Coagulation and inflammation in diabetes mellitus
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Summary and conclusions
Chapter 1
The past decennia, the prevalence of diabetes mellitus type 2 has increased dramatically in the Netherlands. One of the major clinical problems for these patients is the high risk for cardiovascular disease. It has been shown that in addition to hyperglycemia other classical risk factors such as hypercholesterolemia, hyperinsulinemia, hypertension and obesity contribute to the development of vascular disease in diabetic patients. However, there is also evidence that non-traditional risk factors such as increased coagulation activation might be involved in the increased risk for cardiovascular disease in patients with type 2 diabetes.

Myocardial infarction and stroke are both examples of acute arterial complications of cardiovascular disease that are more prevalent in patients with type 2 diabetes. These complications are the result of an occluding thrombus in an atherosclerotic artery, leading to downstream ischemia. Coagulation activation is essential for the development of these thrombotic complications. It is generally considered that increased coagulation activation will result in a higher risk for an acute thrombotic complication like a myocardial infarction. Diabetes mellitus type 2 is associated with increased coagulation activation in blood, which might increase the risk for an acute thrombotic complication. In addition, there is evidence that diabetes mellitus type 2 is associated with changes in the vessel wall that might accelerate thrombus formation. In this thesis the coagulation activation in blood and changes in the vessel wall and kidney that might influence thrombus formation have been studied in relation to the development of cardiovascular complications in patients with type 2 diabetes and in mice with experimental diabetes.

Chapter 2
Increased levels of markers of coagulation and vascular activation are associated with a higher risk for cardiovascular disease. In this chapter the relation between these markers and the presence and development of cardiovascular complications have been studied in patients with type 2 diabetes. Therefore the levels of markers of coagulation and vascular activation were compared between diabetic patients with and without microvascular, macrovascular and neurogenic complications and compared with age-matched controls without diabetes. Higher levels of plasminogen activator inhibitor 1 (PAI-1), tissue type plasminogen activator (tPA) and soluble TF (sTF) were observed in patients with type 2 diabetes compared to
the age-matched controls. Furthermore, sTF was found to be independently associated with prevalent microvascular disease (retinopathy and nephropathy) and neurological complications. This finding suggests that sTF is a promising marker of microvascular disease in patients with type 2 diabetes.

Chapter 3
Cholesterol-lowering with HMG-CoA reductase inhibitors, “statins”, reduces the risk for cardiovascular complications in patients with type 2 diabetes with about 25 percent. There is evidence that this protective effect is the result not only from the cholesterol-lowering action, but also from anti-inflammatory and anticoagulant properties of these agents. In this chapter, the anti-inflammatory and anticoagulant properties of statins have been studied in patients with type 2 diabetes. Patients were treated with pravastatin 40 mg for 8 weeks, where after blood was taken for the measurement of coagulation and inflammation markers. These measurements were compared with those done in the same patients after 8 weeks without treatment. The results revealed that pravastatin has indeed anti-inflammatory and anticoagulant properties in patients with type 2 diabetes. High sensitivity C reactive protein, von Willebrand factor antigen, soluble tissue factor and prothrombin fragment 1+2 were reduced after pravastatin treatment. It is likely that these reductions play a role in the prevention of cardiovascular complications in patients with type 2 diabetes.

Chapter 4
In this chapter the effect of pravastatin on microparticles has been studied. Microparticles are small particles, shed from cellular membranes that circulate in blood. These particles are considered to play a role in thrombus formation since they expose proteins that are mediate platelet-vessel wall and platelet-coagulation enzyme interactions.. There is evidence that cholesterol-lowering treatment reduces the number of microparticles in blood, which, theoretically, would reduce the risk for an acute thrombotic complication. Therefore level and composition of microparticles were studied after an eight week pravastatin treatment period and compared with an eight week non-treatment period in type 2 diabetic patients. This study revealed that cholesterol-lowering with pravastatin has no effect on the number of microparticles in patients with type 2 diabetes. However, the composition of platelet derived microparticles was changed after
Pravastatin treatment. Pravastatin treatment resulted in a reduction of the exposure of glycoprotein (GP) IIIa on platelet derived microparticles. This is probably due to reduced platelet activation from pravastatin since a reduction of platelet activation is associated with reduced GPIIIa exposure. GPIIIa is part of the fibrinogen receptor. A reduced exposure of GPIIIa may therefore have an anticoagulant effect and may lower the risk for an acute arterial complication.

Chapter 5
An increased expression of procoagulant genes and a reduced expression of anticoagulant genes in the atherosclerotic vessel wall may increase the risk for acute arterial complications in patients with type 2 diabetes. In this study the presence of tissue factor and endothelial protein C receptor were investigated in symptomatic plaques from patients with type 2 diabetes and age-matched controls. In addition, the presence of advanced glycosylated endproducts, macrophages, T-cells, smooth muscle cells and the stage of the plaques were compared. This study showed that symptomatic plaques from type 2 diabetic patients have more signs of earlier complications than plaques from control patients, which is consistent with the fact that diabetes is associated with an increased risk for cardiovascular complications. However, the number of the different cell types and the level of advanced glycosylated endproducts did not differ markedly between patients with and without type 2 diabetes. Also the expression of tissue factor and endothelial protein C receptor were similar in plaques from diabetic and non-diabetic patients. Thus, in this end stage of atherosclerotic disease, local differences in coagulation and inflammation activation do not seem to be involved in the increased risk for cardiovascular complications in diabetes mellitus type 2.

Chapter 6
Tissue factor, the main initiator of the coagulation cascade, is upregulated in the kidney in various nephropathies and seems to be involved in the pathology of these diseases. It is unknown whether tissue factor is upregulated in diabetic nephropathy. In this study the expression of tissue factor and other coagulation proteins, thrombin and fibrinogen, was evaluated in a model of diabetic nephropathy. Therefore kidneys were studied from mice with streptozotocin induced diabetes. This study showed that the activation of tissue factor is increased in the diabetic kidneys compared to the control kidneys. In addition, thrombin
and fibrinogen were upregulated in the kidneys from the diabetic mice. Whether the increased activation of tissue factor may be induced by the hyperglycemia in the diabetic mice was studied in an in vitro study in which tubular epithelial cells were incubated with high and low amounts of glucose. Cells incubated with high glucose levels produced significantly more tissue factor than cells with normal amounts of glucose. It was concluded that hyperglycemia may have induced the increased tissue factor activity in the model of diabetic nephropathy.

Chapter 7, 8 and 9
In these chapters the effect of diabetes mellitus on vascular gene expression and arterial thrombus formation was studied. Therefore mice were used with streptozotocin induced diabetes in which vascular mRNA levels of various genes involved in coagulation and inflammation and ferric chloride induced arterial thrombus formation was evaluated.

In chapter 7 first the effect of hyperglycemia on vascular gene and arterial thrombus formation was studied. This revealed that hyperglycemia for 10 weeks had no effect on vascular activation. However, coagulation activation was slightly increased in the diabetic mice; increased levels of thrombin anti-thrombin complexes and accelerated thrombus formation were observed in the diabetic mice. In addition, in the diabetic mice the anticoagulant and anti-inflammatory effect of statins was studied by treating them for two weeks with simvastatin. Simvastatin resulted in reduced expression of vascular adhesion molecule, a proinflammatory protein, in the vessel wall, but did not affect thrombus formation. It was concluded that statins prevent for cardiovascular disease by there cholesterol-lowering effect and probably by there anti-inflammatory effect more than by there anti-coagulant effect.

In chapter 8 the effect of hyperglycemia induction was studied on vascular gene expression and coagulation activation in the presence of low grade inflammation. Therefore mice with and without streptozotocin induced diabetes were treated with a low dose endotoxin. Twenty four hours after endotoxin treatment a prolonged thrombus formation period was observed in control mice compared to non-endotoxin treated mice, propably due to endothelial tolerance. However, in the diabetic mice this prolonged thrombus formation did not appear after endotoxin. In addition, vascular gene expression after endotoxin differed in the diabetic mice compared to the control mice, in particular the expression of endothelial nitric oxide synthase was enhanced in the diabetic vessel wall. It was
therefore concluded that a changed vascular response to inflammation could be involved in the increased risk for cardiovascular complications in diabetes mellitus. In chapter 9 the effect of hyperglycemia on vascular gene expression and thrombus formation on a background of atherosclerosis was studied. Therefore streptozotocin diabetes was induced in mice with a LDL receptor deficiency and on an atherogenic diet. Diabetes induction resulted in changed vascular gene expression in these mice, which was correlated with cholesterol levels but not with glucose levels. Furthermore, the diabetic mice had greater plaques than control mice. However thrombus formation was similar in the diabetic mice compared to the control mice. It was concluded that on a background of atherosclerosis diabetes induction results in vascular activation and increased atherosclerosis and thereby probably in a higher risk for thrombotic complications.

Chapter 10
In this chapter we present various methods that can improve measuring coagulation activation in mice. First, it is described that intravenous injection of sodiumcitrate can induce a better coagulating effect in mice plasma than the retrospective administration of sodiumcitrate. Furthermore, the contamination of heparin in mice plasma can be neutralized with heparins, which makes this plasma become available for coagulation tests. In conclusion, we describe the development of a specific mice enzyme linked imunosorbent assay (ELISA) to indicate the amount of thrombin-antithrombin complexes in the blood. This ELISA appears to measure thrombin-antithrombin complexes at an equal level as commercially available humane assays.