thrombin also induces the release of FVIII from VWF which leads to activation of FVIII to FVIIIa. During the propagation phase, the FVIIIa/IXa complex activates FX on the platelet surface, leading to formation of FXa/FVa complexes which may initiate the large burst of thrombin generation needed for conversion of fibrinogen to fibrin and the subsequent formation of a stable fibrin clot (Hoffman 2001).

The coagulation system is tightly regulated by inhibiting factors. Antithrombin, protein S and Tissue factor pathway inhibitor (TFPI) are three major important inhibitors (Versteeg 2013).

The fibrinolytic system (Fig 3) is designed to resolve the stable fibrin clot and thus prevent fibrin accumulation in the vessels. The most important compounds in the fibrinolytic system are plasmin, tissue plasminogen activator (tPA) and PAI-1. Plasmin is the protease that cleaves fibrin and is activated by tPA from its inactive form plasminogen. PAI-1 is the most important fibrinolysis inhibitor and forms a stable complex with tPA which blocks further tPA-dependent plasmin generation.

#### **1.2.4 Platelet-leukocyte aggregates**

Platelet-leukocyte interactions form a link between coagulation and inflammation (Li 2000, Ghasemzadeh 2013). Platelets and leukocytes may influence each other via direct cellular conjugation (to form platelet-leukocyte aggregates; PLA) and/or via the release of soluble mediators without PLA formation (Li 2000). Platelet binding increases the ability of leukocytes to adhere to and invade the vessel wall, and may thus promote the atherosclerotic process. Platelet-released substances (e.g.,  $\text{TxA}_2$  and ADP) can activate leukocytes and, vice versa, leukocyte-released substances (e.g., platelet activating factor, superoxide anions, and enzymes such as elastase and cathepsin G) can activate platelets. There may also be reciprocal inhibition among these cell types via release of NO. As noted above, platelet binding to monocytes also facilitates thrombin generation. Thus, platelets and leukocytes interact in many important ways, and platelet-leukocyte aggregation seems to be important in atherothrombotic diseases (Li 2000, Cerletti 2012, Ghasemzadeh 2013).

P-selectin is stored in the  $\alpha$ -granules of the platelets and is released upon platelet activation. P-selectin mediates interactions between platelets, leukocytes and endothelial cells and is the most important receptor for platelet-leukocyte conjugation through its ligand P-selectin glycoprotein 1 (PSGL-1). CD40-CD40L interactions also enhance PLA formation (Cerletti 2012). Increased PLA formation has been found in conditions with high cardiovascular risk and increased inflammatory activity, such as acute coronary syndromes (Brambilla 2008), stable coronary artery disease (Furman 1998) and in DM (Hu 2004).

#### **1.2.5 The CD40-CD40L system**

The CD40-CD40L system represents an interesting connection between platelets and inflammation (Antoniades 2009). This system is a key mediator of cell communication in the immune system and may also be involved in the progression of established atherosclerotic lesions to more advanced and unstable lesions. Both CD40L and its soluble form sCD40L

interact with CD40 expressed on macrophages, endothelial cells and vascular smooth muscle cells, resulting in proinflammatory and prothrombotic effects that include increased platelet activation and aggregation, and platelet-leukocyte interactions. Activated platelets are found to be the main source of sCD40L in plasma (Antoniades 2009, Lievens 2010), and elevated plasma levels of sCD40L predict future CV risk (Antoniades 2009). CD40-CD40L interactions seem to promote atherosclerosis and atherothrombosis (Lievens 2010). Patients with DM (Lim 2004) and patients with CKD (Schwabe 1999) have been found to have elevated levels of sCD40L, compared to healthy controls.

### **1.2.6 Microparticles**

Microparticles (MPs) are small (0.1-1.0 µm in diameter) particles found in the blood stream, which are released from cell membranes upon activation and/or apoptosis. MPs are derived from various cell types such as platelets, endothelial cells, leukocytes or erythrocytes, and may contain various cytokines, growth factors and proteases depending on their origin. Interestingly, MPs can display biological activities associated with thrombosis, inflammation and immune responses, and they appear to be both a contributor to, and a consequence of, inflammation (Burger 2013). MPs have procoagulant activity through the expression of negatively charged phospholipids (phosphatidylserine, PS), which facilitates the assembly of coagulation factors and promotes thrombin generation (Burger 2013), and they can also expose TF, the primary initiator of blood coagulation (Owens 2011). Platelet derived MPs (PMPs) are the most abundant type of MP in the circulation, and upon release they can expose several proteins and cytokines such as P-selectin, CD40L and TF.

Elevated plasma levels of platelet, endothelial or leukocyte derived MPs have been found in patients with cardiovascular risk factors and disorders, such as hypertension, DM, acute coronary syndromes and stroke (Burger 2013). CKD has also been associated with elevated levels of MPs (Ando 2002, Amabile 2005, Faure 2006, Amabile 2012).

## **1.3 HEMOSTASIS IN DIABETES MELLITUS**

DM is a prothrombotic state with increased platelet activation, activation of the coagulation system, decreased fibrinolytic capacity and increased inflammatory activity (Ferreiro 2010, Hess 2011). Diabetic patients have hyperreactive platelets with exaggerated adhesion, aggregation and thrombin and TxA<sub>2</sub> generation, higher levels of soluble P-selectin in plasma (Yngen 2004, Ferreiro 2010), and increased platelet-leukocyte cross-talk (Hu 2004).

## **1.4 HEMOSTASIS IN CHRONIC KIDNEY DISEASE – BLEEDING AND CLOTTING**

Patients with CKD have major disturbances in their hemostasis, which result either in a hypercoagulable state with an increased risk of suffering thrombotic complications (myocardial infarction, acute coronary syndromes, cerebrovascular events, and venous thrombosis), or in an increased risk of bleeding (Lutz 2014). The reasons for these disturbances are complex and involve several different components of the coagulation system

such as the coagulation cascade, the fibrinolytic system, the platelets, the endothelium and the vessel wall (Lutz 2014). Factors involved in the procoagulant state in patients with chronic kidney disease are shown in Table 1. The exact protrombotic mechanisms responsible for the increased cardiovascular risk in CKD are, however, still not fully understood.

#### **1.4.1 Platelets in CKD**

The increased risk of bleeding frequently seen in CKD, especially in patients with severe renal impairment, has been associated with altered platelet physiology, leading to platelet dysfunction and impaired platelet-vessel wall interactions with increased risk of cutaneous, mucosal, and serosal bleeding as typical manifestations (Boccardo 2004). Several mechanisms are thought to contribute to platelet dysfunction in severe CKD, such as altered platelet granule contents, altered ADP and serotonin release, impaired function of GPIIb/IIIa, decreased GPIb, defective VWF and decreased binding of VWF and fibrinogen to activated platelets (Boccardo 2004).

During the last 10 years, when the connection between CKD and atherothrombotic disease has been in focus, an increasing number of studies have shown hyperreactive platelets with increased platelet activation and aggregation in patients with CKD (Landray 2004, Thijs 2008, Woo 2011, Gremmel 2013, Yagmur 2013), suggesting that platelet activation may play a role in atherothrombosis in CKD. A majority of these studies has included high-risk patients with known cardiovascular disease. The pathophysiology behind increased platelet activation and aggregation in CKD is, however, not fully known. Increased exposure of PS on the platelet surface, and increased levels of P-selectin and GPIIb/IIIa are possible contributors (Lutz 2014, Bonomini 2004).

#### **1.4.2 The coagulation system in CKD**

Several disturbances in the coagulation system have been found in patients with CKD, such as elevated levels of fibrinogen, TF, PAI-1, VWF and coagulation factors XIIa and VIIa, as well as reduced levels of tPA and antithrombin activity, suggesting a prothrombotic state with impaired fibrinolysis (Jalal 2010, Lutz 2014).

<b>Table 1. Factors involved in the procoagulant state in CKD</b>	
Decreased NO production	Moody 2012 (Review)
Hypoxia	Moody 2012
Shear stress	Moody 2012
Chronic inflammation	Stenvinkel 2002, Landray 2004
Increased VWF	Jalal 2010 (Review)
Increased platelet phosphatidylserine	Lutz 2014 (Review), Bonomini 2004
Increased platelet p-selectin	Jalal 2010 (Review), Lutz 2014
Increased GPIIb/IIIa	Lutz 2014, Jalal 2010
Increased sCD40L	Schwabe 1999
Increased MP formation	Ando 2002, Faure 2006,
Increased TF	Pawlak 2009
Increased factor XIIa	Lutz 2014
Increased factor VIIa	Lutz 2014
Increased fibrinogen	Lutz 2014, Jalal 2010
Increased tPA	Lutz 2014, Jalal 2010
Decreased PAI-1	Lutz 2014, Jalal 2010
Increased activated protein C	Lutz 2014, Jalal 2010
Increased thrombin-antithrombin complexes	Lutz 2014
Increased D-dimer levels	Lutz 2014, Jalal 2010
Decreased antithrombin	Lutz 2014

Plasma fibrinogen is an independent marker for cardiovascular disease (Ariens 2013), and elevated levels of fibrinogen have been shown in patients with CKD (Jalal 2010). In addition, activation of the renin-angiotensin-aldosterone system (RAAS) has been associated with elevated levels of fibrinogen, D-dimer and PAI-1 (Jalal 2010, Lutz 2014). Thus, there seem to be close links between blood coagulation, platelets, endothelial dysfunction and inflammation which lead to the hypercoagulable state in CKD.

## **1.5 THE ENDOTHELIUM IN DM AND CKD**

The endothelium is essential in vascular biology and serves as a barrier that separates blood from the underlying tissue. It regulates a complex balance of factors to maintain vascular hemostasis which involve the regulation of vascular permeability, vessel tone, cell adhesion and blood hemostasis. A balanced production/expression of vasodilating (i.e., NO and prostacyclin) and vasoconstricting substances (i.e., endothelin-1, angiotensin II and TxA<sub>2</sub>) characterizes the healthy, normal endothelium. The endothelium also produces adhesion molecules for platelets (i.e, VWF and P-selectin) and leukocytes (ICAM-1, VCAM-1 and E-selectin), anticoagulants (TFPI), and fibrinolytic factors (tPA, thrombomodulin and protein S) and inhibitors (PAI-1).

At sites of vessel injury or during long exposure to risk factors such as chronic inflammation, hypertension, hyperlipidemia or hyperglycemia, the properties of the endothelium change from a vasodilatory, anticoagulant state, towards a vasoconstrictive, proinflammatory, procoagulant state. This endothelial dysfunction (or activation) leads to increased adhesiveness of leukocytes and platelets, increased permeability and endothelial production of cytokines, growth factors and vasoactive molecules (Ross 1999). Endothelial dysfunction plays an important role in the development of atherosclerosis and correlates with cardiovascular diseases (Ross 1999). Circulating endothelial MPs shed from endothelial cells upon activation are emerging as an interesting marker of endothelial function.

Disturbed endothelial function is very common in both DM and CKD (Moody 2012, Roberts 2013), and is considered to be a major contributor to both micro- and macrovascular complications in DM (Roberts 2013). Endothelial dysfunction caused by chronic inflammation and oxidative stress is thought to be an early and important feature in CKD, and is considered to be one of the major pathophysiological mechanisms connecting CKD and CVD (Moody 2012). However, this relationship has not been fully proven in clinical studies, especially not in early CKD stages, and data is confounded by coexisting diseases such as DM and hypertension, which are independently associated with endothelial dysfunction (Moody 2012). However, elevated levels of biomarkers for endothelial dysfunction such as VCAM-1, VWF, thrombomoduline, tPA and PAI-1 have been associated with CKD and diabetic nephropathy (Jalal 2010, Navarro-Gonzalez 2011, Moody 2012).

## **1.6 DYSLIPIDEMIA IN DM AND CKD**

Both DM and CKD are associated with hypertriglyceridemia, lower HDL-cholesterol, higher VLDL-cholesterol, normal levels of LDL-cholesterol, but elevated levels of small dense LDL particles (Siegel 1996, Tsimihodimos 2008).

## **1.7 INFLAMMATION IN DM AND CKD**

DM and CKD are both clearly associated increased inflammatory activity (Schmidt 1999, Stenvinkel 2002, Landray 2004). Inflammation is a major pathogenic mechanism in the development of diabetic nephropathy involving increased chemokine production (i.e, MCP-

1), increased proinflammatory cytokine production (i.e., interleukin (IL)-1, IL6, IL 18, TNF $\alpha$ ), increased levels of adhesion molecules (i.e, VCAM-1, ICAM-1), infiltration of inflammatory cells in the kidney, and tissue damage (Navarro-Gonzalez 2011).

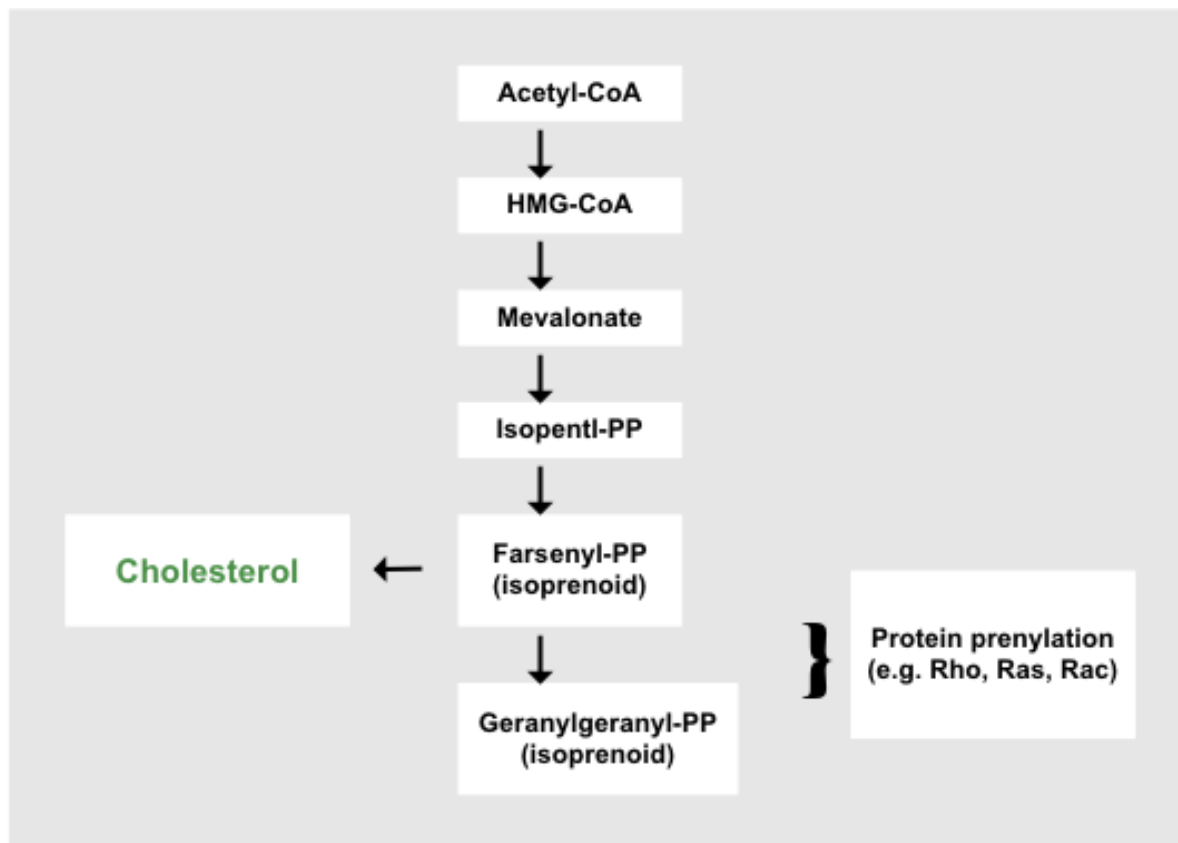
## **1.8 INFLAMMATION IN ATHEROSCLEROSIS AND THROMBOSIS**

Inflammation is closely involved in the pathogenesis of atherosclerosis (Ross 1999). In the initiation phase, infiltration and accumulation of modified LDL particles activates the endothelium and starts an inflammatory process in the artery wall with expression of leukocyte adhesion molecules, in response to pro-inflammatory cytokines. The modified LDL particles are taken up by scavenger receptors on macrophages, which then evolve into foam cells. After adhesion to the vascular endothelium, chemokines stimulate leukocyte migration into the subendothelial site of inflammation. The chemokine monocyte chemoattractant protein-1 (MCP-1) is important for the infiltration of monocytes into early atherosclerotic lesions. The activated immune cells then produce proinflammatory cytokines, which synergistically amplify the inflammatory response and tissue damage (Hansson 2005).

Under normal physiological conditions, platelets do not adhere to the vascular endothelium, but under inflammatory conditions they can adhere to the activated but intact endothelium (Gawaz 2005). After platelet adhesion to the endothelium, platelets secrete proinflammatory cytokines stored in the granules, such as interleukin I $\beta$  and CD40L, adhesion molecules and chemokines, leading to endothelial inflammation (Henn 1998). Adherent platelets then recruit circulating leukocytes, activate them and thereby initiate leukocyte transmigration through the vessel wall with ensuing foam cell formation (Gawaz 2005). Thus, platelets contribute to the inflammatory milieu that promotes atherosclerotic plaque formation.

## **1.9 ANTIINFLAMMATORY AND ANTICOAGULANT EFFECTS OF LIPID-LOWERING TREATMENT**

The question of possible additional effects of statins beyond their lipid-lowering effect, i.e., pleiotropic effects, has been intensely debated. Statins are suggested to have favourable effects on platelet adhesion, thrombosis, endothelial function, inflammation and plaque stability, independently of their cholesterol lowering effect (Bonetti 2003). Statins block the conversion of HMG-CoA to mevalonic acid, leading to reduced synthesis of cholesterol (Fig 4). Many of the pleiotropic effects observed in various studies have been shown to be related to inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway (Bonetti 2003), which may, e.g., influence cellular proliferation.



**Figure 4.** The mevalonate metabolism pathway

Statins have also been found to exert antithrombotic effects through reduced monocyte TF expression (Owens 2014) and reduced thrombin generation (Undas 2005). One of the best documented non-lipid effects of statins is improvement of parameters associated with endothelial function (Bonetti 2003). This is likely achieved by both enhancement of vasodilator and attenuation of vasoconstrictor activity in the vessel wall.

Ezetimibe may also have additional, pleiotropic effects on inflammation and coagulation independently of the lipid-lowering effect, but this remains controversial (Kalogirou 2010).

## 2 AIMS OF THE PROJECT

The overall aim of this project was to contribute to the improvement of cardiovascular risk reduction management in patients with DM and CKD.

### **Specific aims of this work were:**

- To evaluate the prognostic importance of impaired renal function in patients with stable angina pectoris (Paper I).
- To study the impact of CKD on platelet function and platelet-leukocyte interactions, inflammatory parameters and circulating microparticles in patients with DM (Papers II-IV).
- To study effects of lipid-lowering treatment with simvastatin alone or with the combination of simvastatin+ezetimibe in patients with diabetes DM with and without CKD with regard to:
  - platelet function and platelet-leukocyte aggregation, and inflammatory parameters (Paper II).
  - proinflammatory cytokines, chemokines and adhesion molecules (Paper III).
  - circulating microparticles (Paper IV).



## 3 PATIENTS & METHODS

### 3.1 STUDY DESIGN AND POPULATION

#### 3.1.1 Paper I

This was a *post hoc* analysis from the Angina Prognosis Study in Stockholm (APSIS) with the purpose to evaluate the prognostic implications of renal dysfunction in patients with stable angina pectoris. The APSIS was a prospective, randomized, single center trial investigating the prognosis of 809 patients (248 females) with stable angina pectoris treated double-blindly with metoprolol or verapamil (Rehnqvist 1996). Patients aged < 70 years with clinically diagnosed angina pectoris were included and followed at the Heart Research Laboratory at Danderyd Hospital during 1987-1993; the mean follow-up was 40 months. Exclusion criteria included myocardial infarction (MI) within the last 3 years ( $\beta$ -blocker treatment was considered to be indicated among such patients), anticipated need for revascularisation within 1 month, significant valvular disease, severe congestive heart failure or risk for poor compliance. The primary end-point was CV death or nonfatal MI.

At baseline, serum creatinine (SCr) was measured and the estimated creatinine clearance (eCrCl) was calculated by the Cockcroft-Gault formula in 808 of the 809 patients. Outcomes were compared for three groups according to their renal function: eCrCl >90 ml/min, eCrCl 60-89 ml/min and eCrCl 30-59 ml/min.

#### 3.1.2 Papers II-IV

The study had a randomized, double blind, cross-over design with an initial single-blind 6-week wash-out period with placebo followed by two 8-10 weeks double blind periods of LLT. Investigations were performed at baseline and after each treatment period (Fig 5). The target dose for simvastatin was 40 mg daily. If necessary, patients could start with a lower dose. Dose titration was completed after within 4-6 weeks. The ezetimibe dose was 10 mg daily. To minimize confounding by variable co-treatment, all patients received 75 mg aspirin (Trombyl®) daily, and patients not on an ACE-inhibitor or an angiotensin II receptor blocker (ARB) also received enalapril 5-10 mg daily during the entire study.

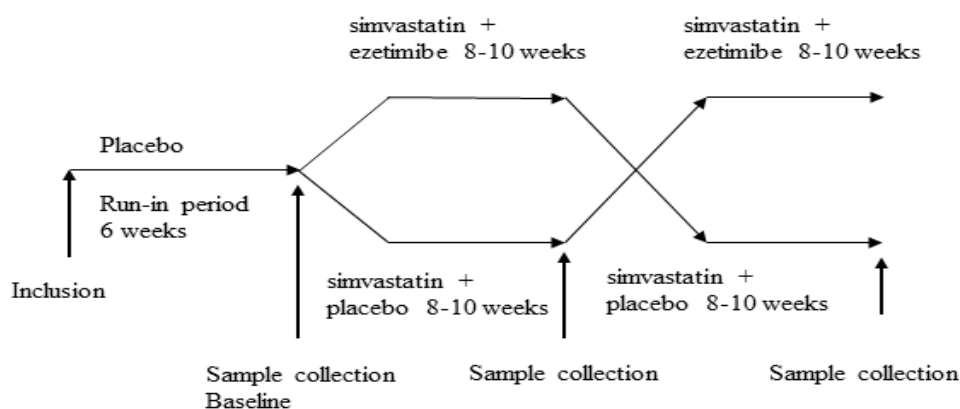


Figure 5. Study design papers II-IV

The MDRD formula (Levey 1999) was used to calculate the eGFR. A total of 39 DM patients were included; 18 patients with an eGFR of 15-59 ml/min x 1.73m<sup>2</sup>, i.e., CDK stages 3-4, (DM-CKD group) and 21 patients with eGFR >75 ml/min x 1.73 m<sup>2</sup> (DM-only group). Patients were recruited from the Department of Nephrology and the Diabetology Unit, Danderyd Hospital, and from the Diabetology Unit, South Hospital, Stockholm, Sweden. Major exclusion criteria were previous MI, coronary revascularisation, or stroke, or poor metabolic control (HbA1c >83mmol/mol). The patient groups were matched for age and sex.

### **3.2 LABORATORY INVESTIGATIONS**

#### **3.2.1 Blood sampling**

- *Paper I*

Venous blood was collected between 8 and 10 am, after an overnight fast.

- *Papers II-IV*

The patients were asked to have a light breakfast and to abstain from alcohol, tobacco and caffeine on days of sampling. Antecubital venous blood was collected in citrate, EDTA and heparin tubes by the vacutainer technique after 30 min of semi-recumbent rest. Plasma was collected after centrifugation at 1400 x g for 10 min at 4° C (for EDTA tubes after an additional centrifugation at 1400 x g for 15 min at 4°) and frozen at -80°C until analysis.

#### **3.2.2 Assessment of platelet function (*Paper II*)**

Unstimulated and agonist-induced platelet P-selectin expression and PAC-1 (activated fibrinogen receptor antibody) binding were determined by whole blood flow cytometry (Li 1999). Within 5 min of collection, aliquots of 5 µl of citrated whole blood were added to 45 µl of Hepes buffered saline containing appropriately diluted antibodies and agonists and incubated at room temperature for 20 min. Samples were fixed and diluted with 0.5% formaldehyde in saline before analysis using a Coulter EPICS XL-MCL flow cytometer. Platelets were identified by their light scattering signal and by staining with FITC-conjugated anti CD42a (GPIX) MAb (Becton Dickinson). Platelet activation was determined using RPE-conjugated anti-P-selectin MabAC1.2 (Becton Dickinson) and FITC-conjugated PAC-1 binding (Becton Dickinson). Platelet agonists were ADP (0.1-10 µM), a collagen related peptide (CRP-18/I; 0.025-10 µg/mL) which activates platelet GPVI, and human α-thrombin (0.01-0.08 U/mL). In thrombin-stimulated samples, GPRP (Gly-Pro-Arg-Pro peptide, Sigma, St Louis, MO, USA) at a final concentration of 0.8 mM was added to prevent clotting. Results are presented as percentages of platelets expressing P-selectin or binding PAC-1 in dose-response curves induced by ADP, thrombin and CRP-18/i (a collagen-related peptide stimulating GPVI).

### 3.2.3 Platelet-leukocyte aggregation (*Paper II*)

PLAs were analysed using whole blood flow cytometric methodology causing little or no artificial generation of PLAs in vitro, thus reflecting circulating PLAs (Li 1999). Leukocytes were identified by anti CD45-PE (Beckman Coulter), and further discriminated by their side scattering characteristics into lymphocytes, monocytes and neutrophils. Monocytes were also identified by anti CD14-PC5 (Beckman Coulter). Total leukocytes and different subgroups were then subjected to two-colour analysis (RPE-CD45 versus FITC-GPIX (CD42a)) to discriminate platelet-coupled and platelet-free leukocytes. Data are presented as percentages of platelet-conjugated leukocytes in the total leukocyte population (PLA), and among neutrophils (P-Neu), monocytes (P-Mon) and lymphocytes (P-Lym) without and with ex vivo stimulation by ADP (1  $\mu$ M), CRP-18/I (1  $\mu$ g/mL) and thrombin (0.04 U/mL).

### 3.2.4 Microparticles analyses (*Paper IV*)

Platelet poor plasma samples (double centrifuged EDTA tubes) that had been stored at -80°C were thawed and centrifuged at 2000 x g for 20 min at room temperature. The supernatant was then re-centrifuged at 13 000 x g for 2 min at room temperature. 20  $\mu$ l from the supernatant were then stained and prepared for flow cytometric analysis. MPs were measured by a Beckman Gallios flow cytometer (Beckman Coulter, Brea, CA, USA). The MP gate was determined using Megamix beads (BioCytex, Marseille, France). MPs were initially defined as particles less than 1.0  $\mu$ m in size and negative to phalloidin in order to exclude cell membrane fragments (Mobarrez 2010). All MPs, regardless of cellular origin were defined as phosphatidylserine positive (PS+) MPs. Platelet-MPs (PMPs) were defined as PS+CD41+, endothelial-MPs (EMPs) as PS+CD62E+ (E-selectin positive) or PS+CD144+, and monocyte-MPs (MMPs) as PS+CD14+. Further phenotyping included measurements of TF (CD142) on PMPs, EMPs and MMPs and CD40L (CD154) on PMPs. Due to incompatibility of flow cytometric dyes, lactadherin could not be used when TF (CD142) expression was measured (both are only available as FITC labeled). Thus, the TF positive MPs were defined by size (<1.0  $\mu$ m) and co-expression of CD142 together with CD41, CD62E or CD14. The absolute numbers of MPs were calculated by the following formula: (MPs counted x standard beads/L)/standard beads counted (FlowCount, Beckman Coulter). Data are expressed as 10<sup>6</sup> MPs/L.

### 3.2.5 Biochemical analyses

#### *Paper I*

SCr was analysed by the Department of Clinical Chemistry, Danderyd Hospital, using an automated Jaffe method.

#### Papers II-IV

HbA1c, creatine kinase (CK), creatinine, potassium, urea nitrogen, cystatin-C, ALAT, total, LDL and HDL cholesterol, triglycerides, blood glucose, C-reactive protein (hsCRP; high sensitivity assay), hemoglobin, platelet counts, leukocyte differential counts and the urinary

albumin/creatinine ratio (ACR) were assessed by standard methods (Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden). Enzyme immunoassays were used to determine plasma sCD40L (R&D Systems), VWF antigen (Asserachrom, Diagnostica Stago, France), elastase (DPC Biermann GmbH, Bad Nauheim, Germany) and soluble vascular cell adhesion molecule-1 (sVCAM-1) (R&D Systems) levels. Interferon- $\gamma$  (IFN $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and monocyte chemoattractant protein-1 (MCP-1) were analyzed using a high throughput automated biochip immunoassay system (EvidenceH with the Evidence Investigator TM equipment, Randox Laboratories Ltd, Crumlin, UK).

### **3.3 STATISTICAL ANALYSES**

#### *Paper I*

Data are presented as mean values  $\pm$  SD or percentages. Statistical comparisons were performed by nonparametric tests (Mann-Whitney U-test, chi-square test). Associations between measured variables and events were investigated by univariate proportional hazard (Cox) analyses, and Kaplan-Meier plots with chi-square tests and log rank statistics. In a second step, variables that showed some relationship to events were further evaluated using a multivariate Cox proportional hazard model including adjustments for known risk factors (sex, age, previous MI, hypertension and DM). Analyses were performed according to the principle of intention-to-treat.

#### *Papers II-IV*

Data are presented as mean  $\pm$  SD (SEM in the figures for increased clarity), or median values and inter-quartile ranges for skewed variables. Multiple comparisons of continuous data were performed by repeated measures ANOVA, after validation for normal distribution by use of the Shapiro Wilk test, and with Fisher's post hoc test to control for multiplicity. Skewed data were logarithmically transformed before the analysis. Student's t-test or Mann-Whitney's U-test, as appropriate, were used to evaluate differences between two independent groups. Correlations between variables were assessed by linear regression analyses and calculations of the Pearson correlation coefficient. Variables in contingency tables were tested by the chi-square test.

Analyses were performed using the SAS system for Windows 9.2 (SAS Institute Inc., Cary, NC, USA) or STATISTICA software (Statsoft, Tulsa, OK, USA). A p-value of  $<0.05$  was considered to be statistically significant.

## 4 RESULTS & DISCUSSION

### 4.1 PAPER I

#### Estimated CrCl <60 ml/min was common in patients with stable angina

In the present study population of 808 patients with clinically diagnosed stable angina pectoris who were treated with metoprolol or verapamil, the mean eCrCl was  $78 \pm 21$  ml/min ( $68 \pm 17$  for women and  $82 \pm 21$  for men) and the mean sCr was  $97 \pm 18$   $\mu$ mol/L at baseline. Their mean age was  $59.4 \pm 7.4$  years. 164 patients (91 women; 20% of the total study population) had an eCrCl below 60 ml/min and the lowest eCrCl was 34 ml/min, i.e., CKD stage 3 according to current guidelines.

Patients with low eCrCl were older, and more likely to be women and non-smokers. The prevalence of previous MI, hypertension and DM was similar in the three renal function groups. The age of male and female patients in the lowest eCrCl group did not differ ( $65 \pm 4$  and  $64 \pm 5$  years respectively). Baseline characteristics of the patients in the three renal function groups are shown in Table 1, Paper I (Appendices).

#### Prognostic implications of eCrCl regarding CV death and MI

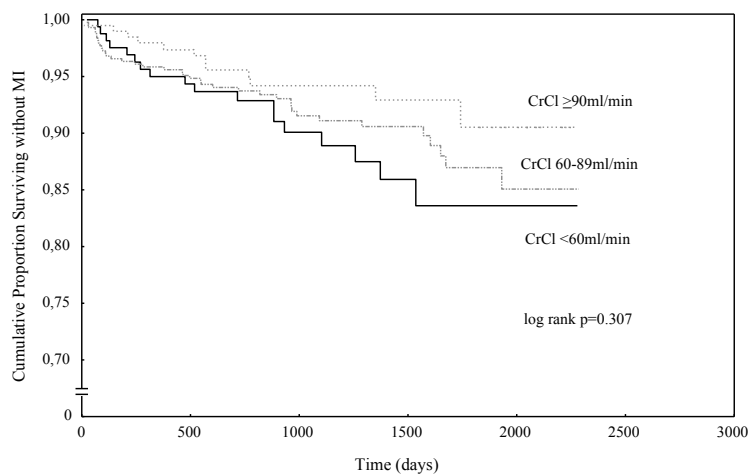
During follow up, 38 patients suffered CV death and 31 patients a nonfatal MI. In the univariate analysis a low eCrCl was related to a higher risk for the combined end-point of CV death and MI for men ( $p=0.036$ ), but not for all patients ( $p=0.307$ ). There was a graded inverse relationship between eCrCl and CV-death or MI among men (Fig 6). No separate analysis of female patients was performed, since only 10 women suffered CV death or MI. Univariate analyses of the relationship between eCrCl and outcomes for all patients were confounded by the higher prevalence of low eCrCl among women, who had a better prognosis in the APSIS study. When analysed with a multivariate Cox-model including known risk factors such as sex, age, previous MI, hypertension and DM, we found an independent prognostic importance of eCrCl for both CV death ( $p=0.046$ ) and the combined endpoint CV death and nonfatal MI ( $p=0.042$ ) for all patients (Table 2).

**Table 2.** Results of the Cox proportional hazard analysis. The following co-variables were used: age, sex, previous MI, hypertension and diabetes mellitus. Results are presented for subgroup comparisons, and for eCrCl as a continuous variable.

	CV death + nonfatal MI			Cv death		
	HR	95% CI of HR	p-value	HR	95% CI of HR	p-value
Continuous variable analysis						
eCrCl (1ml/min diff)	0.984	0.969-0.999	0.042	0.979	0.959-1.000	0.046
Renal function subgroups						
eCrCl $\geq 90$ ml/min	1.000			1.000		
eCrCl 60-89 ml/min	1.529	0.768-3.045	0.227	2.011	0.769-5.257	0.155
eCrCl <60 ml/min	1.986	0.865-4.557	0.106	2.824	0.898-8.883	0.076

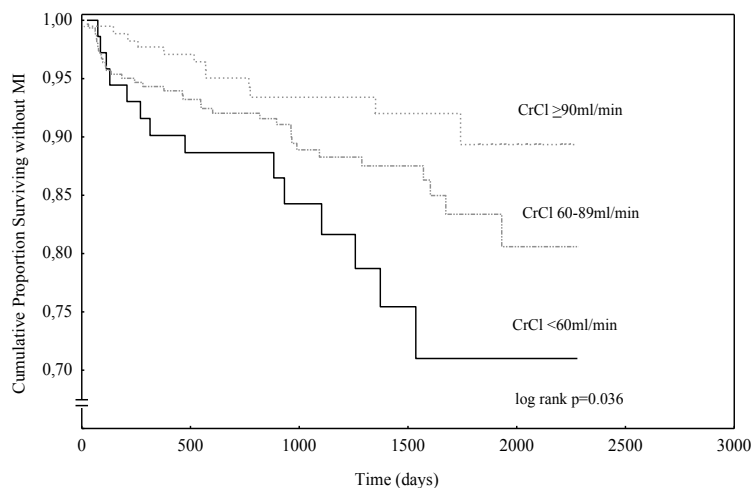
eCrCl, estimated creatinine clearance; CV, cardiovascular; MI, myocardial infarction; CI, confidence interval; HR, hazard ratio

A



**Figure 6.** Kaplan-Meier plots for the cumulative proportion of all patients without CV death or MI (A) and for the cumulative proportion of male patients without CV death or MI (B).

B



When analysed as a continuous variable, a 1 ml/min decrease in eCrCl was associated with a 1.6 (0.1 to 3.1) % increase in the risk of suffering CV death or nonfatal MI, and a 2.1 (0 to 4.1) % increase in the risk for CV death alone. Patients with eCrCl <60 ml/min had a doubling of the risk of suffering CV death or nonfatal MI, compared to patients with eCrCl  $\geq$  90 ml/min. For CV death alone the relationship was steeper with a three-fold increase in risk among patients with the lowest compared to the highest eCrCl (Table 2).

The randomized study treatment (metoprolol vs. verapamil) did not confound the relationships between renal function and CV events (see Paper I for further details).

## Discussion

This study showed that impaired renal function carried significant prognostic information regarding CV death and non-fatal MI in patients with stable angina pectoris. This is in agreement with previous findings in patients with other categories of coronary artery disease (Al Suwaidi 2002, Best 2002, Anavekar 2004, Gibson 2004), as well as in the normal population (Go 2004, Weiner 2004), but had not been shown before in patients with stable

angina. Impaired renal function, as estimated by GFR or CrCl is also associated with an increased risk in other cardiovascular diseases such as atrial fibrillation (Alonso 2011) and heart failure (Kottgen 2007). The prognostic impact of impaired renal function was graded, in agreement with findings of an exponentially increasing risk for CVD with declining renal function (van der Velde 2011).

Twenty % of the patients in our study had an eCrCl <60 ml/min. In the normal population, the prevalence of moderately impaired renal function (eGFR 30-59 ml/min/1.73m<sup>2</sup>) has been estimated to be between 3.2 to 5.6 % (McCullough 2012). Impaired renal function was thus more common among the APSIS patients, and especially among women, as 37 % of them had an eCrCl <60 ml/min, compared to 13 % of the male patients. An increased prevalence of renal dysfunction has also been found among women in other studies of CV risk (Al Suwaidi 2002, Best 2002, Anavekar 2004, Go 2004, Weiner 2004), and has usually been related to the age difference between men and women in populations with MI and acute coronary syndromes. However, there was no age difference between men and women in our study, and no difference in renal function between healthy men and women has been found when measuring GFR directly by Cr-EDTA clearance (Granerus 1981, Hamilton 2000). There is a correction factor in the Cockcroft-Gault formula for gender-related differences, due to the lower muscle mass and creatinine production among women, and also in other creatinine based formulae commonly used for estimating GFR, such as the Modification of Diet in Renal Disease (MDRD) formula (Levey 1999). However, there has been no large evaluation of possible differences between men and women in the estimation of GFR by creatinine based formulae. Whether the equations are less precise in women needs to be further investigated.

In the APSIS study in patients with stable angina pectoris, the long-term CV prognosis was favourable, as the annual incidence of CV death was 1.2 % and that of non-fatal MI was 1.0 % during a median follow-up of 40 months (Rehmqvist 1996). A long term follow-up after the study verified this favourable prognosis (Hjemdahl 2005), and throughout the 9 years of observation women had a better prognosis than men, with similar mortality rates as matched female reference individuals.

There is a 10-20 year delay of the onset of coronary artery disease in women compared to men (Kannel 1995), and in the APSIS study men had a significantly higher prevalence of previous MI or revascularisation than women at inclusion, at the same mean age (59±7 years). However, among the elderly, CVD is the leading cause of death both in women and men (Kannel 1995). The rather low mean age in the APSIS study with no age difference between men and women might therefore, to some extent, have contributed to the better prognosis among women. Interestingly, the small subgroup of female patients with DM had a poor prognosis in the long-term follow up of the APSIS study (Hjemdahl 2005). DM is a strong cardiovascular risk factor with accelerated progression of atherosclerosis, and several studies report that the gender difference in CV risk is lost in the presence of DM, and that

DM increases the CV risk more markedly in women than in men (Kannel 1979, Lee 2000, Juutilainen 2004).

The higher prevalence of impaired renal function among women was a confounder in the univariate analyses, because of their better prognosis, and the association between eCrCl and the risk for CV death or MI was strengthened by multivariate analysis. In a population with older patients, the association between eCrCl and CV risk would probably have been stronger due to the higher risk among women. In the HERS study, moderate renal insufficiency was associated with an increased risk of suffering CV events among postmenopausal women with documented coronary artery disease (Shlipak 2001).

## 4.2 PAPER II

Baseline patient characteristics of the 18 patients in the DM-CKD group and the 21 patients in the DM-only group are presented in Table 3. In the DM-CKD group, five patients had CKD stage 4 and 13 patients had CKD stage 3. The mean dose of simvastatin was 34±9 mg daily in DM only and 30±10mg daily in DM-CKD patients.

**Table 3.** Patients characteristics and biochemical analyses at baseline (after the placebo treatment).

Variable	DM only group (n=21)	DM-CKD group (n=18)	p
Sex (male/female)	13/8	10/8	0.69
Age (years)	64 ±7	67±6	0.16
Smoking (yes/no)	4/17	1/17	0.21
DM type (type 2/type 1)	13/8	10/8	0.69
Hypertension (yes/no)	18/3	17/1	0.37
Plasma creatinine (µmol/L)	73±13	148±65	<0.01
eGFR (ml/min x 1.73m <sup>2</sup> ) <sup>a</sup>	87±11	42±13	<0.001
HbA1c (mmol/mol) <sup>b</sup>	57±3	59±1	0.99
Systolic BP (mmHg)	131±13	143±19	0.03
Diastolic BP (mmHg)	72±9	70±11	0.54
Total cholesterol (mmol/L)	5.6±1.0	6.0±1.0	0.30
HDL-cholesterol (mmol/L)	1.3±0.4	1.3±0.4	0.75
LDL-cholesterol (mmol/L)	3.5±0.8	3.8±1.0	0.31
Triglycerides (mmol/L)	1.9±1.4	1.9±1.3	0.92
hsCRP (mg/L) <sup>c</sup>	1.1 (0.7;3.6)	1.9 (1.3;4.7)	0.68
Urinary albumin-creatinine ratio (mg/mmol) <sup>c</sup>	0.8 (0.5;2.1)	10.2 (0.7;32.1)	0.01
Hemoglobin (g/L)	137±12	126±14	<0.01
Leukocyte counts (x10 <sup>9</sup> /L)	6.5±1.7	6.3±2.1	0.81
Platelet counts (x10 <sup>9</sup> /L)	264±68	245±40	0.32

Data are means ± SD and p-values from Student's t-test for independent samples.

<sup>a</sup>eGFR estimated with the MDRD formula. <sup>b</sup>HbA1c = international IFCC (mmol/mol).

<sup>c</sup>Median values (25<sup>th</sup> and 75<sup>th</sup> percentiles) and p-values by the Mann-Whitney *U*-test.

## Influence of CKD on platelet function, platelet-leukocyte aggregation (PLA) and inflammatory parameters in DM patients

At baseline DM-CKD patients had elevated levels of PLAs and sCD40L (Fig 8), as well as of plasma elastase ( $39.2 \pm 8.9$  vs  $31.5 \pm 8.9$  ng/ml;  $p=0.01$ ) and VWF ( $1.4 \pm 0.4$  vs  $1.0 \pm 0.4$  U/ml;  $p<0.01$ ), compared to DM-only patients: This indicates increased inflammatory activity, leukocyte activation and endothelial dysfunction among the DM-CKD patients.

However, there were no significant group differences regarding platelet P-selectin expression (reflecting platelet secretion) or PAC-1 binding (reflecting fibrinogen binding and aggregability) in either resting or ADP-, thrombin-, or CRP-18/I stimulated samples (see Fig 2, Paper II, Appendices).

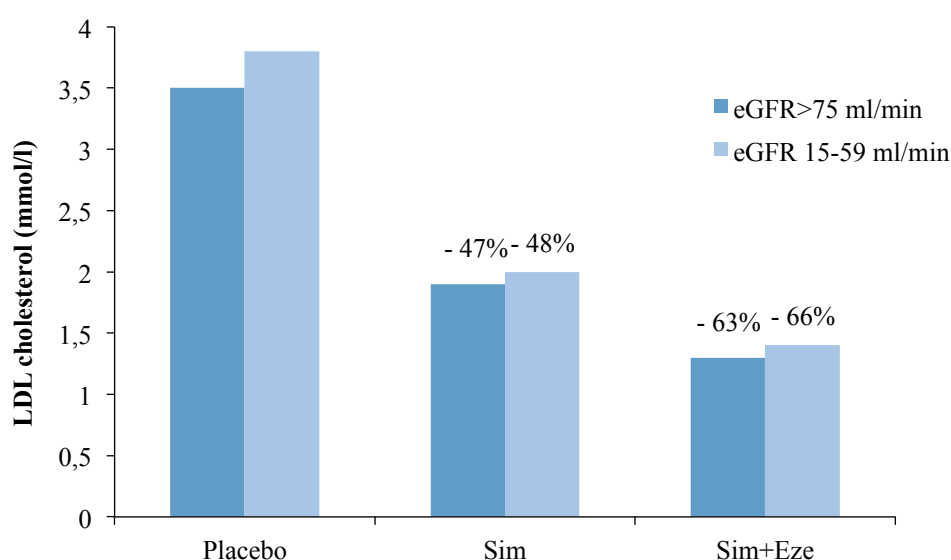
### Correlations

PLAs were positively correlated with sCD40L ( $r=0.33$ ,  $p=0.04$ ) and VWF ( $r=0.34$ ,  $p=0.03$ ) and sCD40L was positively correlated with elastase ( $r=0.39$ ,  $p=0.01$ ) at baseline among all patients. CD40-CD40L interactions have previously been found to increase platelet-leukocyte aggregation and leukocyte activation (Lievens 2010) and elevated plasma elastase in DM-CKD patients might indicate leukocyte activation at least in part induced by CD40-CD40L interactions.

PLAs ( $r=-0.46$ ,  $p<0.01$ ), sCD40L ( $r=-0.42$ ,  $p<0.01$ ) and plasma elastase ( $r=-0.45$ ,  $p<0.01$ ) were all inversely correlated to eGFR at baseline.

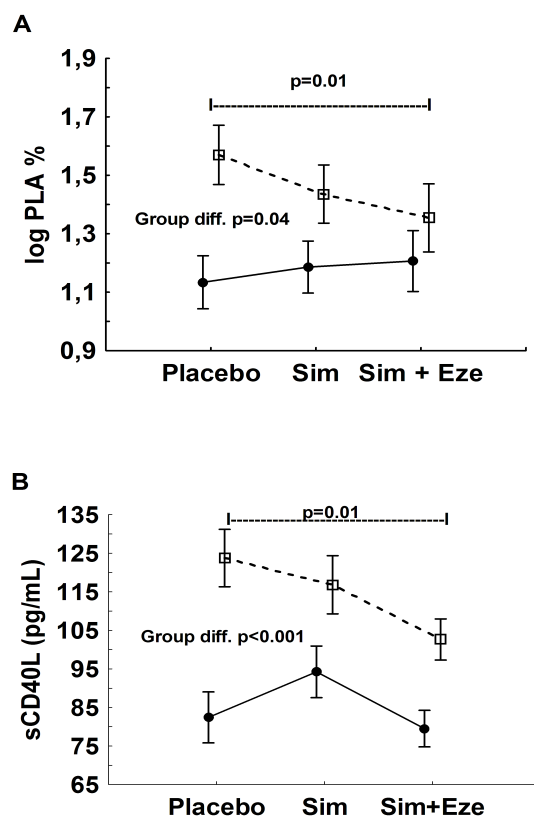
### Lipid-lowering treatment reduced PLAs and sCD40L levels in DM-CKD

Lipid levels did not differ between the two patient groups either before or during LLT (Fig 7) (for details see Table 2, Paper IV, Appendices).



**Figure 7.** LDL-cholesterol levels at baseline and after lipid-lowering treatment

Combined LLT with simvastatin and ezetimibe significantly reduced PLAs and sCD40L levels (Fig 8) as well as P-Neus ( $p=0.03$  by ANOVA; *post hoc* test  $p=0.01$ ) among DM-CKD patients. The combination treatment significantly reduced LDL cholesterol by 63% in DM-only and 66% in DM-CKD patients ( $p<0.001$  for both). Simvastatin alone, which lowered LDL cholesterol by 47% in DM-only and by 48% in DM-CKD patients ( $p<0.001$  for both), reduced P-Neus in DM-CKD patients ( $p=0.03$  by ANOVA; *post hoc* test  $p=0.02$ ), but only tended to reduce total PLAs and sCD40L. In DM-only patients there were no significant effects of either LLT regimen on PLAs or sCD40L. No carry-over effect between treatment periods was found for any variable, and HbA1c levels were not affected by LLT in either patient group (data not shown).



**Figure 8.** Platelet-leukocyte aggregates (PLA%) in the total leukocyte population in unstimulated samples (panel A) and plasma levels of sCD40L (panel B) in the DM-CKD (□ dotted line) and DM only (• solid line) groups.

Measurements were at baseline (placebo) and during treatment with simvastatin (Sim), and simvastatin+ezetimibe (Sim+eze). P-values are *post hoc* test in the ANOVA. Group differences by repeated measures ANOVA are shown in the figure. Data are means  $\pm$  SEM.

## Discussion

CKD and/or the presence of albuminuria are associated with increased inflammatory activity and endothelial dysfunction, which may explain the elevations of PLAs, sCD40L, VWF and plasma elastase in the DM-CKD group. Elevated levels of sCD40L and VWF have previously been found in patients with CKD, compared to healthy controls (Schwabe 1999)

as well as in diabetic nephropathy compared to DM patients with normoalbuminuria (Lajer 2010).

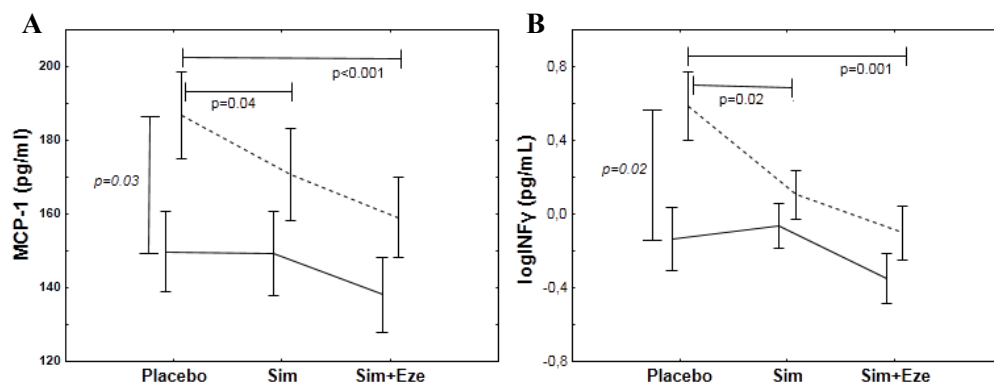
Intensive LLT with simvastatin and ezetimibe reduced PLA formation and sCD40L in the DM-CKD group whereas simvastatin alone only tended to reduce these parameters. Both platelet-leukocyte interactions and the CD40-CD40L system form links between platelets and inflammation. Activated platelets are the main source of sCD40L in plasma (Antoniades 2009), and CD40-CD40L interactions promote PLA formation (Lievens 2010). Since there were no other signs of reduced platelet activation (i.e., P-selectin expression or PAC-1 binding), it might be suggested that the reduced levels of sCD40L during combination treatment were involved in the reduction of PLA formation among our DM-CKD patients. Interestingly, neither sCD40L nor PLAs decreased with increasing LLT intensity in the DM-only group.

### 4.3 PAPER III

In this extended analysis of study II we found elevated levels of IFN $\gamma$ , TNF $\alpha$ , MCP-1 and sVCAM-1 among DM-CKD compared to DM-only patients (see Table 2, Paper III, Appendices). These parameters were all inversely correlated to eGFR ( $r=-0.53$ ,  $r=-0.53$ ,  $r=-0.43$ ,  $r=-0.64$ , respectively;  $p<0.01$  for all).

#### Effects of lipid-lowering treatment

In DM-CKD patients, simvastatin alone reduced the plasma levels of MCP-1 and IFN $\gamma$ , and combined LLT with simvastatin and ezetimibe decreased them further (Fig 9). In the DM-only group, there were no significant effects of either LLT regimen on MCP-1 or IFN $\gamma$ . Differences between the groups at baseline thus disappeared during treatment. Combined LLT reduced sVCAM-1 levels overall, with a significant effect in the DM-only group, and the difference between the groups thus remained during LLT ( $p=0.81$  for interaction term in the ANOVA).



**Figure 9.** Plasma levels of MCP-1 (A) and IFN $\gamma$  (B) in the DM-CKD group (dotted line) and in DM-only groups (solid line). Measurements were at baseline (placebo) and during treatment with simvastatin (Sim), and simvastatin+ezetimibe (Sim+Eze). *P*-values are group differences at baseline (Student's *t*-test). *P*-values are post hoc test in the ANOVA. Data are means  $\pm$  SEM.

## Discussion

Since CKD and diabetic nephropathy are associated with increased inflammatory activity, the elevation of inflammatory parameters in our DM-CKD patients was not a surprising finding, as discussed above. IFN $\gamma$  and TNF $\alpha$  are powerful proinflammatory cytokines with key roles in the development and progression of atherosclerosis (McLaren 2009, Ait-Oufella 2011), and the chemokine MCP-1 is important for the infiltration of monocytes into early atherosclerotic lesions. Oxidized LDL cholesterol, but not native LDL cholesterol, induces MCP-1 production in endothelial and smooth-muscle cells and may be a molecular link between oxidized lipoproteins and foam cell formation in the vessel wall (Charo 2004). MCP-1 may also activate tissue factor and thus contribute to thrombin generation and thrombus formation (Charo 2004). Thus, elevations of IFN $\gamma$ , TNF $\alpha$  and MCP-1 among DM-patients with CDK may be of importance for their increased cardiovascular risk. MCP-1 may also contribute to the pathogenesis of diabetic nephropathy by facilitating the migration of monocytes and macrophages into the kidney (Galkina 2006).

Several studies have shown reduced levels of proinflammatory mediators, including IFN $\gamma$  and MCP-1, during statin treatment (Bonetti 2003, Schonbeck 2004), in agreement with our results. Antiinflammatory effects of statin treatment are believed to be of importance for the reduction of atherosclerotic complications (Hansson 2005) due to a combination of LDL-related effects and pleiotropic anti-inflammatory effects. Reduced levels of MCP-1 and IFN $\gamma$  during simvastatin treatment might be beneficial with regard to the progression of both atherosclerosis and diabetic nephropathy in patients with DM and concurrent CKD.

## 4.4 PAPER IV

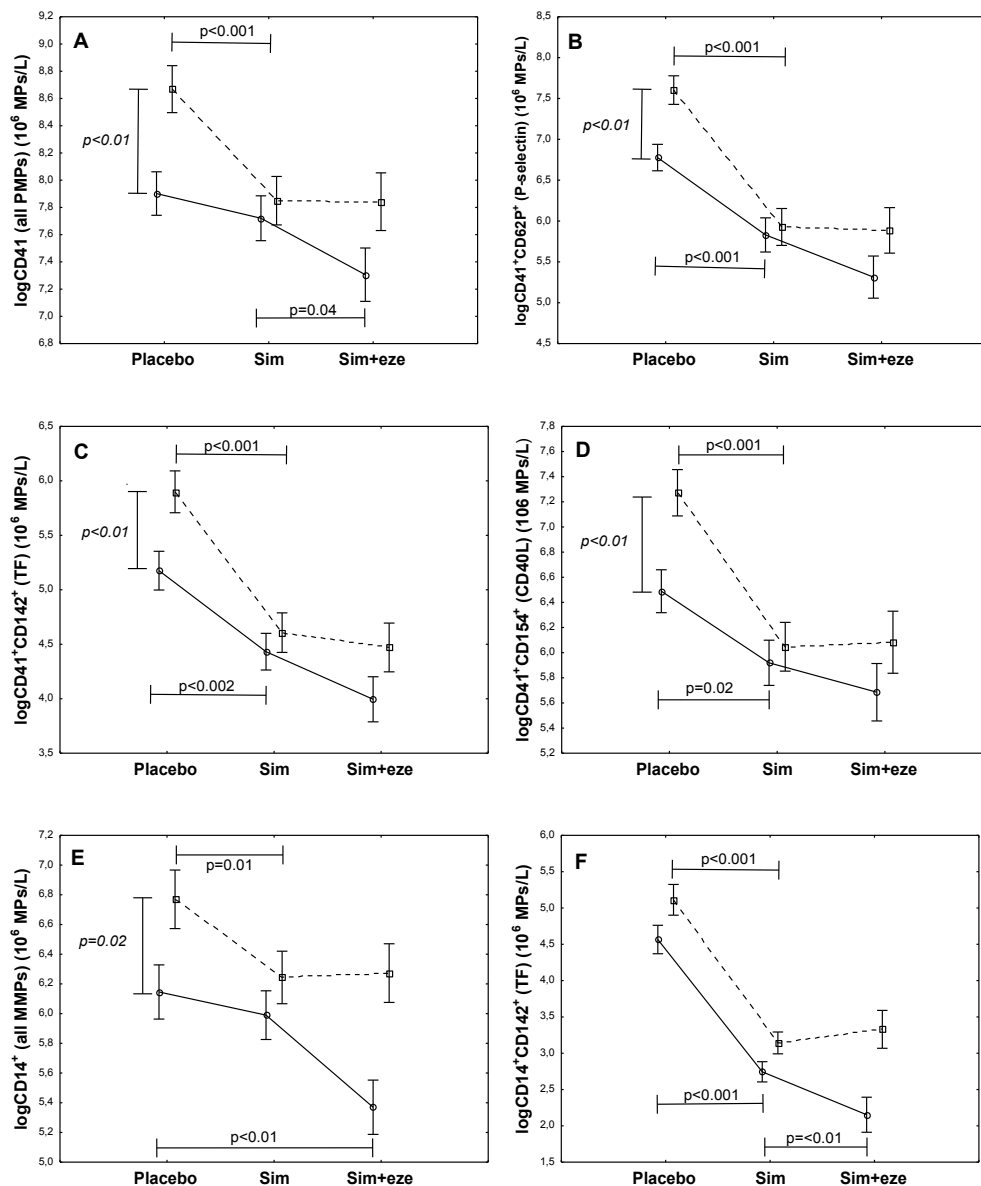
### Elevated levels of circulating microparticles (MPs) in DM-CKD patients

The total counts of all types of MPs studied (total PS+MPs, PMPs, MMPs, EMPs) were elevated in DM patients with CKD stages 3-4 compared to DM-only patients at baseline. The expressions of P-selectin, CD40L and TF on PMPs, as well as of TF on EMPs were also higher in DM-CKD compared to DM-only patients (Fig 10) (Table 4).

### Lipid-lowering treatment reduces procoagulant MPs

In DM patients with CKD stages 3-4, LLT with simvastatin alone significantly reduced the counts of total procoagulant PS+MPs, MMPs and PMPs, and the expression of P-selectin, CD40L and TF on PMPs as well as of TF on MMPs, compared to placebo (for statistics see Table 4). Combination treatment with simvastatin and ezetimibe had no further effect. Addition of ezetimibe, however, reduced the expression of TF on EMPs in the DM-CKD group. In DM-only patients simvastatin reduced the expression of P-selectin, TF and CD40L on PMPs and of TF on MMPs, compared to placebo. Combination treatment was required to achieve reductions of all PMPs, MMPs and TF expression of EMPs in the DM-only group. The difference between DM-CKD and DM-only seen at baseline disappeared with simvastatin treatment (Fig 10) (Table 4).

DM-CKD patients thus responded better to simvastatin treatment than DM-only patients, with no further effect of adding ezetimibe (Fig 10). DM-only patients demonstrated a more dose-response like pattern for LDL-lowering, since combination treatment resulted in a trend towards further decreases of activation markers on PMPs and MMPs and was required to significantly reduce the total counts of PMPs and MMPs in this patient group (Fig 10). Changes of lipid levels and MP formation were not correlated in either patient group.



**Figure 10.** Levels of all PMPs (A), P-selectin expressing PMPs (B), TF expressing PMPs (C), CD40L expressing PMPs (D), all MMPs (E) and TF expressing MMPs (F) in the DM-CKD (dotted line) and DM-only (solid line) groups. Measurements were at baseline (placebo) and during treatment with simvastatin (Sim), and simvastatin+ezetimie (Sim+eze). *P*-values are group differences at baseline (Student's t-test). *P*-values are post hoc test in the ANOVA. Data are means  $\pm$  SEM.

	DM-only group (n=21)		*p		DMK-CDK group (n=18)		❖p
Microparticles							
All MPs (Lactadherin)	6852±5172	6409±3458	4576±3380	<0.01	12945±8294	7181±4194 §	8575±5179# <0.001
PMPs							
All PMPs (CD41)	3009 (1824;4334)	1953 (1420;3060)	1931 (844;2801)#	<0.01	5847 (3625;9450)	3394 (1053;4575)°	3202 (1598;4061)# <0.001
CD41+CD62P (P-selectin)	903 (578;1191)	320 (187;640)	212 (77.5;558)	<0.01	1946 (1284;3308)	386 (158;809)	340 (218;525) <0.001
CD41+CD142(TF)	187 (100;228)	78 (57;122)	82 (31;111)	<0.01	373 (184;695)	120 (44;183)	74 (48;162) <0.001
CD41+CD154(CD40L)	657 (456;840)	313 (228;625)	322 (160;728)	<0.01	1432 (798;2582)	521 (159;1040)	410 (616;749) <0.001
EMPs							
CD144 (VE-cadherin)	600 (416;774)	1167 (717;1561)	1168 (830;1507)	<0.01	1055 (727;1576)	1483 (983;3007)	1821(957;3106) <0.001
CD62E (E-sel)	600 (329;731)	824 (538;1569)	1147 (624;1603)	<0.01	1135 (662;1778)	1109 (828;2151)	1128 (620;2241) <0.001
CD62E+CD142(TF)	94 (60;120)	110 (81;190)	53 (31;81)	<0.01	197 (137;298)	218 (84;401)	84 (54;143) <0.001
MMPs							
All MMPs (CD14)	579 (223;859)	486 (239;701)	213 (113;441)	0.02	827 (436;1941)	600 (397;712)	770 (271;958) <0.001
CD14+CD142(TF)	98 (53;199)	14 (10;29)	9 (3;26)	0.06	168 (92;349)	20 (18;32)	30 (12;57) <0.001

Data presented as mean±SD or median values (25th and 75th percentiles) (for skewed data)

\*p-values are group difference at baseline (Student's t-test)

❖p-values are overall treatment effects by repeated measures ANOVA

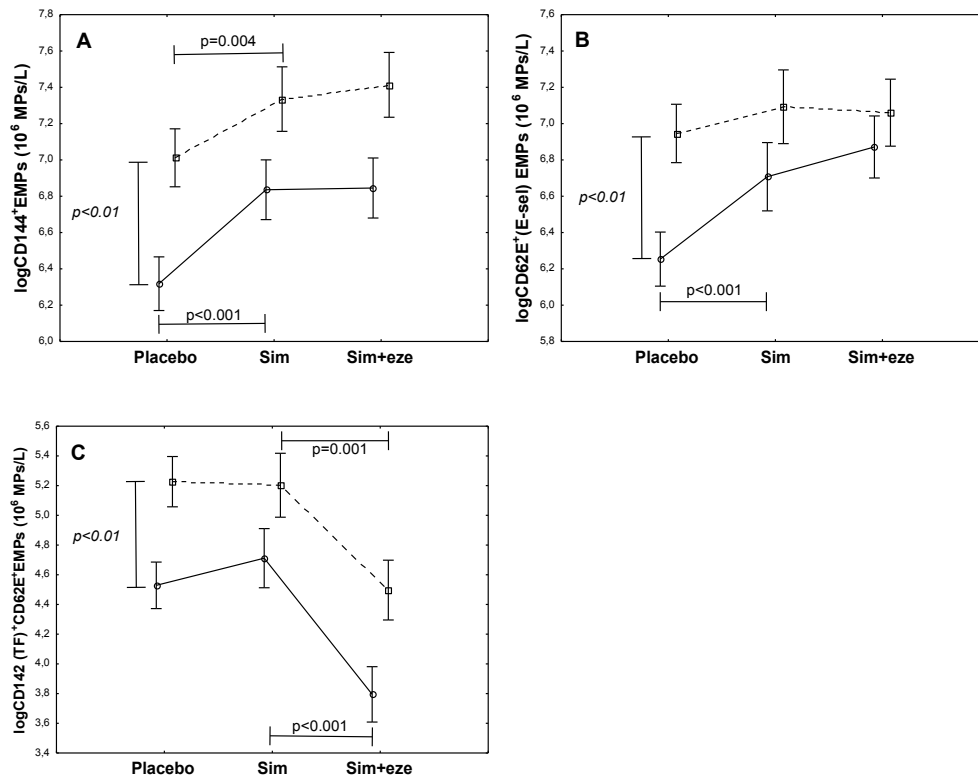
§p<0.05 post hoc test, treatment effects within group (placebo vs. Sim)

# p<0.05 post hoc test, treatment effect within group (placebo vs. Sim+eze)

**Table 4.** Levels of microparticles at baseline (after the placebo run-in) and after lipid-lowering treatment.

## Elevations of EMPs with lipid-lowering treatment

Simvastatin significantly elevated the levels of EMPs expressing VE-cadherin (PS+CD144+) in both patient groups, compared to placebo (Fig 11). Combination treatment with simvastatin and ezetimibe did not elevate the levels of VE-cadherin+ EMPs any further than did simvastatin alone in either of the patient groups. Simvastatin alone elevated EMPs expressing E-selectin (PS+CD62E+) in DM-only patients compared to placebo (*post-hoc* test  $p<0.001$ ) (Fig 11). The expression of TF on CD62+ EMPs (CD62E+CD142+) was reduced during combination treatment in both patient groups (Table 4).



**Figure 11.** Levels of CD144<sup>+</sup> (VE-cadherin) EMPs (A), CD62E<sup>+</sup> (E-selectin) EMPs (B) and CD142<sup>+</sup> (TF) CD62<sup>+</sup> EMPs (C) in the DM-CKD (dotted line) and DM-only (solid line) groups. Measurements were at baseline (placebo) and during treatment with simvastatin (Sim), and simvastatin+ezetimibe (Sim+eze). *P*-values are group differences at baseline (Student's *t*-test). *P*-values are post hoc test in the ANOVA. Data are means  $\pm$  SEM.

## Discussion

MPs are released from various cells upon activation and/or apoptosis and appear to both contribute to, and a consequence of inflammation (Burger 2013). Increased inflammatory activity in the DM-CKD group was thus most likely of importance for the difference in MP formation between DM-CKD and DM-only patients. Circulating PMPs are the most abundant microparticles in the circulation, and seem to be markers of inflammation, platelet activation and hypercoagulability (Burger 2013). Like platelets, MPs have procoagulant activity through the exposure of negatively charged PS on the surface which allows coagulation factors to bind and thereby enable the formation of tenase and prothrombinase complexes and the

promotion of thrombin generation (Burger 2013). TF expressed on MPs may also contribute (Owens 2011).

Reduced levels of PMPs as well as decreased expression of procoagulant proteins on PMPs during simvastatin treatment suggest that such treatment reduces platelet activation and hypercoagulability. The mechanisms through which statin treatment reduced the formation of PMPs need further clarification.

Statins are also suggested to have anticoagulant effects through reductions of TF via both lipid- and non-lipid dependent pathways (Owens 2014). MMPs are considered to be the main MP-related source of TF which may be transferred to platelets and PMPs from monocytes (Owens 2011, Osterud 2012). The expression of TF on MPs has been much debated, especially whether or not it is active (Owens 2011). Recent studies suggest that TF may contribute to the procoagulant effects of MPs (Owens 2014), and the markedly reduced expression of TF on MMPs and PMPs during simvastatin treatment in our study indicates a less procoagulant milieu. However, as shown by our coworkers, the main procoagulant effects of MPs are most likely an interaction between PS expression and TF expression (Mobarrez 2011). As TF is dependent on PS to activate FVII, one can speculate that TF expression on MPs can help initiate the coagulation, and later the exposed PS on MPs can maintain or even further promote coagulation.

As discussed above, the CD40-CD40L system promotes proinflammatory and prothrombotic alterations in atherothrombosis. Statins have been found to reduce the plasma levels of sCD40L and the CD40L expression of human endothelial and smooth muscle cells (Antoniades 2009). The reduced expression of CD40L on PMPs by simvastatin treatment in both patients groups in our study, may contribute to beneficial reductions of inflammatory activity and hypercoagulability.

EMP release is proposed to reflect endothelial cell activation and dysfunction (Burger 2013). The current findings of elevated EMPs in plasma during simvastatin treatment are surprising since improvement of endothelial function is thought to be one of the major pleiotropic effects of statins (Bonetti 2003). However, the present findings are in line with previous results from our coworkers showing an increased release of EMPs during treatment with atorvastatin (80 mg/day) in patients with peripheral arterial occlusive disease (Mobarrez 2012). In patients with type-1 DM, high-dose atorvastatin treatment caused impaired endothelium-dependent function of microvessels and a tendency toward elevated levels of EMPs (Tehrani 2013). In addition, the EMP release from human umbilical endothelial cells has been shown to be increased by simvastatin in vitro (Diamant 2008). Some in vitro studies have suggested that statins may contribute to apoptosis by inducing expression of the proapoptotic enzymes caspase-3 and -9, and by interfering with Ras prenylation (Schonbeck 2004). However, further research is needed to clarify how statins influence EMP release and, most importantly, the possible clinical consequences of this.

## **5 GENERAL DISCUSSION**

### **5.1 RENAL FUNCTION AS A PROGNOSTIC PREDICTOR**

The burden of CV mortality and morbidity is a growing, worldwide health problem. CKD (i.e,  $\text{eGFR} < 60 \text{ ml/min/1.73m}^2$ ) is now considered to be an independent predictor of all-cause and CV mortality in both the general population and in populations with increased risk for CKD, defined as a history of hypertension, diabetes, or cardiovascular disease (Chronic Kidney Disease Prognosis 2010, van der Velde 2011). As reported in Paper I, we found that impaired renal function carried independent prognostic information regarding the risk of suffering CV death or nonfatal MI in patients with stable angina pectoris. The prognostic impact was graded and the risk of CV death or non-fatal MI was doubled at  $\text{eCrCl} < 60 \text{ ml/min}$  compared to  $\text{eCrCl} \geq 90 \text{ ml/min}$ . This is in line with data from large cohort studies in which patients with moderately reduced renal function ( $\text{eGFR} 30\text{-}59 \text{ ml/min/1.73m}^2$ ) were found to have a doubled risk, and patients with severely decrease renal function ( $\text{eGFR} 15\text{-}29 \text{ ml/min/1.73m}^2$ ) had a trippled risk for cardiovascular mortality compared to individuals with normal renal function. There seems to be a threshold for increased CV risk around  $\text{eGFR} 60 \text{ ml/min/1.73m}^2$ , and below this threshold there is an exponential increase in risk as  $\text{eGFR}$  declines (Chronic Kidney Disease Prognosis 2010, van der Velde 2011).

Thus, patients with CKD should be considered to belong to one of the highest-risk groups for CV events and disease, and considerable attention should be paid to risk factor management and CV risk reduction therapies in these patients. However, despite the increasing attention to the close relationship between CKD and CV risk during recent years, studies repeatedly point out that the CVD risk in patients with impaired renal function is poorly recognised and often undertreated. Patients with CKD and CVD are not receiving adequate treatment with aspirin, beta-blockers, ACE-inhibitors and statins according to current guidelines to the same extent as patients without CKD (Tonelli 2001, Wright 2002, Szummer 2010). They are also less frequently treated with anticoagulants and revascularization in non-ST-elevation MI or reperfusion therapy in ST-elevation MI (Wright 2002, Szummer 2010). Thus, patients with reduced renal function should be identified early, and there is a considerable need for improved risk factor management and improved CVD risk reduction therapy. In the development of new clinical guidelines for CV treatment and prevention, patients with CKD should receive special attention.

### **5.2 SIGNS OF INCREASED INFLAMMATION AND HYPERCOAGULABILITY IN DM-CKD PATIENTS**

The increased CV risk in patients with CKD is in part due to the high prevalence of traditional risk factors such as diabetes and hypertension but the strong association between impaired renal function and CV risk cannot only be explained by this. Thus, "non-traditional" risk factors seem to play an important role in the excessive CV risk in patients with CKD. Chronic inflammatory activation is considered to be an important such non-traditional risk

factor. Inflammation is highly involved in both atherosclerosis and thrombosis (Ross 1999) and is common among patients with CKD.

In our study, reported in Papers II-IV, we found signs of increased inflammatory activity with increased PLA formation and MP formation, as well as elevated levels of proinflammatory cytokines and sCD40L, in DM patients with concurrent CKD compared to DM patients with normal renal function. Similar to CKD, DM is also associated with increased inflammatory activity, as well as endothelial dysfunction and oxidative stress, factors which are considered to be associated with the high CV risk in these patients (Goldberg 2009). Furthermore, both DM and CKD are considered to be prothrombotic states with major hemostatic disturbances (Hess 2011, Lutz 2014). Thus, DM and CKD share many features that act as risk factors for CVD, and when DM and CKD are concurrent, these risk factors may amplify each other and result in an excessive risk of suffering CV events. Thus, our findings with signs of increased inflammatory activity and endothelial dysfunction in DM patients with concurrent CKD stages 3-4, compared to DM patients with normal GFR, were expected and are probably of importance for the increased CV risk in these patients.

There are many links between inflammation, platelets and coagulation, and our observations of elevated levels of PMPs and increased expression of P-selectin, CD40L and TF on PMPs in Paper IV may, together with the increased PLA formation and sCD40 levels reported in Paper II, indicate not only increased inflammatory activity but also suggest that there is a state of increased platelet activation and hypercoagulability in the current DM-CKD patients.

Whether CKD is associated with increased platelet activation is controversial. Impaired renal function has most commonly been associated with platelet dysfunction causing reduced aggregability with an increased risk of bleeding (Lutz 2014). However, there is an increasing number of studies showing hyperreactive platelets or increased platelet activation in patients with CDK (Thijs 2008, Landray 2004, Woo 2011, Gremmel 2013, Yagmur 2013), and Baber et al found that platelet reactivity in patients undergoing percutaneous coronary intervention was higher among patients with both DM and CKD compared to those with one condition alone (Baber 2013). In paper II we found no signs of increased platelet activity as evaluated by single platelet P-selectin expression or fibrinogen receptor binding in DM-CKD compared with DM-only patients. It is worth noting that the patients in our study had no history of previous MI or stroke and that they were well-controlled regarding blood pressure, lipid-levels and HbA1c etc., with no differences between the two patient groups. This relatively low risk profile at baseline might be associated with less activated platelets and therefore a limited possibility of detecting group differences. However, platelet activity is a complex issue and different parameters reflecting it may be differentially altered by disease states and treatments. Measuring PLA formation and PMP release illustrate other aspects of platelet activity than activity markers on single platelets, and these measures suggested that platelet activity indeed was increased in our DM-CKD patients.

We found no differences between men and women in any of the evaluated parameters in Papers II-IV, but the groups were too small to reveal limited gender differences. The

association between CKD and CV mortality has been found to be at least as strong in women as in men and the relationship between reduced eGFR and increased mortality is steeper in women than in men (Nitsch 2013). Data concerning gender differences in inflammatory activity and hypercoagulability in CKD are sparse, and further research in this area would be of interest.

### **5.3 EFFECTS OF LIPID-LOWERING TREATMENT**

We found markedly reduced levels PMPs and MMPs and their expression of prothrombotic markers and reduced levels of IFN $\gamma$  and MCP-1 with simvastatin treatment, and reduced PLA formation and sCD40L levels with combination LLT with simvastatin and ezetimibe in DM patients with CKD. These findings suggest reduced inflammatory activity, as well as reduced platelet activation and less hypercoagulability, which might contribute to the beneficial effects of LLT on atherothrombotic complications in DM patients with concurrent CKD. The exact mechanisms responsible for the antithrombotic properties of statin treatment are still not fully understood. Reductions of lipids and lipoprotein levels are most likely important but so-called pleiotropic effects may well contribute.

Statins have been shown to interfere with CD40-CD40L interactions at several levels which seem to be both lipid-dependent and -independent (Schonbeck 2004), and may thereby influence platelet activity and hypercoagulability (Antoniades 2009). Activated platelets are the main source of sCD40L (Antoniades 2009), and we found reduced levels of sCD40L with combined LLT as well as reduced CD40L expression on PMPs with simvastatin treatment in our DM-CKD patients. As discussed above, the reduced levels of sCD40L during combination treatment might have been involved in the reduction of PLA formation seen among our DM-CKD patients.

Circulating microparticles seem to have a strong association with endothelial damage, platelet activation and hypercoagulability and are emerging as interesting players in the complex process of atherosclerosis and atherothrombotic complications (Shantsila 2010). There is growing interest in the possibility to modulate the levels of MPs and their expression of prothrombotic markers with pharmacological agents, such as statins. Previous studies have suggested that statin treatment may decrease MP production (Nomura 2004, Sommeijer 2005), and our coworkers have previously found that atorvastatin treatment reduced the expression of TF, P-selectin and GPIIIa on PMPs in patients with peripheral arterial occlusive disease (Mobarrez 2011), and in patients with type-1 diabetes (Tehrani 2010). The study presented in paper IV is, to our knowledge, the first to investigate statin associated effects on MP formation in patients with CKD. The results of this study suggest that MPs may be involved in the hypercoagulable state in CKD and that influences of LLT on MP production can lead to a less procoagulant milieu. However, surprisingly we found elevated levels of EMPs in plasma during simvastatin treatment. Thus, different MP subpopulations may behave differently during statin treatment, and further research is needed to clarify these mechanisms and their possible importance.

## 5.4 THE INFLUENCE OF CKD WAS COUNTERACTED BY LIPID-LOWERING TREATMENT

Interestingly, the differences between DM-CKD and DM-only patients at baseline regarding PLA-formation, inflammatory parameters and procoagulant PMPs and MMPs, were counteracted by LLT. PLA formation and sCD40L levels were reduced with simvastatin + ezetimibe, and IFN $\gamma$  and MCP-1 were reduced with simvastatin alone in DM-CKD patients but not in DM-only patients. The effect of simvastatin on PMP levels, as well as on the expression of P-selectin, CD40L and TF on PMPs was more prominent in DM-CKD patients than in DM-only patients. Thus DM-only and DM-CKD patients responded differently to LLT despite similar LDL reductions and similar lipid-levels at baseline. Why these responses to LLT were enhanced when CKD was present is not known, but the effects may be related to beneficial effects on the increased inflammatory activity among CKD patients.

Statins effectively decrease blood lipid levels by inhibiting the enzyme HMG-CoA reductase and thereby cholesterol synthesis, but they may also influence inflammatory activity and atherothrombotic mechanisms independently of their lipid-lowering effect, mainly due to inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway and thereby altered protein prenylation, as discussed before. However, since hyperlipidemia itself is associated with increased inflammatory activity (Siasos 2011), and oxidized LDL particles play an important role in the triggering of inflammation in atherosclerosis (Hansson 2005), the reduction of LDL most likely influences inflammatory activity in concert with possible pleiotropic effects of statin treatment. Oxidized LDL particles may also, apart from activating endothelial cells and inducing an inflammatory response in the arterial wall, be directly procoagulant by supporting thrombin generation on their PS expressing, negatively charged surfaces (Griffin 2001). The exact mechanisms through which statins reduce cardiovascular complications are still uncertain, and a most interesting question is to what extent their effects are related to the LDL reduction or to other pleiotropic effects.

In Papers II-IV we studied the effects of simvastatin alone or with ezetimibe and we could hence evaluate whether additional LDL reduction with the same statin dose would provide additional effects. Since combination treatment with simvastatin and ezetimibe was required to achieve significant reductions of PLA formation and sCD40L, with only a trend towards decreased levels with simvastatin alone in DM-CKD patients, it is difficult to invoke pleiotropic effects of statin treatment as the primary mechanisms behind these findings. Furthermore, the reductions of IFN $\gamma$  and MCP-1 were significant with simvastatin alone, but increased when ezetimibe was added. Since pleiotropic effects of ezetimibe are uncertain (Kalogirou 2010) the additional 18% lowering of LDL cholesterol achieved with combination treatment might explain these further reductions. This is in line with a meta-analysis by Kinlay et al., which included 23 studies with 57 groups treated with different LLT regimens and concluded that most of the anti-inflammatory effect of LLT is related to the magnitude of change in LDL (Kinlay 2007). However, the meta-analysis was dominated by statin studies and it is difficult to separate directly LDL-related effects from pleiotropic effects. A most interesting study for the future would be to compare the antiinflammatory and antithrombotic

effects of statins with those of new lipid-lowering drugs, such as the proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors which causes marked reductions of LDL (Koren 2014), in order to distinguish between LDL related and possibly LDL independent pleiotropic effects.

## **5.5 LIPID-LOWERING TREATMENT IN CKD AND CLINICAL OUTCOMES**

The Study of Heart and Renal Protection (SHARP) was the first trial specifically powered to investigate atherosclerotic outcomes in non-dialysis CKD patients. It showed a 17% reduction of major atherosclerotic events with combined simvastatin and ezetimibe treatment compared to placebo in patients with CKD and no prior MI or coronary revascularization (i.e, primary prevention) (Baigent 2011). Unfortunately, the trial lacked a group treated with simvastatin only but a recent large meta-analysis including data from 51099 patients with CKD (including SHARP) concluded that statin treatment reduces cardiovascular mortality and cardiovascular events in CKD patients who are not receiving dialysis (Palmer 2012). The proportional decreases in major cardiovascular and mortality outcomes in this meta-analysis were similar to or larger than those observed in broader populations with established CVD (Baigent 2005). There was also an additional analysis after excluding SHARP which found treatment effects for statins alone to be similar to those of combined therapy with statin+ezetimibe (Palmer 2012). Thus, statin treatment has beneficial effects on cardiovascular morbidity and mortality in patients with CKD similar to other high-risk populations, as DM patients, but the addition of ezetimibe has not yet been shown to improve clinical outcomes in any of these populations.

The Kidney Disease: Improving Global Outcomes (KDIGO) organization has recently developed clinical practice guidelines on lipid management in CKD (Tonelli 2014). These guidelines are mainly based on the results of the SHARP study and post hoc analyses of trial from the general population focusing on participants with CKD. A key recommendation in these new guidelines is to use statin or statin/ezetimibe treatment for primary prevention, regardless of blood cholesterol levels, in patients aged  $\geq 50$  years with  $\text{eGFR} < 60 \text{ ml/min/1.73m}^2$  who are not treated with dialysis. Furthermore, statins are recommended for all DM patients with non-dialysis-dependent CKD. These guidelines will likely increase the use of statins in CKD patients worldwide and hopefully contribute to reduced CV mortality and morbidity in the future. The findings in our study, suggesting beneficial effects of LLT on inflammatory activity and hypercoagulability in DM patients with CKD stages 3-4, could be of importance with regard to CV risk reduction in these high-risk patients.

## **5.6 LIMITATIONS**

The major limitation of our DM-CKD study presented in Papers II-IV is the small number of patients which limited the ability to reveal modest differences in treatment effects. Another possible limitation is the lack of a control group without DM. However, we did not study patients with CKD only or healthy individuals since the influence of DM has been studied previously and our aim was to elucidate the influence of CKD in DM. We included patients

with both type 1 and type 2 DM. The lesions underlying renal dysfunction in type 1 and type 2 DM may differ, but the present study focused on prothrombotic mechanisms in DM, irrespective of its type. We found no differences between type 1 and type 2 DM patients in any of the evaluated parameters in Papers II-IV, but the groups were small which limited the ability to reveal differences depending on the type of DM.

## 6 FUTURE PERSPECTIVES

Based on the present findings it would be of interest to:

- Further characterize procoagulant mechanisms in different CKD-stages with a focus on the connection between inflammation, platelet function and MPs.
- Compare the antiinflammatory and antithrombotic effects of statins with those of new lipid-lowering drugs, such as the proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, in order to distinguish between LDL related and possibly LDL independent pleiotropic effects of statin treatment.
- Further evaluate MP formation in patients with CKD without DM and possible relationships to CKD stages and types of underlying renal disease.
- Evaluate in larger trials whether MP measurements could be used for prognostic purposes and risk assessment of patients with CKD.



## 7 SUMMARY AND CONCLUSIONS

The following conclusions can be drawn from this work:

- Impaired renal function provided independent prognostic information regarding the risk of suffering cardiovascular death or MI in patients with chronic stable angina pectoris, in agreement with findings in patients with other categories of coronary artery disease as well as in the normal population.
- The prognostic impact was graded and patients with eCrCl <60 ml/min had a doubled risk of suffering CV death or MI, compared to patients with eCrCl ≥90 ml/min.
- Patients with DM and concurrent CKD stages 3-4 (eGFR 15-59 ml/min/1.73m<sup>2</sup>) had signs of increased inflammatory activity, platelet activation and hypercoagulability compared to DM patients with normal eGFR. This may be of importance for their increased cardiovascular risk.
- Lipid-lowering treatment (LLT) with simvastatin alone markedly reduced the levels of prothrombotic microparticles derived from platelets and monocytes and their expression of activation markers, and reduced the plasma levels of IFN $\gamma$  and MCP-1 in DM patients with CKD stages 3-4.
- Combination LLT with simvastatin and ezetimibe reduced platelet-leukocyte aggregation and sCD40L levels in DM patients with CKD stages 3-4.
- These findings suggest reduced inflammatory activity, as well as reduced platelet activation and less hypercoagulability, which might contribute to the beneficial effects of LLT on atherothrombotic complications in DM patients with concurrent CKD.
- Influences of CKD on markers associated with increased inflammation and platelet activation were counteracted by LLT in patients with DM.
- LLT elevated the levels of microparticles derived from endothelial cells, which could imply deterioration of some aspects of endothelial function. Further research is needed to clarify these mechanisms and their possible importance.
- Neither the LDL lowering effect alone nor possible pleiotropic effect of statin treatment could independently explain the findings in this work. Thus, LDL-dependent and -independent mechanisms likely act in concert with regard to anti-inflammatory and anti-thrombotic effects of LLT.

## 8 SVENSK SAMMANFATTNING

Njurarna har en livsviktig funktion när det gäller att rena blodet från restprodukter som kroppen inte behöver samt att avlägsna överflödigt vatten. Njursvikt innebär att njurarna av någon orsak inte klarar av detta, och skadliga ämnen stannar kvar i kroppen. Kronisk njursvikt innebär att njurarna långsamt blir sämre, ofta under flera år. Diabetes mellitus (sockersjuka) är en av de vanligaste orsakerna till kronisk njursvikt, till stor del beroende på den snabba ökningen av typ 2 diabetes och fetma världen över. Personer med diabetes eller kronisk njursvikt löper en mycket hög risk att utveckla hjärt-kärlsjukdom (som t.ex. hjärtinfarkt eller stroke) och risken är ännu högre om man har båda tillstånden samtidigt.

Hjärt-kärlsjukdom är den vanligaste dödsorsaken hos personer med kronisk njursvikt och en person med måttlig kronisk njursvikt har dubbelt så stor risk att dö i hjärt-kärlsjukdom jämfört med en person med normal njurfunktion.

Både diabetiker och personer med kronisk njursvikt har en förändrad/störd hemostas (kroppens förmåga att bilda och bryta ner blodproppar) och aktiverade ”kladdiga” blodplättar (trombocyter). Detta ger en ökad benägenhet att koagulera (levra) blodet och därmed en ökad risk att bilda blodproppar. Personer med diabetes och njursvikt har också tecken på kronisk inflammation, vilket också bidrar till den störda balansen i hemostasen, till aktivering av blodplättarna samt till ökad åderförkalkning i blodkärlen.

Behandling med en grupp av läkemedel som sänker blodfetterna, så kallade *statiner*, minskar risken för hjärt-kärlsjuklighet hos personer både med diabetes och kronisk njursvikt.

Statinerna hämmar ett enzym i kolesterolsyntesen i levern, med minskad kolesterolproduktion som följd. Statinerna har också visat sig ha effekter som inte enbart kan förklaras av den blodfettsänkande effekten, som t.ex. att minska inflammatorisk aktivitet och minska benägenheten för blodkoagulation. Dessa effekter spelar sannolikt en viktig roll, tillsammans med själva blodfettsänkningen, för statinernas skyddande effekter.

*Ezetimibe* är en annan typ av blodfettsänkande läkemedel som verkar genom att hämma upptaget av kolesterol från maten. Vid kombinationsbehandling med ezetimibe och en statin uppnår man en kraftigare blodfettssänkning än med enbart en statin.

Syftet med den här avhandlingen har varit att närmare undersöka effekten av blodfettsänkande behandling med statinen simvastatin enbart eller i kombination med ezetimibe hos patienter med diabetes, med eller utan kronisk njursvikt. Vi ville studera hur blodplättar, andra faktorer i hemostasen samt inflammatorisk aktivitet påverkas av statinbehandling. Vi ville också studera om ytterligare kolesterolsänkning genom tillägg av ezetimibe till simvastatin påverkar dessa parametrar och därmed risken för blodpropp. I denna avhandling har vi också studerat om nedsatt njurfunktion har någon prognostisk betydelse hos patienter med kärlkramp (angina pectoris).

Med hjälp av en ekvation kan njurfunktionen skattas utgående från mängden av ett ämne i kroppen som heter kreatinin. Vid njursvikt ansamlas kreatinin i ökande mängd i blodet.

I **delstudie 1** skattade vi njurfunktionen på detta sätt hos 808 patienter med kärlkramp som alla deltog i en studie som utvärderade risken för hjärtinfarkt och hjärt-kärl död vid behandling med två olika typer av läkemedel. Vi fann att patienter med kärlkramp relativt ofta har måttligt nedsatt njurfunktion, framför allt kvinnor. Vi fann också att nedsatt njurfunktion har prognostisk betydelse för risken att drabbas av hjärt-kärl död eller hjärtinfarkt hos patienter med kärlkramp, vilket betyder att ju sämre njurfunktionen är desto större är risken att drabbas. I denna studie hade patienter med måttligt nedsatt njurfunktion dubbelt så stor risk att drabbas av hjärt-kärl död som patienter med normal njurfunktion, och risken fortsatte att öka hos de med sämre njurfunktion. Detta samband mellan nedsatt njurfunktion och risken för hjärt-kärlsjuklighet och död är även påvisat i många andra studier. Patienter med kronisk njursvikt är således en högriskgrupp. Det är mycket viktigt att upptäcka kronisk njursvikt tidigt för att kunna vidta åtgärder som minskar risken för att njurarna försämras ytterligare och minska risken hjärt-kärlsjukdom, t.ex. sänka blodtrycket, sluta röka, gå ner i vikt eller behandla höga blodfetter.

I den studie som ligger till grund för resultaten i **delstudie II-IV** inkluderade vi 18 diabetespatienter med kronisk njursvikt stadium 3-4 (måttlig till grav njursvikt) samt 21 diabetespatienter med normal njurfunktion. Efter en 6 veckor lång inledande period med ”sockerpiller” (placebo) behandlades patienterna med simvastatin enbart i en 8-10 veckors lång period samt därefter med simvastatin+ezetimibe i en 8-10 veckors lång period. Efter varje behandlingsperiod undersöktes funktionen av så kallade mikropartiklar, blodplättar, vita blodkroppar, samt inflammations markörer. Mikropartiklar är mycket små membranpartiklar som avknoppas från ett flertal olika celler såsom blodplättar, vita blodkroppar och endotelceller (celler som täcker blodkärlens insida), vid retning eller celldöd. Mikropartiklar verkar ha en viktig betydelse för bland annat blodproppsbildning och inflammation. Vi fann att diabetespatienter med kronisk njursvikt hade tecken på ökad inflammatorisk aktivitet, ökad bildning av aggregat av blodplättar och vita blodkroppar (PLA) samt ökad mängd mikropartiklar från blodplättar, vita blodkroppar och kärlvägg jämfört diabetespatienter med normal njurfunktion. Detta kan vara av betydelse för den ökade hjärt-kärl risken hos dessa patienter.

Vi fann att blodfettsänkande behandling med enbart simvastatin minskade nivåerna av mikropartiklar från blodplättar och vita blodkroppar. Simvastatin minskade också nivåerna av två specifika inflammatoriska molekyler (IFN $\gamma$  och MCP-1) och behandling med simvastatin + ezetimibe minskade PLA-bildning hos patienterna med njursvikt men inte hos diabetespatienterna med normal njurfunktion. Dessa resultat talar för att den blodfettsänkande behandlingen bidrog till minskad inflammatorisk aktivitet, minskad aktivering av blodplättar samt minskad tendens till blodkoagulering, vilket kan vara av betydelse för att minska risken för hjärt-kärl sjukdom hos diabetespatienter med kronisk njursvikt.

## 9 ACKNOWLEDGEMENTS

There are many people to whom I wish to express my sincere gratitude for helping me in making this thesis possible, especially all the patients participating in the clinical studies included in this work. In particular I wish to thank:

**Paul Hjemdahl**, my main supervisor, for support and guidance during all these years and for introducing me to scientific thinking. In particular I am thankful for all you have taught me about writing. You are an inspiring research and writing model, with your profound scientific knowledge and clear thinking. You have a wonderful way of improving any text, making it clear, elegant, and precise.

**Stefan Jacobson**, my co-supervisor, for all your support, enthusiasm and encouragement, and for being such an inspiring person and researcher. Thank you for sharing all your optimism, energy and great knowledge, and for all the laughs any conversation with you always contains.

**Per-Eric Lins**, my co-supervisor, for sharing your great scientific ideas and for always being so encouraging, friendly and supportive.

**Jonas Berglund**, my former co-supervisor and former boss, first of all for employing me at the Department of Nephrology and also for being one of the initiators of this project.

**Fariborz Mobarrez**, co-author, for all your help and assistance, for introducing me into the fields of microparticles, and for being such a nice person. Also, **Håkan Wallén**, co-author, for being so encouraging and supportive, always taking time to answer my questions and for interesting discussions.

**Lennart Forslund**, co-author, for helping me with my first stumbling steps into statistics and writing a scientific paper. Also, **Nina Rehnqvist** and **Richard Farndale**, co-authors, for valuable constructive criticism of the manuscript to Paper I. Also **Per Näsman**, co-author, for invaluable statistical support and advice.

**Maud Daleskog**, for all the hours you have spent on this project, your great ability to take care of the patients when they come to the lab, and your excellent laboratory expertise. Without you this project would not have been possible! Also, **Maj-Christina Johansson**, for invaluable laboratory expertise during the first years of the project. Also, **Ragnhild Stålesen**, for your excellent laboratory expertise and for your help with making the figures in Paper II. **Charlotte Ander**, **Kajsa Sundqvist**, **Kathinka Leine**, for expert technical assistance.

**Nailin Li**, for expert knowledge in the flow cytometric method.

All the **APSIS investigators**, without your great job, the first part of my project would not have been possible.

**Lena Landstedt-Hallin**, Danderyd Hospital and **Nils Adner**, Södersjukhuset, for helping me in my search for patients to recruit.

**Björn Samnegård**, head of the Department of Nephrology, Danderyd Hospital, for believing in me, encouraging me, providing research time and showing to everyone that you think my research is important. You are a great boss!

All my colleagues at the Department of Nephrology, Danderyd Hospital, **Guna Germanis**, **Anders Danielsson**, **Anna Fagergren**, **Boa Grönros**, **Elena Fedchenko**, **Eva Bång Bernstone**, **Josefin Mörtberg**, **Lysann Essinger**, **Annika Alvelius**, **Lars Edwall** and **Senka Sendic** for your friendship, care and interest in my project. Having you as colleagues is a privilege, with all your knowledge and experience, and with all our laughs and inspiring conversations about almost anything, even when things are tough and we have a very busy working schedule.

**Sakke Rekola**, my former ST supervisor, for introducing me to the field of nephrology, sharing all your broad experience and for your great subtle humor. I miss you!

**All the staff at the Department of Nephrology** Danderyd Hospital, for making our clinic such a great and valuable place to work in. A special thanks to, **Jeanette Wallin**, research nurse, for help, support and inspiring conversations and to **Anna Ström**, for being so helpful, service minded and competent.

**Eva Wikström Jonsson**, for saving me this summer when I was in urgent need for someone to examine me in Clinical Pharmacology.

**Jan and David**, my brothers and **their families**, for being such great, caring, interesting, funny, smart and inspiring people.

**Magnus**, my best friend, husband and soul mate, for always believing in me, always standing by me, all your immense love and support during all this time and for being such a great listener. Without you this thesis could not have happened. I promise you that I will now grumble less and live more!

**Johan**, **Sofia** and **Gustaf**, my beloved, wonderful children, for putting up with me, loving me, supporting me and being so loyal, all these years. Every time I was ready to quit, you did not let me. A special thanks for all your expert help and support regarding computers and other technical stuff. You are the true meaning of my life, not platelets, I promise you that!

**Nils**, my father, for always believing in me, loving me, supporting me and always accepting me for who I am.

**Åsa**, my mother, you are present in my mind every day. Your smiling spirit hovers over me in my hectic everyday life, giving me confidence and strength.

And of course, **all other friends and relatives** not mentioned here but must not be forgotten. You fill my life with inspiration, laughter and energy!

The study was supported by the Swedish Research Council/Medicine, the Swedish Heart-Lung foundation, the regional Agreement on Medical Training and Clinical Research (ALF) between Stockholm County Council and the Karolinska Insitutet, and a Medical School Grant from Merck, Sharp and Dohme.

## 10 REFERENCES

- Afkarian, M., M. C. Sachs, B. Kestenbaum, et al. Kidney disease and increased mortality risk in type 2 diabetes. *J Am Soc Nephrol* 2013;24:302-8.
- Ait-Oufella, H., S. Taleb, Z. Mallat, et al. Recent advances on the role of cytokines in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2011;31:969-79.
- Al Suwaidi, J., D. N. Reddan, K. Williams, et al. Prognostic implications of abnormalities in renal function in patients with acute coronary syndromes. *Circulation* 2002;106:974-80.
- Alonso, A., F. L. Lopez, K. Matsushita, et al. Chronic kidney disease is associated with the incidence of atrial fibrillation: the Atherosclerosis Risk in Communities (ARIC) study. *Circulation* 2011;123:2946-53.
- Amabile, N., A. P. Guerin, A. Leroyer, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol* 2005;16:3381-8.
- Amabile, N., A. P. Guerin, A. Tedgui, et al. Predictive value of circulating endothelial microparticles for cardiovascular mortality in end-stage renal failure: a pilot study. *Nephrol Dial Transplant* 2012;27:1873-80.
- Anavekar, N. S., J. J. McMurray, E. J. Velazquez, et al. Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. *N Engl J Med* 2004;351:1285-95.
- Ando, M., A. Iwata, Y. Ozeki, et al. Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients. *Kidney Int* 2002;62:1757-63.
- Antoniades, C., C. Bakogiannis, D. Tousoulis, et al. The CD40/CD40 ligand system: linking inflammation with atherothrombosis. *J Am Coll Cardiol* 2009;54:669-77.
- Ariens, R. A. Fibrin(ogen) and thrombotic disease. *J Thromb Haemost* 2013;11 Suppl 1:294-305.
- Baber, U., J. Bander, R. Karajgikar, et al. Combined and independent impact of diabetes mellitus and chronic kidney disease on residual platelet reactivity. *Thromb Haemost* 2013;110:118-23.
- Baigent, C., A. Keech, P. M. Kearney, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* 2005;366:1267-78.
- Baigent, C., M. J. Landray, C. Reith, et al. The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (Study of Heart and Renal Protection): a randomised placebo-controlled trial. *Lancet* 2011;377:2181-92.
- Best, P. J., R. Lennon, H. H. Ting, et al. The impact of renal insufficiency on clinical outcomes in patients undergoing percutaneous coronary interventions. *J Am Coll Cardiol* 2002;39:1113-9.
- Boccardo, P., G. Remuzzi and M. Galbusera. Platelet dysfunction in renal failure. *Semin Thromb Hemost* 2004;30:579-89.
- Bonetti, P. O., L. O. Lerman, C. Napoli, et al. Statin effects beyond lipid lowering--are they clinically relevant? *Eur Heart J* 2003;24:225-48.

- Bonomini, M., S. Dottori, L. Amoroso, et al. Increased platelet phosphatidylserine exposure and caspase activation in chronic uremia. *J Thromb Haemost* 2004;2:1275-81.
- Brambilla, M., M. Camera, D. Colnago, et al. Tissue factor in patients with acute coronary syndromes: expression in platelets, leukocytes, and platelet-leukocyte aggregates. *Arterioscler Thromb Vasc Biol* 2008;28:947-53.
- Burger, D., S. Schock, C. S. Thompson, et al. Microparticles: biomarkers and beyond. *Clin Sci (Lond)* 2013;124:423-41.
- Cerletti, C., C. Tamburrelli, B. Izzi, et al. Platelet-leukocyte interactions in thrombosis. *Thromb Res* 2012;129:263-6.
- Charo, I. F. and M. B. Taubman. Chemokines in the pathogenesis of vascular disease. *Circ Res* 2004;95:858-66.
- Chronic Kidney Disease Prognosis, C., K. Matsushita, M. van der Velde, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 2010;375:2073-81.
- Colhoun, H. M., D. J. Betteridge, P. N. Durrington, et al. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. *Lancet* 2004;364:685-96.
- Collins, R., J. Armitage, S. Parish, et al. MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. *Lancet* 2003;361:2005-16.
- Davi, G. and C. Patrono. Platelet activation and atherothrombosis. *N Engl J Med* 2007;357:2482-94.
- Diamant, M., M. E. Tushuizen, M. N. Abid-Hussein, et al. Simvastatin-induced endothelial cell detachment and microparticle release are prenylation dependent. *Thromb Haemost* 2008;100:489-97.
- Faure, V., L. Dou, F. Sabatier, et al. Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost* 2006;4:566-73.
- Ferreiro, J. L., J. A. Gomez-Hospital and D. J. Angiolillo. Platelet abnormalities in diabetes mellitus. *Diab Vasc Dis Res* 2010;7:251-9.
- Furman, M. I., S. E. Benoit, M. R. Barnard, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998;31:352-8.
- Galkina, E. and K. Ley. Leukocyte recruitment and vascular injury in diabetic nephropathy. *J Am Soc Nephrol* 2006;17:368-77.
- Gawaz, M., H. Langer and A. E. May. Platelets in inflammation and atherogenesis. *J Clin Invest* 2005;115:3378-84.
- Ghasemzadeh, M. and E. Hosseini. Platelet-leukocyte crosstalk: Linking proinflammatory responses to procoagulant state. *Thromb Res* 2013;131:191-7.
- Gibson, C. M., R. L. Dumaine, E. V. Gelfand, et al. Association of glomerular filtration rate on presentation with subsequent mortality in non-ST-segment elevation acute coronary

syndrome; observations in 13,307 patients in five TIMI trials. *Eur Heart J* 2004;25:1998-2005.

Go, A. S., G. M. Chertow, D. Fan, et al. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004;351:1296-305.

Goldberg, R. B. Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications. *J Clin Endocrinol Metab* 2009;94:3171-82.

Granerus, G. and M. Aurell. Reference values for 51Cr-EDTA clearance as a measure of glomerular filtration rate. *Scand J Clin Lab Invest* 1981;41:611-6.

Gremmel, T., M. Muller, S. Steiner, et al. Chronic kidney disease is associated with increased platelet activation and poor response to antiplatelet therapy. *Nephrol Dial Transplant* 2013;28:2116-22.

Griffin, J. H., J. A. Fernandez and H. Deguchi. Plasma lipoproteins, hemostasis and thrombosis. *Thromb Haemost* 2001;86:386-94.

Groop, P. H., M. C. Thomas, J. L. Moran, et al. The presence and severity of chronic kidney disease predicts all-cause mortality in type 1 diabetes. *Diabetes* 2009;58:1651-8.

Hamilton, D., P. Riley, U. Miola, et al. Total plasma clearance of 51Cr-EDTA: variation with age and sex in normal adults. *Nucl Med Commun* 2000;21:187-92.

Hansson, G. K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-95.

Henn, V., J. R. Slupsky, M. Grafe, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391:591-4.

Hess, K. and P. J. Grant. Inflammation and thrombosis in diabetes. *Thromb Haemost* 2011;105 Suppl 1:S43-54.

Hjemdahl, P., S. V. Eriksson, C. Held, et al. Favourable long-term prognosis in stable angina pectoris: an extended follow-up of the Angina Prognosis study In Stockholm (APSIS). *Heart* 2005;

Hoffman, M. and D. M. Monroe, 3rd. A cell-based model of hemostasis. *Thromb Haemost* 2001;85:958-65.

Hu, H., N. Li, M. Yngen, et al. Enhanced leukocyte-platelet cross-talk in Type 1 diabetes mellitus: relationship to microangiopathy. *J Thromb Haemost* 2004;2:58-64.

Jalal, D. I., M. Chonchol and G. Targher. Disorders of hemostasis associated with chronic kidney disease. *Semin Thromb Hemost* 2010;36:34-40.

Jurk, K. and B. E. Kehrel. Platelets: physiology and biochemistry. *Semin Thromb Hemost* 2005;31:381-92.

Juutilainen, A., S. Kortelainen, S. Lehto, et al. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. *Diabetes Care* 2004;27:2898-904.

Kalogirou, M., V. Tsimihodimos and M. Elisaf. Pleiotropic effects of ezetimibe: do they really exist? *Eur J Pharmacol* 2010;633:62-70.

Kannel, W. B. and D. L. McGee. Diabetes and cardiovascular disease. The Framingham study. *JAMA* 1979;241:2035-8.

- Kannel, W. B. and P. W. Wilson. Risk factors that attenuate the female coronary disease advantage. *Arch Intern Med* 1995;155:57-61.
- Kinlay, S. Low-density lipoprotein-dependent and -independent effects of cholesterol-lowering therapies on C-reactive protein: a meta-analysis. *J Am Coll Cardiol* 2007;49:2003-9.
- Koren, M. J., P. Lundqvist, M. Bolognese, et al. Anti-PCSK9 monotherapy for hypercholesterolemia: the MENDEL-2 randomized, controlled phase III clinical trial of evolocumab. *J Am Coll Cardiol* 2014;63:2531-40.
- Kottgen, A., S. D. Russell, L. R. Loehr, et al. Reduced kidney function as a risk factor for incident heart failure: the atherosclerosis risk in communities (ARIC) study. *J Am Soc Nephrol* 2007;18:1307-15.
- Lajer, M., I. Tarnow, A. D. Michelson, et al. Soluble CD40 ligand is elevated in type 1 diabetic nephropathy but not predictive of mortality, cardiovascular events or kidney function. *Platelets* 2010;21:525-32.
- Landray, M. J., D. C. Wheeler, G. Y. Lip, et al. Inflammation, endothelial dysfunction, and platelet activation in patients with chronic kidney disease: the chronic renal impairment in Birmingham (CRIB) study. *Am J Kidney Dis* 2004;43:244-53.
- Lee, W. L., A. M. Cheung, D. Cape, et al. Impact of diabetes on coronary artery disease in women and men: a meta-analysis of prospective studies. *Diabetes Care* 2000;23:962-8.
- Levey, A. S., J. P. Bosch, J. B. Lewis, et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999;130:461-70.
- Levey, A. S. and J. Coresh. Chronic kidney disease. *Lancet* 2012;379:165-80.
- Li, N., A. H. Goodall and P. Hjerdahl. Efficient flow cytometric assay for platelet-leukocyte aggregates in whole blood using fluorescence signal triggering. *Cytometry* 1999;35:154-61.
- Li, N., H. Hu, M. Lindqvist, et al. Platelet-leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol* 2000;20:2702-8.
- Li, N., N. H. Wallen and P. Hjerdahl. Evidence for prothrombotic effects of exercise and limited protection by aspirin. *Circulation* 1999;100:1374-9.
- Lievens, D., A. Zerneck, T. Seijkens, et al. Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. *Blood* 2010;116:4317-27.
- Lim, H. S., A. D. Blann and G. Y. Lip. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation* 2004;109:2524-8.
- Lutz, J., J. Menke, D. Sollinger, et al. Haemostasis in chronic kidney disease. *Nephrol Dial Transplant* 2014;29:29-40.
- Mackman, N. The many faces of tissue factor. *J Thromb Haemost* 2009;7 Suppl 1:136-9.
- McCullough, K., P. Sharma, T. Ali, et al. Measuring the population burden of chronic kidney disease: a systematic literature review of the estimated prevalence of impaired kidney function. *Nephrol Dial Transplant* 2012;27:1812-21.
- McLaren, J. E. and D. P. Ramji. Interferon gamma: a master regulator of atherosclerosis. *Cytokine Growth Factor Rev* 2009;20:125-35.

- Mobarrez, F., J. Antovic, N. Egberg, et al. A multicolor flow cytometric assay for measurement of platelet-derived microparticles. *Thromb Res* 2010;125:e110-6.
- Mobarrez, F., N. Egberg, J. Antovic, et al. Release of endothelial microparticles in vivo during atorvastatin treatment; a randomized double-blind placebo-controlled study. *Thromb Res* 2012;129:95-7.
- Mobarrez, F., S. He, A. Broijersen, et al. Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease. *Thromb Haemost* 2011;106:344-52.
- Moody, W. E., N. C. Edwards, M. Madhani, et al. Endothelial dysfunction and cardiovascular disease in early-stage chronic kidney disease: cause or association? *Atherosclerosis* 2012;223:86-94.
- Navarro-Gonzalez, J. F., C. Mora-Fernandez, M. Muros de Fuentes, et al. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nat Rev Nephrol* 2011;7:327-40.
- Nitsch, D., M. Grams, Y. Sang, et al. Associations of estimated glomerular filtration rate and albuminuria with mortality and renal failure by sex: a meta-analysis. *BMJ* 2013;346:f324.
- Nomura, S., A. Shouzu, S. Omoto, et al. Effects of losartan and simvastatin on monocyte-derived microparticles in hypertensive patients with and without type 2 diabetes mellitus. *Clin Appl Thromb Hemost* 2004;10:133-41.
- Osterud, B. Tissue factor/TFPI and blood cells. *Thromb Res* 2012;129:274-8.
- Owens, A. P., 3rd, J. R. Byrnes and N. Mackman. Hyperlipidemia, tissue factor, coagulation, and simvastatin. *Trends Cardiovasc Med* 2014;24:95-8.
- Owens, A. P., 3rd and N. Mackman. Microparticles in hemostasis and thrombosis. *Circ Res* 2011;108:1284-97.
- Palmer, S. C., J. C. Craig, S. D. Navaneethan, et al. Benefits and harms of statin therapy for persons with chronic kidney disease: a systematic review and meta-analysis. *Ann Intern Med* 2012;157:263-75.
- Pawlak, K., J. Tankiewicz, M. Mysliwiec, et al. Tissue factor/its pathway inhibitor system and kynurenines in chronic kidney disease patients on conservative treatment. *Blood Coagul Fibrinolysis* 2009;20:590-4.
- Rehnqvist, N., P. Hjendahl, E. Billing, et al. Effects of metoprolol vs verapamil in patients with stable angina pectoris. The Angina Prognosis Study in Stockholm (APSIS). *Eur Heart J* 1996;17:76-81.
- Roberts, A. C. and K. E. Porter. Cellular and molecular mechanisms of endothelial dysfunction in diabetes. *Diab Vasc Dis Res* 2013;10:472-82.
- Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999;340:115-26.
- Sarnak, M. J. Cardiovascular complications in chronic kidney disease. *Am J Kidney Dis* 2003;41:11-7.
- Schmidt, M. I., B. B. Duncan, A. R. Sharrett, et al. Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. *Lancet* 1999;353:1649-52.

- Schonbeck, U. and P. Libby. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* 2004;109:II18-26.
- Schwabe, R. F., H. Engelmann, S. Hess, et al. Soluble CD40 in the serum of healthy donors, patients with chronic renal failure, haemodialysis and chronic ambulatory peritoneal dialysis (CAPD) patients. *Clin Exp Immunol* 1999;117:153-8.
- Shantsila, E., P. W. Kamphuisen and G. Y. Lip. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. *J Thromb Haemost* 2010;8:2358-68.
- Shlipak, M. G., L. F. Fried, M. Cushman, et al. Cardiovascular mortality risk in chronic kidney disease: comparison of traditional and novel risk factors. *JAMA* 2005;293:1737-45.
- Shlipak, M. G., J. A. Simon, D. Grady, et al. Renal insufficiency and cardiovascular events in postmenopausal women with coronary heart disease. *J Am Coll Cardiol* 2001;38:705-11.
- Siasos, G., D. Tousoulis, E. Oikonomou, et al. Inflammatory markers in hyperlipidemia: from experimental models to clinical practice. *Curr Pharm Des* 2011;17:4132-46.
- Siegel, R. D., A. Cupples, E. J. Schaefer, et al. Lipoproteins, apolipoproteins, and low-density lipoprotein size among diabetics in the Framingham offspring study. *Metabolism* 1996;45:1267-72.
- Sommeijer, D. W., K. Joop, A. Leyte, et al. Pravastatin reduces fibrinogen receptor gpIIIa on platelet-derived microparticles in patients with type 2 diabetes. *J Thromb Haemost* 2005;3:1168-71.
- Stenvinkel, P. and A. Alvestrand. Inflammation in end-stage renal disease: sources, consequences, and therapy. *Semin Dial* 2002;15:329-37.
- Szumner, K., P. Lundman, S. H. Jacobson, et al. Relation between renal function, presentation, use of therapies and in-hospital complications in acute coronary syndrome: data from the SWEDEHEART register. *J Intern Med* 2010;268:40-9.
- Tehrani, S., F. Mobarrez, A. Antovic, et al. Atorvastatin has antithrombotic effects in patients with type 1 diabetes and dyslipidemia. *Thromb Res* 2010;126:e225-31.
- Tehrani, S., F. Mobarrez, P. E. Lins, et al. Impaired endothelium-dependent skin microvascular function during high-dose atorvastatin treatment in patients with type 1 diabetes. *Diab Vasc Dis Res* 2013;10:483-8.
- Thijs, A., P. W. Nanayakkara, P. M. Ter Wee, et al. Mild-to-moderate renal impairment is associated with platelet activation: a cross-sectional study. *Clin Nephrol* 2008;70:325-31.
- Tonelli, M., C. Bohm, S. Pandeya, et al. Cardiac risk factors and the use of cardioprotective medications in patients with chronic renal insufficiency. *Am J Kidney Dis* 2001;37:484-9.
- Tonelli, M., C. Wanner and M. Kidney Disease: Improving Global Outcomes Lipid Guideline Development Work Group. Lipid management in chronic kidney disease: synopsis of the Kidney Disease: Improving Global Outcomes 2013 clinical practice guideline. *Ann Intern Med* 2014;160:182.
- Tsimihodimos, V., E. Dounousi and K. C. Siamopoulos. Dyslipidemia in chronic kidney disease: an approach to pathogenesis and treatment. *Am J Nephrol* 2008;28:958-73.
- Tuttle, K. R., G. L. Bakris, R. W. Bilous, et al. Diabetic Kidney Disease: A Report From an ADA Consensus Conference. *Am J Kidney Dis* 2014;64:510-33.

- Undas, A., K. E. Brummel-Ziedins and K. G. Mann. Statins and blood coagulation. *Arterioscler Thromb Vasc Biol* 2005;25:287-94
- van der Velde, M., K. Matsushita, J. Coresh, et al. Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int* 2011;79:1341-52.
- Versteeg, H. H., J. W. Heemskerk, M. Levi, et al. New fundamentals in hemostasis. *Physiol Rev* 2013;93:327-58.
- Weiner, D. E., H. Tighiouart, M. G. Amin, et al. Chronic kidney disease as a risk factor for cardiovascular disease and all-cause mortality: a pooled analysis of community-based studies. *J Am Soc Nephrol* 2004;15:1307-15.
- Woo, J. S., W. Kim, S. R. Lee, et al. Platelet reactivity in patients with chronic kidney disease receiving adjunctive cilostazol compared with a high-maintenance dose of clopidogrel: results of the effect of platelet inhibition according to clopidogrel dose in patients with chronic kidney disease (PIANO-2 CKD) randomized study. *Am Heart J* 2011;162:1018-25.
- Wright, R. S., G. S. Reeder, C. A. Herzog, et al. Acute myocardial infarction and renal dysfunction: a high-risk combination. *Ann Intern Med* 2002;137:563-70.
- Yagmur, E., R. D. Frank, J. Neulen, et al. Platelet Hyperaggregability is Highly Prevalent in Patients With Chronic Kidney Disease: An Underestimated Risk Indicator of Thromboembolic Events. *Clin Appl Thromb Hemost* 2013;
- Yngen, M., C. G. Ostenson, H. Hu, et al. Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia* 2004;47:537-40.