Coagulation and inflammation in diabetes mellitus
Sommeijer, D.W.

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General Overview and outline of the thesis

Dirkje W. Sommeijer¹, Petra G.C. van Os¹, Pieter H. Reitsma¹
and Hugo ten Cate²

¹Laboratory for Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, ²Department of Internal Medicine and Cardiovascular Research Institute Maastricht, Academic Hospital and University of Maastricht, Maastricht, The Netherlands
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8. Outline of the thesis
1. Introduction

The prevalence of diabetes mellitus has dramatically increased during the past decades and this disease is now affecting about half a million people in the Netherlands (1). The growing number of diabetic people is mainly due to an increase in type 2 diabetes (about 90% of diabetic patients has type 2, the other 10% has type 1 diabetes), and has been attributed to an increased prevalence of obesity. Obesity is associated with dysregulations in metabolism that strongly increase the risk for the development of type 2 diabetes (2), as discussed below. The increasing age of the Dutch population and a better awareness of diabetes in an elderly population has further contributed to the high prevalence of type 2 diabetes (1). It has been estimated that over 10% of the Dutch population 50 years of age and older have type 2 diabetes or its precursor, impaired glucose tolerance (Figure 1) (3).

![Figure 1. Mean prevalence of diabetes mellitus type 1 and 2 in the Netherlands divided in men and women (2003). Prevalence increases with age.](image)

A major clinical problem for patients with diabetes is the high risk for cardiovascular disease (4). For instance, the risk of developing a myocardial infarction is 2 to 3 times higher in diabetic patients than in non-diabetic individuals of comparable age (5). In addition, the outcome of cardiovascular complications is worse in patients with diabetes compared to patients without diabetes (6). The high risk for cardiovascular disease and the worse outcome accounts for about 70% of deaths in these patients (7).
There has been increased interest in mechanisms that accelerate vascular disease in diabetes the past 10 years (8). The research has clarified that multiple factors are involved in the development of diabetic cardiovascular disease, but the molecular pathogenesis is still not completely understood. The incomplete understanding of this complex disease limits the options for prevention of vascular disease in diabetics, which remains a major health care problem in the Netherlands and the rest of the Western world.

This thesis addresses the pathogenesis of cardiovascular disease in patients with type 2 diabetes from the perspective of the coagulation system. As we will make clear, several components from the coagulation system play important roles in the pathogenesis of atherothrombosis and the development of organ damage. In the following chapters, different aspects of the blood coagulation system, vessel wall and kidney in relation to hyperglycemia and the increased risk for cardiovascular disease will be explored.

2. Diabetes mellitus type 1 and 2

Two main types of diabetes mellitus can be distinguished. Type 1 diabetes, occurring at relatively young age, is an autoimmune disease caused by destruction of beta cells in the pancreas and subsequent loss of insulin production. Due to lack of insulin production, glucose is not adequately transported from the blood into peripheral tissue. This results in high glucose levels in blood and urine and to low glucose levels in the muscles and other tissues. The cornerstone of treatment for type 1 diabetes is insulin administration. Specific life style factors that increase the risk for type 1 diabetes have not been identified yet, which is in contrast to type 2 diabetes.

Type 2 diabetes is strongly associated with a sedentary lifestyle and obesity (2). In particular central and visceral obesity, characterized by fat deposition in the upper and central part of the body are responsible for the development of the so-called insulin resistance syndrome (9) which precedes overt type 2 diabetes. Insulin resistance is characterized by relative insensitivity of insulin receptor signaling, leading to a relatively deficiency of insulin to transport glucose sufficiently into the peripheral tissue. To compensate this relative shortage the pancreas enhances the amount of insulin production. The resulting state of hyperinsulinemia with relatively normal glucose levels can last for years.
Genera ll overview

Gradually, however, the beta cells in the pancreas lose their capacity to produce large amounts of insulin and glucose levels start to increase above normal levels. A state of type 2 diabetes has than developed, typically characterized by relatively high insulin as well as high glucose levels in blood. Therefore, and in contrast with type 1 diabetes, therapy for type 2 diabetes is focused on treatment of insulin resistance, by a combination of weight loss and specific medication. When these measures do not result in normoglycemia, combined treatment of oral medication and insulin may be warranted.

In spite of major differences in etiology of type 1 and type 2 diabetes, which is reflected by the specific treatment of these conditions, there are also two important common features, hyperglycemia (glucose > 7 mmol/L) and an increased risk for cardiovascular disease.

3. Diabetes mellitus and cardiovascular disease

Although in essence an endocrine disorder, the main phenotypic characteristic of type 2 diabetes is cardiovascular disease. This involves both complications in the smaller vessels, leading to nephropathy, neuropathy and retinopathy, and complications in the larger vessels, leading to coronary artery disease, stroke and peripheral artery disease. Until recently it was thought that the increased risk for cardiovascular disease in diabetes could be reduced by effective treatment of hyperglycemia. Several large clinical studies including The United Kingdom Prospective Diabetes Study (10), have now demonstrated that treatment of hyperglycemia indeed significantly reduces small vessel disease. In contrast, it appears that the risk for large vessel disease remains substantially increased compared to non-diabetics. Such studies suggest that the remaining risk for large vessel complications is determined by additional cardiovascular risk factors, including hypercholesterolemia, obesity, hyperinsulinemia, hypertension, increased activity of inflammation and coagulation pathways, which comprise the metabolic syndrome in type 2 diabetes (11; 12). The current consensus opinion is that prevention of cardiovascular disease in type 2 diabetic patients requires treatment of each of these risk factors (11; 13-16). While type 1 diabetes is also associated with dyslipidemia and changes in the coagulation and inflammation systems, the contribution of these risk factors to cardiovascular events is not as clear as in type 2 diabetes.
4. Atherosclerosis and atherothrombotic events

In general, atherosclerosis is the underlying pathological process of cardiovascular disease, already starting in young adulthood. An early feature of the atherosclerotic process is dysfunction of vascular endothelium, the inner layer of the vessel wall (Figure 2). Under influence of specific triggers, such as oxidized LDL, endothelial cells produce proinflammatory cytokines and adhesion molecules that in turn are responsible for the capture of activated leukocytes from the streaming blood. Expression of chemotactic cytokines causes migration of the adherent leukocytes through the endothelial layer into the vessel wall proper. These leukocytes, in particular macrophages, scavenge lipids to form foam cells and the vessel wall lesion becomes a “fatty streak” (Figure 3). Cycles of accumulation of leukocytes,
migration and proliferation of smooth muscle cells and the formation of fibrous tissue lead to enlargement and remodeling of the lesion. This process finally leads to a "complicated plaque", characterized by a large lipid core rich in tissue factor (TF) and a high risk to rupture (Figure 4). If a plaque ruptures TF, the initiator of the coagulation cascade, and atherosclerotic debris is exposed to the blood stream resulting in the formation of a thrombus, a blood clot consisting of platelets and threads of fibrin. This thrombus can completely occlude the vessel lumen, leading to ischemia in the downstream tissue and this represents a so-called acute atherothrombotic event (Figure 5). The main acute atherothrombotic complications of cardiovascular disease, such as a myocardial infarction result from a thrombus formed on a ruptured plaque or from a thrombus formed on an eroded plaque.
atherosclerotic vessel wall (17; 18). The development of atherothrombotic complications, is considered to be determined by a combination of factors including blood flow, (altered) blood composition and pre-existing vascular changes, in totality known as Virchow’s triad (19-24). In patients with diabetes mellitus type 2 two of these elements have been identified to be abnormal and are supposed to increase the risk for a thrombotic event: first, an imbalance of hemostatic, procoagulant and fibrinolytic factors in blood and second, changes in vessel wall composition.

5. Prothrombotic factors in blood contributing to increased risk for atherothrombotic events

Since coagulation is an essential element in the development of atherothrombotic complications many investigators believe that a condition of activated coagulation, and/or inhibited fibrinolysis, i.e. the hypercoagulable state, may be involved in the pathogenesis of such complications (25). Indeed, a large body of evidence indicates that patients at risk to develop atherothrombotic complications, such as patients with type 2 diabetes, have increased levels of procoagulant proteins and enzymes in blood as compared to age matched controls (26-28).

The blood coagulation system consists of a primary hemostatic mechanism, designed by nature to arrest bleeding. This system consists of vasoconstriction and blood platelets, which by adhesion to the vessel wall and by aggregation, stop bleeding or cover a wound surface (such as in the case of a ruptured atherosclerotic plaque). The secondary mechanism, that operates simultaneously with the primary system, consists of plasmatic and vessel wall proteins, indicated in Figure 6. This system of linked reactions operates by stepwise enzymatic cleavage of precursor proteins leading to the formation of fibrin, which acts as a “glue” to establish the blood clot (or the thrombus in case of pathological clotting like on ruptured plaques). To limit the extent of clotting, the blood contains a system that degrades fibrin clots, i.e. fibrinolysis as well as a system that has anticoagulant properties, including antithrombin, heparin cofactor II, tissue factor pathway inhibitor, and the protein C anticoagulant pathway. The activity of coagulation and fibrinolytic systems is determined by the availability (concentration, localization, conformation) of the different proteins (including
activators, zymogens, and inhibitors). Elevations of activators such as TF may drive fibrin formation, while elevations in platelet numbers and in adhesive proteins (von Willebrand factor) may enhance primary hemostasis (platelet “stickiness”).

In plasma from patients with type 2 diabetes higher levels of markers that indicate increased plasma coagulation activity such as D-dimer (29; 30), prothrombin fragment 1+2 (F1+2) (25; 31) thrombin anti-thrombin (TAT) complexes (32), fibrinogen (33), factor VII (34) and an increased expression of TF on circulating monocytes (35) have been observed. In addition, circulating plasma markers of fibrinolysis including tissue type plasminogen activator (tPA) (30; 36) and the inhibitor, plasminogen activator inhibitor 1 (PAI-1) (28; 36-39) are higher in patients with type 2 diabetes. Von Willebrand factor antigen (vWFAG) and thrombomodulin (TM), which is a component of the protein C anticoagulant system, are also
reported to be increased in patients with type 2 diabetes (30; 36) (40). A number of these proteins, including vWFag, TM, tPA, and PAI-1, are produced by vascular endothelial cells (41). Detection of increased levels in blood is therefore considered to reflect endothelial activation or damage, as an indication of vascular disease (41).

Whether there is a causal relation between elevation of these markers of coagulation and fibrinolysis, and endothelial dysfunction on the one hand, and existing or developing cardiovascular complications on the other hand in type 2 diabetic patients is not yet established and remains controversial. This uncertainty is mainly due to the lack of clinical studies showing that anticoagulant agents reduce the risk of cardiovascular complications in diabetic patients, while pro-fibrinolytic agents for long-term treatment are not yet available.

Similarly, a number of abnormalities in platelet function, including increased levels of surface adhesion molecules (P-selectin) and the fibrinogen receptor glycoprotein IIbIIIa have been reported in platelets from patients with diabetes (42). In addition, platelets from diabetic patients are more sensitive to aggregating agents and display enhanced synthesis of thromboxane, together contributing to the platelet hyperreactivity that is observed in patients with type 2 diabetes (27). Such changes in platelet functions are generally believed to be of importance in the risk for atherothrombotic complications in diabetes. The importance of platelets is demonstrated by the apparent efficacy of platelet inhibiting agents including aspirin and the more selective glycoprotein IIbIIIa inhibitors in reducing cardiovascular morbidity and mortality in patients with type 2 diabetes (43).

6. Vascular factors contributing to increased risk for atherothrombotic events

The development of atherothrombotic complications is considered to be primarily determined by pre-existent vascular changes, culminating in atherosclerosis (22-24). Abnormalities in vascular function and structure, which may predispose to atherothrombotic complications, have been identified in patients with type 2 diabetes (Table 1). General changes in the vessel walls of diabetic patients are endothelial dysfunction (44), arterial hypertrophy and distensibility (45). Evidence suggests that these structural and functional alterations already exist before the
Table 1. Vascular elements predisposing to atherothrombotic complications

<table>
<thead>
<tr>
<th>Structural and functional characterizations</th>
<th>Vulnerable plaque</th>
<th>Type I diabetic vs. non-diabetic artery plaque</th>
<th>Type II diabetic vs. non-diabetic artery plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>Moderate -69</td>
<td>Moderate -52</td>
<td>Moderate -52</td>
</tr>
<tr>
<td>Lipid content</td>
<td>↑ (49; 70)</td>
<td>↑ ?</td>
<td>↑ ?</td>
</tr>
<tr>
<td>Collagen</td>
<td>Reduced -71</td>
<td>Disturbed -58</td>
<td>Disturbed, Reduced (55; 58)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>↑↓ (49; 72)</td>
<td>↑ -73</td>
<td>↑ -73</td>
</tr>
<tr>
<td>Endothelium</td>
<td>Dysfunction -74</td>
<td>Dysfunction -44</td>
<td>Dysfunction -44</td>
</tr>
<tr>
<td>Distensibility &amp; Stiffness</td>
<td>↑ -51</td>
<td>↑ -75</td>
<td>↑ -45</td>
</tr>
</tbody>
</table>

Inflammation

| Macrophages                                  | ↑ (49; 53; 54; 70; 76; 77) | ↑ -50 | ↑ (50;55;78) |
| T lymphocytes                                | ↑ (53; 79)                  | ↑ -50 | ↑ (55; 78)   |
| IL-6                                         | ↑ (80-82)                   | ↑ -59 | ↑ ?          |
| TNF-α                                       | ↑ (63-85)                   | ↑ -59 | ↑ -62        |
| E-selectin                                   | ↑ (79; 85)                  | ↑ -99 | ↔ -60        |
| P-selectin                                   | ↑ (79; 85)                  | ↑ -99 | ↑ ?          |
| ICAM-1                                       | ↑ (80; 85; 86)              | ↔ -100| ↑ -60        |
| VCAM-1                                       | ↑ (79; 87; 88)              | ↑ (61),(59),(99) | ↑ -60 |
| MCP-1                                        | ↑ -89                      | ↑ -59 | ↑ ?          |
| MMP                                          | ↑ -90                      | ↑ -92 | ↑ -92        |
| AGE                                          | ↑ -61                      | ↑ -92 | ↑ -92        |
| RAGE                                         | ↑ -61                      | ↑ -92 | ↑ -92        |

Coagulation

| Antithrombin                                  | ↓ -93                     | ↓ -64 | ↑ ?          |
| Fibrinogen                                    | ↑ -94                     | ↑ -64 | ↑ ?          |
| TF                                           | ↑ (79; 95)                | ↑ -61 | ↑ ?          |
| PAI-1                                        | ↑ (96; 97)                | ↑ -61 | ↑ -66        |
| u-PA                                         | ↑ -96                     | ↑ -66 | ↑ -66        |

Data concerning type 1 diabetes are partly based on data from animal models of type 1 diabetes. IL=interleukin, TNF-α=tumor necrosis factor-α, ICAM-1= intercellular adhesion molecule-1, VCAM-1= vascular cell adhesion molecule-1, MCP-1= monocyte chemoattractant protein-1, MMP=metalloproteinase, AGE=advanced glycation endproduct, RAGE=receptor for AGE, TF=tissue factor, PAI-1=plasminogen activator inhibitor type 1, u-PA=urokinase-type plasminogen activator.

↑=increased, ↓=decreased, ↔=unchanged, ?=unknown

Clinical signs of type 2 diabetes become apparent (46), i.e. during the phase of insulin resistance. This also suggests that the enhanced process of atherosclerosis is not solely dependent on one of the main biochemical characteristics of type 2 diabetes, hyperglycemia.

An important player in the increased risk for atherothrombosis in diabetes is oxidative stress induced endothelial dysfunction. Oxidative stress is the result of
the generation of free radicals, such as superoxide (47) and causes vascular dysfunction by activating signalling pathways in endothelial and other vascular cells leading to reduced nitric oxide (NO) production and increased platelet and leukocyte adhesion (48).

In addition, there is evidence that vascular wall associated factors that predispose to plaque rupture (49) are of importance in diabetic patients. For instance, atherosclerotic plaques from diabetic type 2 patients are associated with a significant increase in lipid pool content (50; 51) and moderate stenosis (52) which increase the risk for rupture and thrombosis. Activation of inflammation is another factor of importance in the development of atherosclerosis and plaque instability (53; 54), particularly in patients with diabetes. Several studies reported increases in macrophage infiltration in plaques from patients with type 2 diabetes compared to non-diabetic patients (50; 55). Macrophages are found in rupture prone plaque areas (53; 56), and play a role in degrading plaque stability via their production of metalloproteinases (MMPs), which are able to digest collagen and elastin (57). It has been shown that plaques from type 2 diabetic patients have increased levels of MMPs and disturbed content and biosynthesis of collagen (55; 58).

There is evidence, although limited and partly based on animal models of diabetes, that other features of a proinflammatory state of the vessel wall are also evident in diabetes mellitus (Table 1). T lymphocytes, intercellular adhesion molecule-1 (ICAM-1) (60), vascular cell adhesion molecule-1 (VCAM-1) (60; 61), monocyte chemoattractant peptide-1 (MCP-1) (59), tumor necrosis factor-α (TNFα) (62), and interleukin-6 (IL-6) (59) are found to be increased in diabetic plaques and arteries, and may predispose to an increased risk for plaque rupture and thrombotic events (55; 59).

A view is emerging that advanced glycation endproducts (AGEs) are important in inducing inflammatory responses and oxidative stress in the diabetic vasculature (63). AGEs are the products of nonenzymatic glycation of proteins and lipids that accumulate in plasma and tissue of patients with diabetes. It was shown that diabetic type 2 plaques are characterized by an increased amount of AGEs and increased expression of RAGE (receptor for AGE) (55).

Few studies addressed the presence of pro- and anticoagulant proteins in diabetic atherosclerosis that may influence plaque vulnerability and thrombosis risk (Table 1). Based on findings in animal studies, it was shown that diabetic atherosclerotic
plagues and arteries are characterized by increased TF (61) and fibrinogen (64). In addition, the anticoagulant serine protease inhibitor antithrombin was demonstrated to be decreased in the vessel wall from rabbits with diabetes (64). Studies on atherectomies and arteries from diabetic patients revealed increases in PAI-1 expression, which may lead to increased thrombogenicity (65; 66). Remarkably, one possible local risk factor, urokinase-plasminogen activator (uPA), found to be increased in advanced atherosclerotic plaques (67), seems to be reduced in diabetic atherosclerotic plaques (66); theoretically, this would suggest a diminished fibrinolytic capacity in diabetic vessel, but it is more likely that uPA is involved in vascular remodelling rather than clot lysis (68).

6. Conclusion
Type 2 diabetes is associated with an accelerated process of atherosclerosis and an increased risk for atherothrombotic complications and organ damage. Although it has become clear that multiple factors are involved in the development of cardiovascular disease in diabetes mellitus, the molecular pathogenesis is still not completely understood. Multiple abnormalities in circulating blood coagulation parameters and in the vascular expression of genes involved in coagulation activation are observed in both patients with type 2 diabetes and animals with experimental diabetes as compared to controls. However, available data on the involvement of these abnormalities in the development of atherothrombotic complications and organ damage remain in part conflicting and have not yet led to a clear understanding of their potential significance. In part this is due to a lack of proper clinical studies addressing the value of anticoagulant therapy, which could provide a proof of concept. In addition, there is a shortage in studies on mechanisms and agents that change in vivo expression of genes involved in coagulation activation and their effect on arterial thrombosis and organ damage in diabetes mellitus. However, with the expanding knowledge on the potential importance of the coagulation system in cardiovascular disease new avenues can be explored. Particularly, the role of coagulation mechanisms in modifying the increased risk for cardiovascular disease in diabetes becomes an important area of research. Against this background, the experimental and clinical studies described in this thesis have been designed.
7. Outline of the thesis

The general objective of this thesis is to gain insight into the pathogenesis of the increased risk of cardiovascular disease in diabetes mellitus type 2. Emphasis was put on the role of increased coagulation activation in acute arterial thrombosis and organ damage. We used the following observational and intervention studies in humans and mice with diabetes mellitus to accomplish this objective.

Chapter two evaluates the level of different blood coagulation markers in patients with and without cardiovascular disease and type 2 diabetes and determines the potential importance of soluble tissue factor as a marker of small vessel disease.

Chapter 3 and 4 describe the effect of pravastatin treatment on different hemostatic markers in patients with type 2 diabetes, revealing mild anti-inflammatory and anticoagulant effects of this drug that may fit with the concept of the importance of pleiotropic actions of statins. Chapter 5 analyses the difference in atherosclerotic plaque composition between patients with type 2 diabetes and control subjects. This study shows that symptomatic plaques from type 2 diabetic patients have more signs of thrombosis but similar levels of markers of coagulation and inflammation. Chapter 6 evaluates the expression of TF, the initiator of the coagulation cascade, in kidneys from mice with experimental diabetes. Here, it was shown that expression of TF is increased in tubuli from diabetic mice, probably due to hyperglycemia as suggested by in vitro experiments showing increases in TF level in tubular epithelial cells after incubation with high glucose levels.

The last part of the thesis addresses studies of several factors that are important for the development of acute arterial thrombosis in experimental diabetes mellitus. In these experiments a model of ferric chloride induced arterial thrombosis was studied in streptozotocin induced diabetic mice. Chapter 7 evaluates the effect of hyperglycemia and treatment with simvastatin on arterial thrombosis. This study reveals that hyperglycemia induces a mild prothrombotic phenotype. Simvastatin did not influence thrombus formation, but had significant anti-inflammatory effects on vessel wall gene expression. Chapter 8 evaluates the effect of both endothelial dysfunction and hyperglycemia on arterial thrombosis. To this end mice were studied with endotoxin induced endothelial dysfunction and experimental diabetes. Chapter 9 describes the effect of hyperglycemia on arterial thrombosis in LDLr⁻/⁻ mice, which are prone to develop
atherosclerosis due to a genetic defect in the LDL receptor. This study reveals that experimental diabetes alters vascular gene expression and increases atherosclerosis. Interestingly, these changes had no effect on arterial thrombus formation. Finally, **chapter 10** describes methods to evaluate coagulation activation in mice. The development of a mouse specific assay for the detection of thrombin-antithrombin complexes is reported and different methods for plasma collection are evaluated.

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