

## Activation of platelets and coagulation during haemodialysis

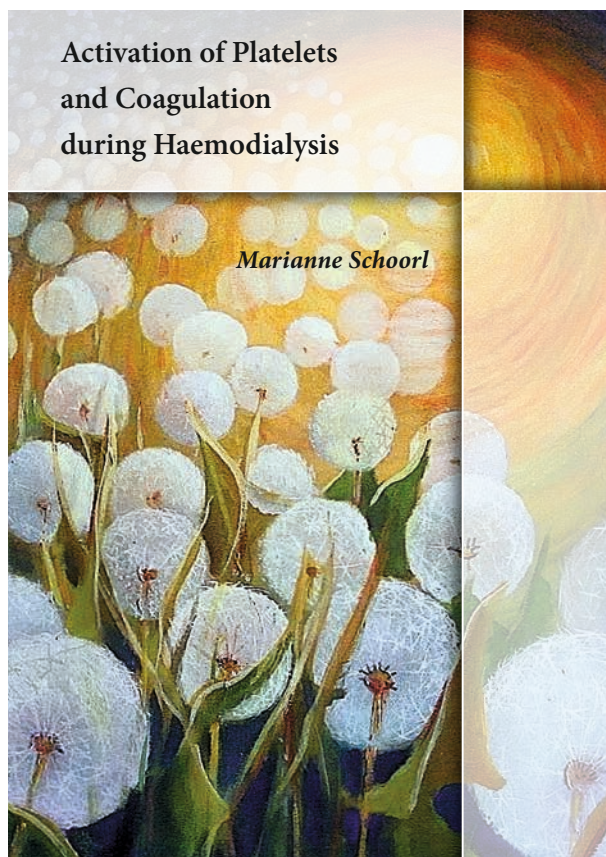
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### 1. Introduction

Chronic kidney disease affects 7% of the population world-wide over the age of 30 years, and 25-35% of people over 65 years (1). Chronic kidney disease is characterized by a strongly reduced glomerular filtration rate, hypertension and a marked activation of the sympathetic nervous system. Patients with chronic kidney disease have an increased risk for the development of end stage kidney disease. In addition, the associated morbidity and mortality from cardiovascular causes in patients with chronic kidney disease is up to 30 times higher than in the general population (2). The vascular endothelium plays a pivotal role in the modulation of vascular tone, initiation of coagulation, fibrinolysis activity and release of inflammatory mediators (3). Maintaining the functional integrity of the endothelium is important in prevention or delay of vascular diseases.

One of the main noncardiovascular factors causing death is infection (20-25%), often characterized by vascular access related infections (2).

Chronic kidney failure is measured in five stages, which are calculated using a patient's glomerular filtration rate. Stage 1 of chronic kidney disease is mildly diminished renal function, with few overt symptoms. Stages 2 and 3 need medical support to slow and treat the renal dysfunction. Patients in stages 4 and 5 usually require active treatment in order to survive. Stage 5 of chronic kidney is called end stage kidney disease and is considered as a severe illness requiring renal replacement therapy, such as haemodialysis. In end stage kidney disease glomerular filtration rate has been reduced to only 10-15%. Haemodialysis is applied to achieve extracorporeal removal of excess fluid and waste products such as uraemic toxins and creatinine from the blood. Despite a rather high efficiency of state of the art dialysers, haemodialysis treatment remains inferior to normal kidney function. Compared to a clearance rate of 90-120 ml/minutes for normal kidneys, haemodialysis treatment results in a weekly clearance of small molecular weight substances amounting to 10-15 ml/minutes. The so-called 'middle molecules' and larger molecules which are normally



excreted or metabolised by the healthy kidney, are cleared inadequately and accumulate in the course of time. Also, various interactions, including both short- and long-term side effects, occur between the subject on maintenance haemodialysis treatment and various components of the extracorporeal haemodialysis circuit (Figure 1) (4, 5).

Because the materials of the extracorporeal circuit are bio-incompatible with respect to various blood cells and components, leukocytes and platelets become activated. Haemodialysis-induced leukocyte activation has been detected, indicating a manifestation of bio-incompatibility which contributes to induction of cardiovascular changes. Platelet activation contributes to side effects of haemodialysis treatment as indicated in a higher risk for thromboembolic disease. Despite systemic anticoagulation during haemodialysis treatment, the extracorporeal circuit will still trigger activation of coagulation.

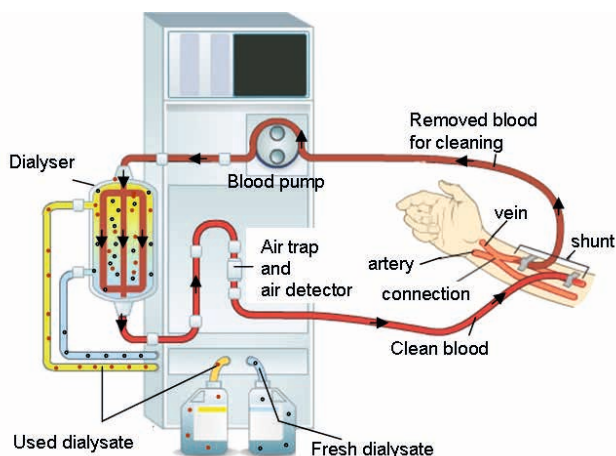
In this chapter the process of haemodialysis is discussed, including interaction with the extracorporeal circuit, application of various types of dialysis membranes and different anticoagulation procedures. Various aspects

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**Figure 1.** Haemodialysis instrumentation and the various components of the extracorporeal circuit.  
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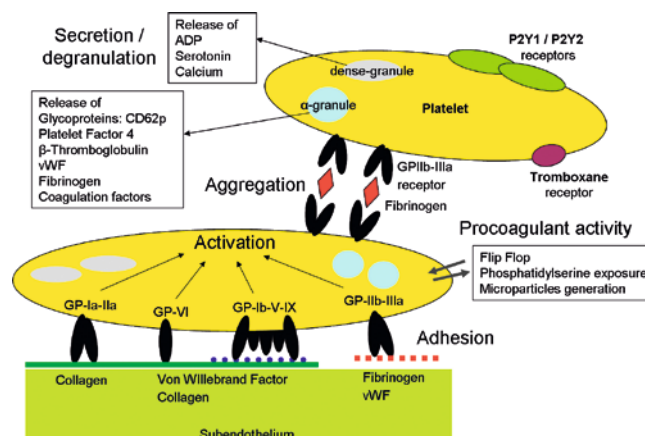
of platelet aberrations are considered with special emphasis on platelet activation, platelet degranulation, as well as endothelial integrity and activation of the intrinsic coagulation pathway in chronic kidney disease. Finally, the objectives of several studies are mentioned, which focus on haemodialysis-induced changes regarding platelet granules depletion, activation of platelets, intrinsic coagulation activation and endothelial integrity.

## 2. Platelets

Platelets belong to the smallest circulating blood cells. The reference range amounts to 150-400 x10<sup>9</sup>/L. Platelets are shedded from megakaryocytes in the bone marrow and exhibit a short life of up to 10 days in the circulation. The platelet cytoplasm contains alpha and dense granules. The α-granules are intracellular storage organelles for a wide range of adhesive proteins, coagulation factors, endothelial and other growth factors which are important for induction of platelet aggregation, inflammation and wound healing. Dense granules provide intracellular stores of serotonin, adenine nucleotides ADP and ATP and other small molecular weight substances.

Non-activated platelets, the so-called resting platelets, are small flattened discs (2-4 x 0.5  $\mu\text{m}$ ). A platelet membrane exhibits an asymmetrical distribution of the lipids between the inner and outer leaflets of the lipid bilayer, with a majority of negatively charged phospholipids on the inner leaflet. The small flattened discs continuously check the integrity of vascular endothelium.

Upon vessel wall injury, platelets undergo a sequence of reactions, including adhesion, activation of the platelet, release of its intracellular constituents, aggregation to other platelets, and generation of procoagulant activity on its surface. Vascular wall injury results in exposure of the subendothelial matrix including collagen. Platelet adhesion is initially mediated by von Willebrand factor in the plasma, which is able to construct a bridge between the exposed collagen and the GPIb-IX-V complex on the platelet membrane. Collagen binding to GPVI and to GPIIb/IIIa results in



**Figure 2.** The role of platelets in haemostasis. Modified from Harrison (7).

platelet activation. GPIb and the activated GPIIb/IIIa complex also serve as receptors and ligand binding sites for von Willebrand factor and fibrinogen. Von Willebrand factor and fibrinogen are important for firm platelet adhesion and platelet-platelet aggregation via the activated GPIIb/IIIa receptor. During platelet activation, granules release their content, such as platelet factor 4, ADP and serotonin, to the exterior. The release reaction involves the fusion of the granular membranes with the outer membrane of a platelet. Thus, originally granular membrane located components such as p-selectin (CD62p) become exposed on the platelet surface. P-selectin is therefore a good example of a receptor which becomes exposed on the platelet surface of the activated platelet. Interaction of p-selectin with its receptor p-selectin ligand on certain types of leukocytes leads to the stimulation of leukocytes to produce cytokines as an initiator of the inflammatory reaction, an example of so-called platelet-leukocyte crosstalk.

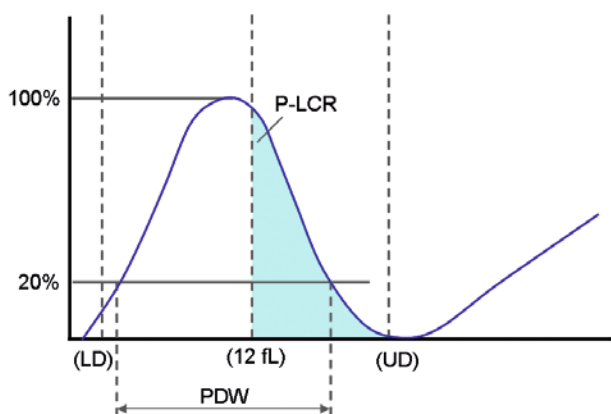
Activated platelets generate procoagulant activity as they shed microparticles from their outer membrane and expose phosphatidylserine on the outer platelet membrane which was located in the inner membrane leaflet of the resting platelet. Procoagulant activity results in generation of thrombin and fibrin formation, and stabilization of the haemostatic plug (Figure 2).

A haemostatic plug occludes the site of vascular damage to prevent blood loss. A cascade of follow-up reactions comprises clot dissolution, regrowth of normal tissue and wound healing. When considering the complexity of the haemostatic process and the essential role of the platelets, it is not surprising that aberrations may result in the impairment of haemostasis with an increased risk of bleeding (6).

## 2.1 Platelet morphology aberrations

### Platelet count and analysis of aberrations

Platelets can routinely be counted with various haematology analysers, for instance the Sysmex XE2100 Haematology Analyzer (Sysmex, Kobe,



**Figure 3.** Platelet Distribution Width and Platelet Large Cell ratio. Modified from Briggs (8).

Abbreviations: PDW = platelet distribution width; P-LCR = platelet large cell ratio; LD / UD = lower / upper discrimination for platelet size distribution

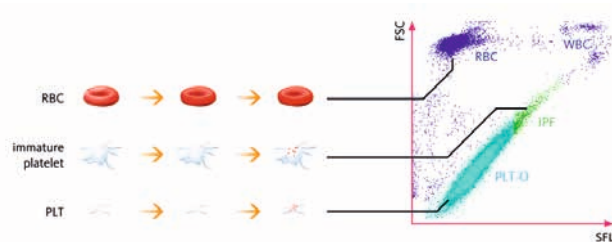
Japan). With application of this equipment a platelet size distribution plot is generated using three thresholds. One threshold is fixed at the 12 fL level and the other two thresholds are allowed to mark the upper and lower ends of the platelet population between certain limits. The lower platelet size threshold may move between 2 and 6 fL, the higher threshold between 12 and 30 fL. In addition to the traditional impedance count methodology (PLT-I), an optical fluorescent platelet count (PLT-O) methodology has been introduced on the Sysmex XE2100 haematology analyzer. The optical platelet count is measured in the reticulocyte channel. A polymethine dye is applied for staining the RNA/DNA of reticulated cells and platelet membrane and granules. The fluorescence intensity distribution of particles is analyzed to discriminate platelets from erythrocytes and reticulocytes. The fluorescent staining procedure of platelets allows the exclusion of nonplatelet particles, and is thus more effective in separating platelets from other potentially interfering particles than impedance methods.

*Additional platelet parameters: mean platelet volume, platelet distribution width, platelet large cell ratio*

Circulating platelets vary in both size and functional activity. Large platelets are probably younger, more reactive and able to produce thrombogenic factors. The Sysmex XE2100 haematology analyzer provides platelet count and platelet indices which are calculated from the platelet size distribution histogram. Platelet parameters supply clinically useful information if methodologic problems are taken into consideration. Mean platelet volume is calculated by dividing the plateletcrit by the platelet count. The platelet distribution width refers to the width of the size distribution curve in fL established at the 20% height level of the peak (Figure 3). The platelet large cell ratio corresponds to the number of cells above the 12-fL threshold divided by the total platelet count (Figure 3).

#### *Reticulated immature platelets*

After labelling with immunocytologic markers and a fluorescent dye binding to RNA, young platelets with a



**Figure 4.** Indication of the location of immature platelet fraction in the optical platelet count cluster of the reticulocyte channel of the Sysmex XE-2100 Haematology analyzer. Immature platelet fractions yield higher fluorescent intensity and are larger in size compared to mature platelets. Provided by Sysmex Europe, Hamburg, Germany.

Abbreviations: IPF = immature platelet fraction; PLT-O = optical platelet count; RBC = red blood cells; WBC = white blood cells; FSC = forward light scatter; SFL = side fluorescence light intensity

high RNA content are discriminated by flowcytometry. Young platelets are indicated as reticulated platelets. Using the Sysmex XE2100 haematology analyzer a reliable method is available to quantify reticulated platelets. The immature platelet fraction is identified by flowcytometry techniques and the application of the nucleic acid specific dyes polymethine and oxazine in the reticulocyte/optical platelet channel (9). Both dyes are able to penetrate the cell membrane for staining RNA in reticulated platelets. Stained particles and cells pass through a semiconductor diode laser beam. Both the forward light scatter intensity (the measure for cell volume) and fluorescence intensity (the measure for RNA content) are measured. A computer algorithm is able to discriminate the immature platelet fraction from the mature platelet fraction on the basis of deviations from the intensity of forward-scattered light and fluorescence. Immature platelet fraction data are expressed as a fraction of the total optical platelet count in order to indicate the platelet production status (Figure 4).

#### **Platelet morphology / Platelet granule density / Electron microscopy**

Blood slide smears, stained according to May-Grünwald-Giemsa methodology, are used routinely for leukocyte typing and can also be used for qualitative screening of morphological aspects of platelets with light microscopy. Using the CellaVision DM96 analyzer (CellaVision, Lund, Sweden), deviations between observations of biomedical personnel using this method is reduced. Platelet granule-containing cytoplasm is stained light purple or pink. The  $\alpha$ -granules correspond with azurophilic granules viewed in light microscopy. After discharge of the granule content, activated platelets are stained grey.<sup>10</sup> In this thesis, modifications of qualitative aspects of platelets are classified in four categories of staining density of the granule-containing cytoplasm, namely platelets with <25%, 25-50%, 50-75% or >75% pink staining density.

Transmission electron microscopy is a specialized technique which has proven to be a useful tool

for studying the internal morphology of platelets (11). Transmission electron microscopy provides magnifications up to a factor of  $10^5$  and a resolution of 0.2 nm. The technique is used to elucidate the platelet ultrastructure, which can be separated into the outer platelet membrane, cytoplasmic organelles (the granules and mitochondria), internal membranes and cytoskeleton (microtubules). Test samples require specialized processing techniques including fixation.

### **Analysis of platelet function and platelet activation**

Flowcytometers are able to determine characteristics of individual cells. Before flowcytometric analysis, cells in suspension are labelled with a fluorescence-conjugated monoclonal antibody. The suspended cells pass through a flow chamber in the flowcytometer and through the laserbeam at a rate of typically 2.000 to 10.000 cells per minute. After activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence light scattering intensity. Monoclonal antibodies are used in flowcytometric assays for detection of the expression of platelet surface antigen. In this respect, antibodies which bind to activated platelets but not to resting platelets are of particular interest.

#### *Flowcytometry based platelet function tests: p-selectin or platelet surface expression of CD62p*

The activated platelet surface acquires additional membrane markers from the inner platelet granular membranes. Therefore, platelet activation results in expression of new markers on the platelet surface. P-selectin (CD62p) is used as a marker for platelet activation in the circulation. As stated before, p-selectin is a component of the  $\alpha$ -granule membrane of resting platelets that is only expressed on the platelet surface membrane after  $\alpha$ -granule content secretion by fusion of the granular membrane to the outer membrane. Therefore the CD62p-specific monoclonal antibody binds to degranulated platelets. The activation-dependent increase in p-selectin expression on the platelet surface is not reversible over time by re-internalization processes. The *in vivo* circulating degranulated platelets rapidly lose their surface p-selectin into the environment, but platelets stay in circulation and keep functioning (12, 13). Because the platelet activation in the circulation is a continuous process in many clinical conditions, the p-selectin expression can still be used as a marker of *in vivo* platelet activation in spite of its reported loss from the surface.

#### *Platelet factor 4 and $\beta$ -thromboglobulin*

In case of platelet activation substances stored in  $\alpha$ -granules, dense bodies and other intracellular granules such as lysosomal granules are secreted immediately to the extracellular environment. Since platelet factor 4 and  $\beta$ -thromboglobulin are secreted in the extracellular milieu only after platelet activation, their plasma levels are considered to reflect the (extent of) platelet activation *in vivo*.

### **2.2 Involvement of platelets in thrombus formation**

Platelets demonstrate a tendency to stick to collagen and fibrin and then acquire procoagulant properties after activation by their exposure of negatively charged phospholipids. Activated platelets release Factor V, von Willebrand factor and fibrinogen, but also contain and release 50% of the plasminogen activator inhibitor 1, the main inhibitor of the activator of the fibrinolytic system in blood. The process resulting in procoagulant activity of activated platelets thus involves release of clotting factors, exposition of phospholipids on the platelet surface and thus exposure of clotting factor binding sites and inhibition of fibrinolysis, which are all essential for the thrombus formation.

### **2.3 Platelet abnormalities and chronic kidney disease**

Patients with end-stage kidney disease are prone to develop complications due to derangements in two opposite directions of the haemostatic process: bleeding and clotting. Bleeding disorders result from insufficient platelet function, inefficient coagulation and/or excessive activation of the fibrinolytic system (14, 15). Bleeding and clotting problems are clinically relevant as fatal bleeding episodes such as prolonged bleeding from the dialysis fistula, gastrointestinal bleeding or cerebral haemorrhage can occur. A prothrombotic status is associated with an increased number of cardiovascular events or recurrent thrombosis of the dialysis access with insufficient dialysis quality (15). Pathogenesis of bleeding in uraemia is considered to be multifactorial and involves the coagulation cascade, the fibrinolytic system, the platelets, the endothelium and the vessel wall with its extracellular matrix. However, major defects involve the so-called primary haemostasis, i.e. platelet adhesion and aggregation, because abnormalities in platelet-platelet and platelet-vessel wall interactions are of crucial importance. The relationship between all these components is influenced by uraemic toxins and metabolic compounds accumulating during renal insufficiency (16). Structural changes in the vessel wall related to arteriosclerosis, due to impaired calcium and phosphate metabolism resulting in increment of vessel wall calcifications, may also influence the proneness to coagulation activation (17).

### **Thrombocytopenia**

Mild thrombocytopenia frequently occurs in uraemia, suggesting inadequate platelet production, overconsumption or increased clearance (18). However, thrombocytopenia which is severe enough to cause bleeding is very rare. The haemodialysis procedure may itself cause thrombocytopenia through the interaction of blood components with the dialysis membranes that may activate complement (e.g. cuprophane) (18-20) or from heparin (used as anticoagulant) which occasionally may induce thrombocytopenia through an immunologic mechanism (21, 22). In addition, a reduced percentage of reticulated platelets has been reported in patients with haemodialysis treatment, indicating reduced production (23).

## Risk of haemorrhage

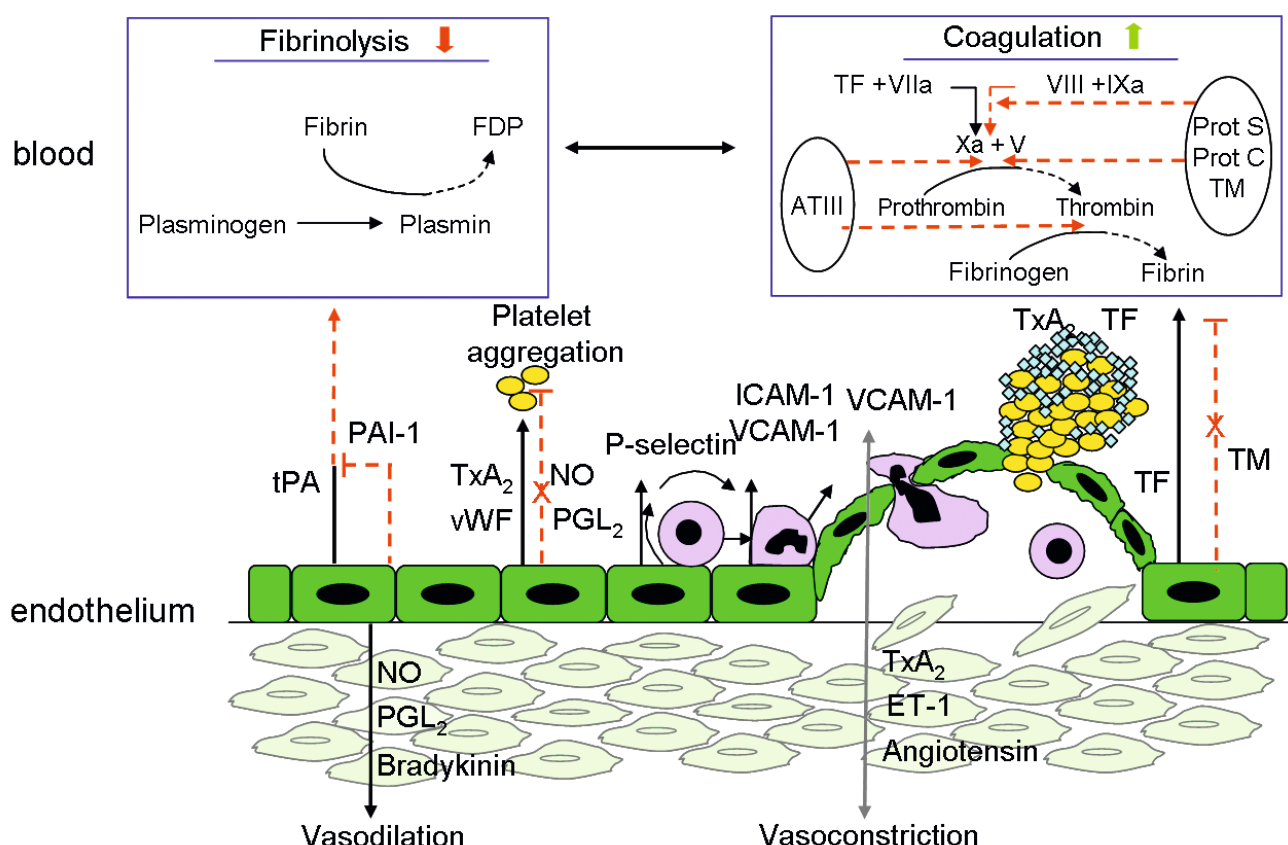
Haemodialysis improves platelet functional abnormalities and reduces the risk of haemorrhage by removing uraemic toxins with a MW of 2 - 40 kD, the so-called middle molecules (24). However, since the introduction of haemodialysis, a varying degree of bleeding has been reported in 25-40% of patients (25-28). Haemodialysis increases the bleeding tendency as platelet activation is induced by interaction of blood with the artificial dialyser membrane surface as well as by the use of systemic anticoagulation. The interaction between blood and artificial dialyser membrane surfaces and application of anticoagulants induce chronic activation of platelets, resulting in platelet exhaustion and aberrations in platelet function. The risk of bleeding may be minimized by using a low-dose of the regular high molecular weight heparin, the use of low-molecular-weight heparin or regional anticoagulation with citrate to prevent clotting in the extracorporeal circulation.

Although a bleeding tendency still represents a problem for uraemic patients, with the advent of modern dialysis techniques and the use of erythropoietin to correct anaemia, the incidence of severe haemorrhages has been reduced substantially.

## 2.4 Platelets and endothelial integrity

Intact endothelium maintains vascular integrity by providing a non-thrombogenic outer layer of the vessel wall. Endothelium regulates vascular tone by balancing the release of vasodilator agents (nitric oxide, prostacyclin, bradykinin) and vasoconstrictor agents (endothelin-1, angiotensin). Nitric oxide and prostacyclin both inhibit the adhesion and aggregation of platelets, whereas thromboxane A<sub>2</sub>, from the activated platelet, and von Willebrand factor promote formation of platelet aggregates.

Endothelium also modulates coagulation by inhibiting activated coagulation factors V and VIII through the protein C and protein S pathway (a pathway initiated when thrombin is bound to the endothelial cell receptor thrombomodulin), and antithrombin III which binds to glycosaminoglycans on the endothelial cell surface. Furthermore, the smooth muscle cells of the endothelium produce tissue factor upon vessel damage, which also becomes exposed on leucocytes trapped in the growing thrombus and activates the coagulation cascade. Endothelium features fibrinolytic activity by the release of tissue type plasminogen activator. Finally, intact endothelium regulates leukocyte adhesiveness and migration through the



**Figure 5.** Antithrombotic and prothrombotic activity of the endothelium. Modified from Viles-Gonzalez (40).

→ = induction; - - - = inhibition; Abbreviations: ATIII = antithrombin III; FDP = fibrinogen degradation products; ICAM-1 = intercellular adhesion molecule-1; NO = nitric oxide; PAI-1 = plasminogen activator inhibitor; PGL<sub>2</sub> = prostacyclin; PROT C = protein C; Prot S = protein S; TF = tissue factor; TM = thrombomodulin; tPA = tissue type plasminogen activator; TxA<sub>2</sub> = thromboxane A<sub>2</sub>; VCAM-1 = vascular cell adhesion molecule-1; vWF = von Willebrand factor.

action of adhesion molecules (p-selectin, ICAM-1, VCAM-1) (Figure 5) (29, 30).

Resting platelets periodically interact reversibly with the intact endothelium to sense its status, but do adhere to activated endothelium, particularly at sites of disturbed shear stress. Activated platelets adhere to both intact and activated endothelium (31). Platelet rolling on activated endothelium is mediated by endothelial selectin. Firm interaction of activated platelets with endothelium involves GPIIb/IIIa-dependent bridging and ICAM-1, GPIb $\alpha$  and  $\alpha$ v/ $\beta$ 3 integrin interaction (32). Activated platelets release CD62p and express CD40 ligand, involved in inflammatory signaling (33). Via CD40 ligand, activated platelets trigger an inflammatory response in endothelial cells and induce expression of adhesion molecules (ICAM-1, VCAM-1), as well as the production of various cytokines (IL6, IL8) and tissue factor (34-36).

Chronic endothelial injury is a trigger in the pathogenesis of atherosclerosis. Cardiovascular risk factors like hypertension, homocysteinemia and inflammation result in increased oxidative stress of the vascular wall, endothelial dysfunction and platelet hyperactivity (37).

Local variations in blood flow partly explain the focal distribution of atherosclerosis. It occurs preferentially at lesion prone sites of large and medium-sized elastic arteries (aorta, carotid) and several large and medium-sized muscular arteries (coronary arteries). Lesion prone sites are characterized by decreased shear stress due to oscillatory blood flow at branching vessels or curvatures. One of the first characteristics of endothelial activation is increased permeability, leading to a decreased production of prostacyclin and nitric oxide (38, 39). Decreased prostacyclin and nitric oxide levels result in decreased vasodilating and anti-adhesive properties of the endothelium. Endothelial injury results in decreased anticoagulant properties by decreased expression of thrombomodulin and increased von Willebrand factor secretion, resulting in increased plasma levels. Reduced tissue type plasminogen activator synthesis and excretion by the activated endothelium impairs fibrinolysis (Figure 5).

### **Risk of development of venous thrombosis**

Atherosclerosis seems to be associated with an increased risk of the development of venous thrombosis in patients with chronic kidney disease (41). The reason for this phenomenon could be an overlap of risk factors such as obesity, hypertension, smoking, diabetes and dyslipidaemia. Furthermore, in patients with chronic kidney disease, platelets and the coagulation system could be activated in atherosclerotic vessels, contributing to the formation of venous thrombosis at different vessel sites. In a recent population-based study, 26% of patients with venous thrombosis also had a history of symptomatic atherosclerosis (42).

### **3. Coagulation**

Coagulation is divided into the intrinsic pathway, initiated by contact with negatively charged surfaces, and the extrinsic pathway initiated by tissue factor (TF). In the coagulation cascade the key event is

activation of the extrinsic pathways promoted by TF, a cell membrane protein which is exposed at the site of vascular injury and which is part of the ensuing TF-FVIIa complex. This complex can activate both FIX and FX. FXa activates FV to FVa, resulting in the FXa/FVa complex which is capable of converting prothrombin to thrombin. Once formed, thrombin activates FVIII, FV, FXI, and converts fibrinogen to fibrin monomers, which polymerize to form fibrin strands (Figure 6). Finally, thrombin-activated FXIII, FXIIIa, forms a three-dimensional stable fibrin network by covalent crosslinking of the fibrin strands. Thrombin also induces positive feedback loops to increase thrombin formation by activating platelets and coagulation FXI, resulting in more fibrin formation.

The coagulation cascade is regulated by several inhibitory mechanisms including antithrombin, the protein C system and tissue factor pathway inhibitor. Antithrombin inhibits FXa, FIXa and thrombin. The inhibitor function of antithrombin is accelerated by heparin and heparin-like glycosaminoglycans present on the endothelial surface. Thrombomodulin is a thrombin receptor expressed on the surface of the endothelial cells. After the formation of the thrombin/thrombomodulin complex, thrombomodulin activates protein C, which, together with its cofactor protein S, inactivates both FVa and FVIIIa. Another anticoagulant localized on the endothelium is the tissue factor pathway inhibitor that inhibits FXa and the TF/FVIIa complex (Figure 5). Main markers of the activated coagulation system and main alterations of coagulation activation are demonstrated in Figure 6.

Fibrinolysis constitutes a protection mechanism which, due to the proteolytic degradation exerted by plasmin, leads to fibrin and thus thrombus dissolution. Fibrinolysis is activated by the action of tPA, urokinase, or by the contact system that converts plasminogen to plasmin (Figure 5).

Plasmin cleaves FV, FVIII, fibrinogen, and the GPIIb receptor on platelets. Fibrin and fibrinogen degradation products interfere with fibrin formation and impair platelet function by GPIIb-IIIa complex occupancy. Plasminogen activator inhibitors (PAI-1 and -2), plasmin inhibitors (alpha-1-antiplasmin, alpha-2-macroglobulin), and thrombin activatable fibrinolysis inhibitor (TAFI) are molecules that counteract fibrinolysis. The main markers of activated fibrinolysis and main alterations of fibrinolysis, as described, are also demonstrated in Figure 6.

### **3.1 Dysbalance of coagulation and fibrinolysis in chronic kidney disease: thrombotic tendency**

Despite decreased platelet function and haemorrhagic tendency, uraemic patients demonstrate activation of the coagulation system. Abnormalities of blood coagulation and fibrinolysis are indicative for the presence of a hypercoagulable state with associated risk of cardiovascular and thrombotic complications (14, 44, 45). Uraemic patients on haemodialysis are prone to thrombotic complications related to vascular access. Percutaneous cannulation, central vein catheters, and native vein or prosthetic arteriovenous fistula are all associated with thrombotic occlusion.

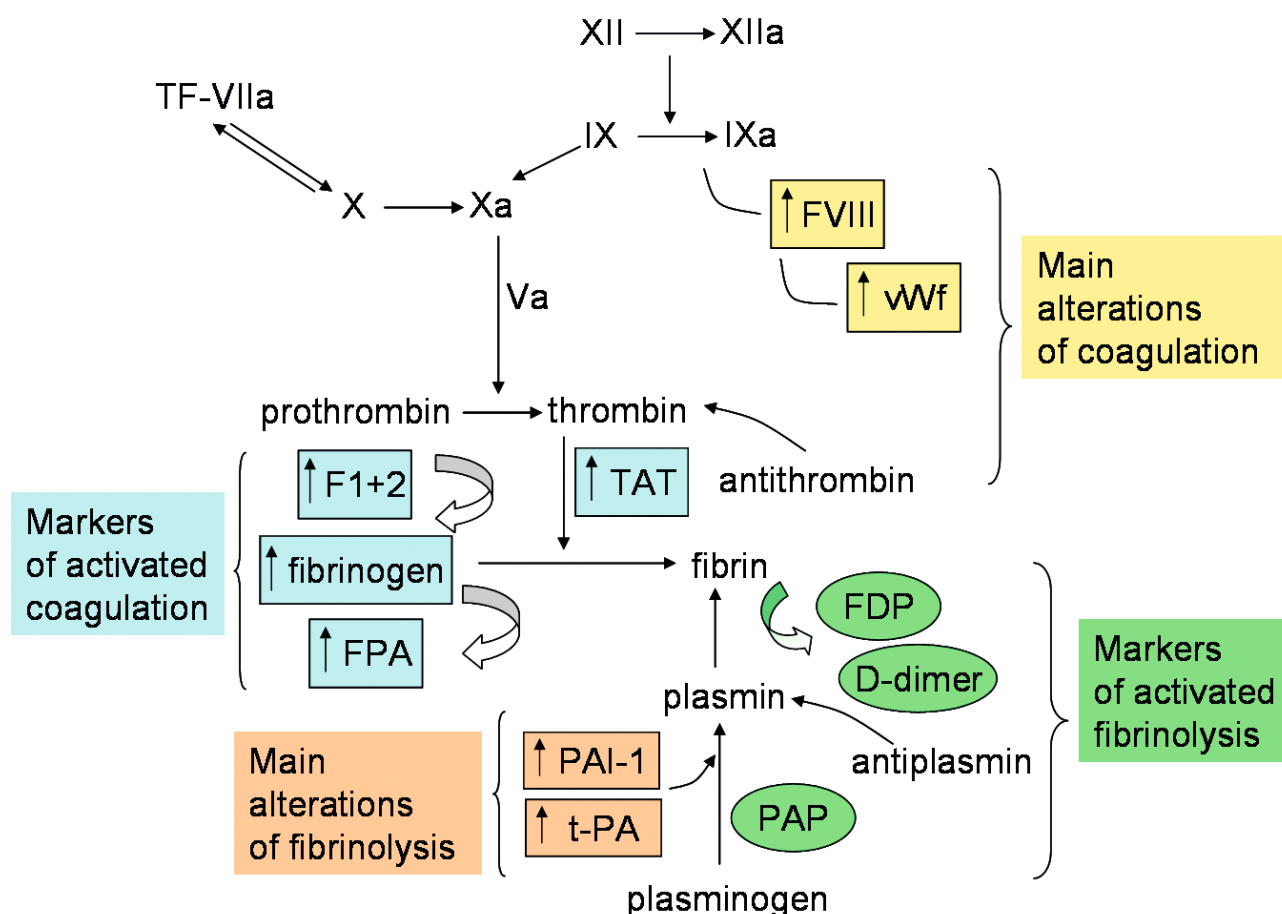
Risk factors for generation of a hypercoagulable state include enhanced platelet aggregability, increased concentrations of plasma fibrinogen, FVIII and von Willebrand factor. Contradictory results have been obtained regarding the fibrinolytic system, indicating both a decreased activity and an activation of fibrinolysis after a haemodialysis session (46, 47).

#### Risk of cardiovascular events

Cardiovascular events related to thrombosis are a predominant cause of death and account also for an important morbidity in patients with end stage kidney disease. Cardiovascular causes of death include myocardial infarction, ischemic or haemorrhagic stroke, sudden death and heart failure. The United States renal data system registry reports 43% of deaths to be cardiovascular (48). In the Dutch CONvective TRANsport STudy (CONTRAST), a randomized controlled trial in 714 chronic haemodialysis patients, the distribution of causes of death was compared to that of the Dutch dialysis registry and of the Dutch general population. In this study 32% of the patients died from cardiovascular disease, 22% due to infection and 23% because of haemodialysis withdrawal. These ratios were similar to those in the Dutch dialysis registry and the cardiovascular mortality was similar to that of the Dutch general population (49).

#### 4. Haemodialysis

Chronic haemodialysis is usually performed three times a week, for about 3-4 hours for each treatment. The principle of haemodialysis involves diffusion of solutes across a semipermeable membrane. Haemodialysis utilizes counter-current flow, i.e. the dialysate is flowing in the opposite direction of the blood flow in the extracorporeal circuit (Figure 1). Counter-current flow maintains the concentration gradient across the membrane at a maximum and increases the efficiency of the dialysis process. Fluid removal (ultrafiltration) is achieved by altering the hydrostatic pressure of the dialysate compartment, causing free water and dissolved solutes to move across the membrane along a created pressure gradient. The dialysis solution consists of a sterilized solution of mineral ions. Urea and other waste products, potassium, and phosphate diffuse into the dialysis solution. Concentrations of sodium and chloride are similar to those of plasma to prevent loss. Sodium bicarbonate is added in a higher concentration than plasma to correct blood acidity. A low concentration of glucose is also commonly used. To gain access to the blood, an arteriovenous fistula or a synthetic graft is made in the arm of the patient by vascular surgery. To create an arteriovenous fistula an artery and a vein are joined together. The advantages of an AV fistula use compared to a synthetic graft



**Figure 6.** Markers of activated coagulation and fibrinolysis together with main alterations of coagulation and fibrinolysis. Modified from Miljic (43).

Abbreviations: F1+2 = prothrombin fragments F1+2; FPA = fibrinopeptide A; PAI-1 = plasminogen activator inhibitor; PAP = plasmin-antiplasmin complexes; TAT = thrombin-antithrombin complexes; t-PA = tissue type plasminogen activator; vWf = von Willebrand factor

use are lower infection rates, because no foreign material is involved, higher blood flow rates through which a more effective dialysis can be reached, and a lower incidence of thrombosis. The fistula causes an increased blood flow from the artery into the joined vein, resulting after some time into an increased size of the vein, which can then be more readily accessed. Heparin is the most commonly used anticoagulant in haemodialysis. Heparin sensitivity can infrequently be a problem, also known as heparin-induced thrombocytopenia. In this category of patients, alternative anticoagulants are applied such as regional anticoagulation with citrate.

#### 4.1 Extracorporeal circuit

The haemodialysis circuit or extracorporeal circuit consists of needles, blood lines, a roller-pump, dialysate fluid and a dialyzer (Figure 1).

The dialyzer is a device consisting of a semi-permeable membrane, which separates the blood from the dialysate fluid. During haemodialysis, blood flow varies between 200-300 ml/min, whereas the dialysate flow is generally fixed at 500 ml/min. Most patients with end stage kidney disease are dialysed for 10-15 hours a week, implying that every week 120-270 litres of blood and 300-450 liters of dialysate are pumped through the dialysis circuit. In the extracorporeal circuit, blood flows through lines which are connected via needles to the arterial (afferent) and venous (efferent) sites of a patients' vascular access (Figure 1). Blood is pushed forward by a roller pump, which clenches a compressible part of the afferent bloodline and pumps the blood through the dialyzer back to the patient via the efferent bloodline (Figure 1). Two air traps (bubble traps, deflation chambers) are located along the bloodlines in order to prevent air, which may have entered the circuit, from entering into the patients's circulation (Figure 1).

#### 4.2 Dialysis membranes

The dialyzer is the piece of equipment that actually filters the blood. The dialyzer consists of a cylindrical bundle of hollow fibers, whose walls are composed of a semi-permeable membrane. The dialysis membrane yields the largest surface area of the extracorporeal circuit (1.0 - 2.0 m<sup>2</sup>). Dialysis membranes with smaller pore sizes are called 'low-flux' and those with larger pore sizes are called 'high-flux'.

In the early days of haemodialysis, cellulose membranes with small pore size and large thickness, were applied. In those days, the dialysis membranes were reused. Dialyzer devices were characterized by an inefficient small solute removal and various undesirable side effects, including complement and leukocyte activation. In the past decades, several developments in membrane manufacturing took place. Modified cellulose membranes have been developed, in which complement-activating hydroxyl groups have been replaced by other moieties (50). In addition, a wide variety of synthetic dialysers have been produced, differing in material polysulfone (PS), polyamide (PAN), polymethylmetacrylate (PMMA), membrane thickness and membrane structure. Most of the modern

dialyzers exhibit considerably less side effects than cellulose membranes and the focus in haemodialysis has shifted mainly to the optimal removal of uraemic toxins with a MW of 2 - 40 kD, the so-called middle molecules (51, 52).

In the studies described in this thesis only biocompatible dialysis membranes were used, made of polyacrylonitril (AN-69 high flux), polymethylmethacrylate (PMMA low flux) and polysulphone (F-60 high flux and F-6 low flux).

#### 4.3 Anticoagulation

The clotting cascade is activated as blood interacts with the dialysis membrane. Without anticoagulation, this would lead to obstruction of the extracorporeal circuit and dysfunction of the dialyzer device. High molecular weight heparin and low molecular weight heparin are the commonly used anticoagulants for haemodialysis. After an initial dose of heparin directly after the start of haemodialysis, continuous administration or small boluses are given during the course of the dialysis session. In the Netherlands, low molecular weight heparins are most often applied and given as a single bolus, based on body weight and duration of haemodialysis. A third modality, with trisodium citrate, represents a procedure of regional anticoagulation within the extracorporeal circuit. Trisodiumcitrate chelates calcium and magnesium. Calcium is a cofactor required in several phases of the coagulation cascade. In clinical haemodialysis, a sterile trisodiumcitrate solution is infused into the arterial line, whereas after passage through the dialyzer Ca<sup>2+</sup> and Mg<sup>2+</sup> levels are corrected by infusion of a CaCl<sub>2</sub>/MgCl<sub>2</sub> solution in the venous line (53). During this anticoagulation procedure, a Ca<sup>2+</sup>-free dialysate is used. Trisodiumcitrate is generally applied on indication, for instance in case of a bleeding tendency, major surgery or heparin-induced thrombocytopenia.

#### 4.4 Bio-incompatibility

The main objective of haemodialysis is the removal of excess fluid and uraemic retention products (54). Despite the relatively high efficiency of modern dialyzers, haemodialysis still entails undesirable side-effects. The material of the dialysis membrane is foreign, which results in the activation of various blood cell elements and protein systems. These activation processes can be best described as an inflammatory response. Depending on the type of dialyzer used, blood-membrane contact results in the generation of the complement activation products C3a and C5a, up-regulation of the adhesion molecule CD11b and down-regulation of the cell surface molecule L-selection (CD62L) on mononuclear and polymorphonuclear cells. These cells adhere to the vasculature of the lungs, resulting in an early and transient leukopenia (55).

Besides the material and structure of the dialysis membrane, the bacterial quality of the dialysate and the anticoagulation mode are important factors influencing bio-incompatibility. Evidence has been obtained that haemodialysis itself contributes to a state of increased oxidative stress and as a consequence to

an acceleration of endothelial dysfunction (56, 57). Apart from activation of complement and leukocytes, platelets and the coagulation system are activated. Activation of platelets results in release of platelet-derived vaso-active substances, such as  $\beta$ -thromboglobulin, thromboxane A<sub>2</sub>, platelet-derived growth factor and serotonin (58-60). In haemodialysis with PAN membranes (e.g. AN-69) an early decrease in Factor XII activity was found, accompanied by a steep transient increase in thrombin/antithrombin complexes and a sustained increase of prothrombin fragments F1+2 as indicators of coagulation activation and fibrinogen as an indicator of the acute phase reaction (61). The results indicate that during haemodialysis hypercoagulability is induced in the efferent line of the dialyzer.

## 5. Objectives of the thesis

In the preceding paragraphs the background for the main topics of this thesis are described. Several aspects of platelet aberrations regarding end stage kidney disease were discussed, with special emphasis on platelet morphology, platelet activation and degranulation, endothelial integrity and activation of the coagulation pathway. The process of haemodialysis was also discussed, including interaction with the extracorporeal circuit, use of different types of dialysis membranes and different modes of anticoagulation.

With respect to the cause of haemodialysis-induced bio-incompatibility, in the past the clinical interest was mainly focussed on type and permeability of dialysis membranes, with special emphasis on complement activation products, activation of mononuclear and polymorphonuclear cells, inflammation and uraemic toxicity (4, 5). Subsequently, the laboratory focus on coagulation and platelet activation met the clinical focus on leukocyte activation when the release of myeloperoxidase demonstrated the same pattern as platelet factor 4 during haemodialysis treatment (62, 63). New studies were organized with special emphasis on the components of the extracorporeal circuit and the anticoagulation procedure (60, 64).

In **Chapter 2 and 3** differences in coagulation activation by different types of dialysis membranes are described. Generation of FXII activity, thrombin-antithrombin complexes, prothrombin fragment F1+2 and thrombus precursor protein polymers have been monitored during haemodialysis treatment with polyacrylonitril (AN-69), polysulphone high-flux (F-60) and low-flux poly-methyl-metacrylate (PMMA) dialysis membranes. For the anticoagulant procedure, bolus injections of low molecular weight heparin are used. Aim of the studies was to evaluate and quantify the effects of different types of dialysis membranes on coagulation activation before and during a session of haemodialysis treatment.

In **Chapter 4** altered characteristics of platelets, indicated by alterations in RNA content and aberrations in platelet volume and platelet morphology, are described before and during haemodialysis treatment

in order to elucidate platelet damage occurring during haemodialysis.

In **Chapter 5** the influence of the extracorporeal circuit is described. Blood is sampled both from the afferent (before the dialyzer) and efferent (after the dialyzer) lines at various time points. Platelet surface expression of CD62p, plasma concentrations platelet factor 4 and  $\beta$ -thromboglobulin and a variety of platelet indices, such as platelet count, mean platelet volume, platelet distribution width, platelet large cell ratio and immature platelet fraction are investigated before, during and at the end of a haemodialysis session. In addition, the influence of low molecular weight heparin on the plasma concentrations platelet factor 4 and  $\beta$ -thromboglobulin is analyzed to determine the involvement of low molecular weight heparin in platelet activation.

During haemodialysis treatment undesirable interactions occur between the extracorporeal circuit and the human body. The total bio-incompatibility is the sum of various side effects during haemodialysis treatment, including amongst others changes on the platelet level. Activation of coagulation is a multifactorial event initiated by the interaction of platelets, plasma von Willebrand factor and the vessels. Within the extracorporeal circuit, endothelium is lacking and activation of platelets and coagulation will be induced by mechanical events and the materials of blood lines and dialyzer. Moreover, immediately after starting haemodialysis, bio-artificial materials of the extracorporeal circuit are coated with circulating plasma proteins. It is unknown whether platelet activation and activation of coagulation in haemodialysis are interrelated. Therefore, in **Chapter 6** deviations in platelet count, immature platelet fraction, platelet morphology, platelet CD62p exposure, platelet factor 4,  $\beta$ -thromboglobulin, serotonin, together with thrombin-antithrombin complexes and prothrombin fragment F1+2 are monitored before and during haemodialysis treatment to investigate directly the timeframe between platelets and coagulation activation.

Platelets are activated and release granule contents during haemodialysis treatment (**Chapter 5**). In **Chapter 7** platelet granule depletion is investigated in more detail to confirm the hypothesis that platelet depletion in patients with haemodialysis treatment is due to frequent platelet activation. Platelet evaluation is performed by light microscopy and electron microscopy in a patient with end stage kidney disease and a healthy reference subject.

During haemodialysis treatment increase of platelet activation and induction of procoagulant activity is demonstrated (**Chapter 2-6**). Although the role of the endothelium and its direct interaction with coagulation and homeostasis is well studied, it is not known how platelet activation markers and activation of coagulation coincide with markers of

endothelial integrity during haemodialysis treatment. In **Chapter 8** uraemia and haemodialysis-induced changes, with particular emphasis on platelet granules depletion, activation of coagulation and endothelial integrity, are investigated.

In **Chapter 9** aspects of platelet disturbances and haemodialysis treatment as well as consequences of repetitive platelet activation are considered in a minireview. Both primary haemostasis with steps mediated by the interaction of platelets, vessel wall and plasma coagulation proteins, and secondary haemostasis process with steps mediated by activated coagulation factors are described. Laboratory parameters reflecting platelet characteristics, platelet activation and degranulation which are of relevance in detecting aberrations in haemostasis in patients with haemodialysis treatment are presented. Furthermore, aspects of the dialysis procedure, dialyzer membranes, extracorporeal circuit and anticoagulation agents are described. Finally, consequences of repetitive platelet activation are discussed with respect to clinical findings of recurrent vascular access failure.

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