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Introduction and objectives of the thesis
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. 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. The vascular endothelium plays a pivotal role in the modulation of vascular tone, initiation of coagulation, fibrinolysis activity and release of inflammatory mediators. 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.

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

Figure 1. Haemodialysis instrumentation and the various components of the extracorporeal circuit. Reproduced from www.nierstichting.nl
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).

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 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 x10⁹/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 cytoplasma 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 µm). 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, acti-
Activation of Platelets and Coagulation during Haemodialysis

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 GPIa-IIa results in 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

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

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
**Reticulated immature platelets**

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

![Figure 4](image)

**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 α-granules correspond with azurophilic granules viewed in light microscopy. After discharge of the granule content, activated platelets are stained grey. 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. 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 α-granule membrane of resting platelets that is only expressed on the platelet surface membrane after α-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. 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 β-thromboglobulin**

In case of platelet activation substances stored in α-granules, dense bodies and other intracellular granules such as lysosomal granules are secreted immediately to the extracellular environment. Since platelet factor 4 and β-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*. 

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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. Bleeding and clotting problems are clinical 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.

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. 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.

Thrombocytopenia

Mild thrombocytopenia frequently occurs in uraemia, suggesting inadequate platelet production, overconsumption or increased clearance. 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) or from heparin (used as anticoagulant) which occasionally may induce thrombocytopenia through an immunologic mechanism. In addition, a reduced percentage of reticulated platelets has been reported in patients with haemodialysis treatment, indicating reduced production.

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. However, since the introduction of haemodialysis, a varying degree of bleeding has been reported in 25-40% of patients. 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 dialyzer membrane surfaces and application of anticoagu-
lants 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 tromboxane A2, 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 endothelia 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 action of adhesion molecules (p-selectin, ICAM-1, VCAM-1) (Figure 5).

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. 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, GP Ibα and αv/β3 integrin interaction. Activated platelets release CD62P and express CD40 ligand, involved in inflammatory signalling. 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.

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. 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. 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).

Figure 5. Antithrombotic and prothrombotic activity of the endothelium. Modified from Viles-Gonzalez.

Abbreviations: ATIII = antithrombin III; FDP = fibrinogen degradation products; ICAM-1 = intercellular adhesion molecule-1; NO = nitric oxide; PAI-1 = plasminogen activator inhibitor; PGL2 = prostacyclin; PROT C = protein C; Prot S = protein S; TF = tissue factor; TM = thrombomodulin; tPA = tissue type plasminogen activator; TXA2 = tromboxane A2; VCAM-1 = vascular cell adhesion molecule-1; vWF = von Willebrand factor.

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. 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 GPIb 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 ran-
domized 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 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 dialyser (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 patient's 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 patient'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 m²). 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. 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.

In the studies described in this thesis only biocompatible dialysis membranes were used, made of polyacrylnitril (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\(^{2+}\) and Mg\(^{2+}\) levels are corrected by infusion of a CaCl\(_2\)/MgCl\(_2\) solution in the venous line.\(^{53}\) During this anticoagulation procedure, a Ca\(^{2+}\)-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 monocellular and polymorphnuclear cells. These cells adhere to the vasculature of the lungs, resulting in an early and transient leukopenia.\(^{55}\)

Beside 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 β-thromboglobulin, tromboxane A\(_2\), platelet-derived growth factor and serotonin.\(^{58,59,60}\) 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. 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. New studies were organized with special emphasis on the components of the extracorporeal circuit and the anticoagulation procedure.

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 polyacrylnitril (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 β-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 β-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, β-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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Activation of coagulation during treatment with haemodialysis

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ABSTRACT

Generation of factor XII, thrombin antithrombin complexes, prothrombin fragment 1+2 and thrombus precursor protein has been monitored in 16 subjects during haemodialysis.

Immediately after starting treatment, contact of blood with the negatively charged surfaces of the polyacrylnitril membrane AN-69 resulted in a 9 - 45% decrease in factor XII activity. Peak concentrations for thrombin antithrombin complexes (50 to 120 µg/L) were observed 30 min after the start of haemodialysis. Establishment of thrombus precursor protein concentrations yielded steadily increasing results without any tendency to decrease during treatment.

Determination of thrombin antithrombin complexes is considered to establish the most sensitive short-term reacting parameter indicating activation of coagulation. A steady generation of fibrin and fibrinogen-fibrin complexes during treatment with haemodialysis is indicated by increasing results for thrombus precursor protein. In order to prevent clotting during haemodialysis, an additional supplementation of anticoagulant is needed.
INTRODUCTION

Extracorporeal circulation of blood disturbs the haemostatic balance in subjects treated with haemodialysis (HD) [1, 2]. In particular, the degree of hypercoagulability is strongly influenced by surface characteristics of the dialyzer membrane and flow conditions within the circuit [3, 4]. Evaluation of conditions concerning the haemocompatibility of membranes, of flow rates and application of anticoagulants is essential in minimizing damage due to blood cell activation [5, 6].

AN-69 membranes are characterized as highly biocompatible. However, contact of blood with negatively charged surfaces like the polyacrylnitril membrane AN-69 may result in specific binding of coagulation factor XII [7]. Because activation of factor XII is induced by mediation of the prekallikrein-kininogen complex, platelet activation may stimulate this conversion by creating a procoagulant surface [8]. As a result, thrombin is generated which converts fibrinogen into fibrin. Fibrin monomers polymerize or are incorporated in complexes with intact fibrinogen, degradation products of fibrinogen and fibrin. Immediate precursors of insoluble fibrin are indicated as thrombus precursor protein (TpP) [9]. The conversion of prothrombin into active thrombin is a key major event in the final stage of the coagulation cascade. Therefore the concentration of prothrombin fragment 1+2 (F1+2) will yield valuable information with regard to the actual amount of thrombin that has been formed.

Until now, time-dependent curves for registration of the accumulation of activation markers in the course of HD have only been published for the initial stage of treatment with AN-69 membranes [5].

The aim of the current study has been to reveal more information with respect to kinetics of factor XII, accumulation of thrombin-antithrombin III complexes (TAT), prothrombin fragment 1+2 and thrombus precursor protein polymers during the complete period of treatment with HD.

SUBJECTS AND METHODS

Subjects

Sixteen subjects between the ages of 20 and 80 years, having undergone HD three times a week, participated in the study. Treatment with HD had been indicated because of renal failure due to pyelonephritis, nephrolithiasis, glomerulosclerosis, membrane proliferative glomerulonephritis, IgA nephropathy or renal cysts. In the remaining cases, nephropathy was of unknown origin. Subjects were treated three times a week in periods ranging from 2 to 12 years.

Subjects were included in the study after informed consent had been received. Criteria for exclusion were application of salicylates or other therapy which might affect platelet function. After venipuncture, blood samples were anticoagulated with sodium citrate (0.129 mol, 1/10). Platelet-poor plasma was prepared by centrifugation of blood at 2500g for 10 min at 4 °C. Small aliquots of plasma were stored in plastic tubes at -70 °C until analysis. Blood samples were collected from the arterial line before dialysis (t=0), after first passage (t=1 min) and subsequently after 5 (t=5), 30 (t=30) and 150 (t=150) min.
Dialysis protocol
The polyacrylnitril membrane AN-69 (Hospal, Uden, The Netherlands) and the biBAG dialysis system (Fresenius, Bad Homburg, Germany) were applied. Blood flow rates ranged between 200 and 250 ml/min and ultrafiltration flow rates between 300 and 1000 ml/min according to the individual need of the patient. Dialysate contained sodium at a concentration of 138 mmol/L, potassium at 2 mmol/L, calcium at 1.75 mmol/L, magnesium at 1.5 mmol/L, chloride at 107 mmol/L, glucose at 5.5 mmol/L and acetate at 38 mmol/L. The dialysis sessions lasted 3 - 4 h, depending on individual needs and efficacy of the treatment.

A continuous intravenous infusion (100 - 800 U h⁻¹) of low molecular weight heparin (Fragmin, Kabi, Stockholm, Sweden) was applied for anticoagulation purposes during HD. Individual amounts were estimated after consideration of the dosage needed at the previous treatment with HD. At the start of dialysis, a priming dosage depending on individual body weight and amounting to 4000 - 6000 units low molecular weight heparin (LMWH) was administered. In one subject a bolus dosage of 2000 units LMWH was supplemented after 2 h. Before starting HD, the extracorporeal system was rinsed with approximately 500 ml of saline.

Factor XII
Factor XII activity was determined by performing a modified activated partial thromboplastin time (APTT) with silica activation. Samples were diluted and added to plasma deficient in factor XII. Correction of the prolonged clotting time of the deficient plasma was proportional to the activity percentage of factor XII in the patient’s plasma. Calibration was performed with application of CALplasma as supplied by the manufacturer (Instrumentation Laboratory, Milano, Italy).

Thrombin antithrombin complexes
The concentration of thrombin-antithrombin III complexes (TAT) was determined by means of a sandwich-type enzyme-linked immunosorbent assay (Enzygnost® TAT micro, Dade Behring, Marburg, Germany).

Prothrombin fragment 1+2
The concentration of prothrombin fragment 1+2 (F1+2) was determined by means of a sandwich-type enzyme-linked immunosorbent assay (Enzygnost® F1+2 micro, Dade Behring, Marburg, Germany).

Thrombus precursor protein
The concentration of thrombus precursor protein polymers (TpP) was determined by application of an enzyme-linked immunoassay (ABS TpP® Assay, Kordia, Leiden, The Netherlands).

Fibrinogen
Fibrinogen concentration was established in accordance with the Clauss method (10) by adding an excess of thrombin to diluted plasma in order to convert fibrinogen to fibrin (Instrumentation Laboratory, Milano, Italy).
Statistics
Statistical evaluation of data was performed by applying multivariate analysis (ANOVA) and Student’s t-test for paired results (SPSS software). Differences were considered to be statistically significant if p<0.05.

Figure 1. Results of factor XII activity (%) established at several stages in 16 subjects before haemodialysis (1), t=1 (2), t=5 (3), t=30 (4) and t=150 min (5) after starting HD. The lower level of the reference range is indicated by the horizontal, dashed line.

Figure 2. Results indicating activation of coagulation established at several stages in 16 subjects before HD (1), t=1 (2), t=5 (3), t=30 (4) and t=150 min (5) after starting HD. A. TAT (μg/L). B. F1+2 (nMol/L). C. TpP (mg/L). The upper level of the reference range is indicated by the horizontal, dashed line. The subject with application of an additional heparin dosage is marked with a - - - - line.
RESULTS

The current study is designed to register kinetics along with intraindividual variations due to activation of coagulation during treatment with HD.

Factor XII

As demonstrated in Figure 1, decreased levels of factor XII concentrations beyond the lower level of the reference range were observed in four subjects. In these cases, levels remained at the lower level of the reference range during treatment with HD. In the complete subjects’ group, a definite decrease occurred in the first minute, amounting to a mean value of 28% (range 9 - 45%). Afterwards, only slight further increases were detected. Finally, towards the end of treatment, results equalled the initial values established before starting HD.

TAT and prothrombin F1+2

Thrombin generation was estimated following the generation of TAT (Figure 2A) and F1+2 (Figure 2B). Determination of TAT concentrations yielded increased results after only 5 min (9.5±1.8 µg/L, mean±SEM), whereas obvious peak values from 50 to 120 µg/L were established 30 min after the start of dialysis. A level of moderately increased values was maintained until the end of dialysis (29.6±3.0 µg/L, mean±SEM).

F1+2 concentrations were demonstrated to be constant during the initial stage (0 - 5 min) of treatment with dialysis; when compared with the reference range, increased results were established in 15 out of 16 participating subjects. Peak values (9.6±0.7 nMol/L, mean ± SEM) were reached at t=30 min. Although peak values decreased slightly, values still remained obviously increased after terminating HD.

Figure 3. Results for fibrinogen concentration (g/L) established at several stages in 16 subjects before HD (1), t=1 (2), t=5 (3), t=30 (4) and t=150 min (5) after starting HD. The upper level of the reference range is indicated by the horizontal, dashed line.
**TpP**

TpP concentrations within the reference range were demonstrated as early as during the initial stage in 12 out of 16 subjects (Figure 2C). At \( t=30 \) min in 11 out of 16 subjects, increased results were observed compared with the reference range. At \( t=150 \) min, a still steadily ongoing increase was observed.

Increased fibrinogen concentrations were initially established for 11 subjects (Figure 3). At the final stage of treatment with HD, values beyond the reference range were observed for 14 subjects.

**DISCUSSION**

For subjects undergoing HD, no standardized procedure for anticoagulation with heparin or other anticoagulants is available to prevent clot formation. Extended monitoring of TAT and D-dimer concentrations can be applied for adjusting the heparin dosage per subject in combination with a biocompatible membrane [11].

Increased TAT concentrations are indicative of activation of coagulation with AN-69 membranes [12]. AN-69 membrane biocompatibility has already been evaluated in healthy volunteers but only for an initial test period of 27 min [5]. When applying AN-69 membranes, only low and comparable levels of activation of coagulation parameters have been reported [5]. In contrast, results from the present study demonstrate obvious deviations due to HD, whereas wide ranges of interindividual variations occur. Concentrations of various coagulation products demonstrated obvious fluctuations in subsequent stages of treatment.

Immediately after starting treatment with HD, contact of blood with the negatively charged artificial surface of the dialysis membrane resulted in an unequivocal decrease of factor XII activity.

Factor XII is activated when bound to negatively charged surfaces and may subsequently cleave prekallikrein to yield kallikrein. Activated factor XII can also activate factor XI, which catalyses the activation of factor IX and hence that of the intrinsic pathway of coagulation. Kallikrein and activated factor XII can activate plasminogen in the intrinsic pathway of fibrinolysis [14].

Blood samples collected from the arterial line during dialysis reflected a state of hypercoagulation in the dialysis circuit.

Distinct peak values for TAT concentrations are observed as little as 30 min after the start of dialysis. A subsequent decrease in TAT concentrations can be explained by a reduction of contact activation due to protein coating within the dialyzer membrane [15].

Owing to the short half-life time (<15 min) it is concluded that TAT is a sensitive and very short-term reacting parameter indicating activation within the coagulation pathway [13]. Simultaneously, only a steadily proceeding increase is demonstrated for F1+2 concentrations with no decline towards lower results at the final stage of dialysis. Before starting HD, F1+2 values still exceeded the upper level of the reference range. Results from our investigations are not in agreement with the conclusion of another study [5] that F1+2 would be an earlier marker of thrombin generation than TAT complexes. Apparently, the effects of essentially different mechanisms have been detected. The theoretical base of these observations has not yet been elucidated.
Fibrin generation, fibrinous deposits and subsequent degradation may result from complex biological processes, including ischaemia, increased platelet aggregability and inflammatory responses [16, 17].

Steadily increasing results for TpP concentrations are indicative of proceeding generation and degradation from fibrin and fibrinogen after thrombin generation due to membrane activation of the coagulation cascade. It has been hypothesized that increased fibrinogen concentrations in the case of HD, which are shown in Figure 3 would contribute to disturbances of haemostatic balance in favour of hypercoagulability. However, a statistically significant relationship between fibrinogen and TAT concentrations cannot be established for the results of this study.

After application of alternative membranes, for instance F500S, F60 and TRICEA, initial contact activation of coagulation appearing from increases in TAT concentration has been observed to some extent (own observation). A reduction of initial coagulation activation is of essential clinical importance. As a consequence of severe clotting, the efficacy of treatment is reduced, resulting in longer duration of dialysis. In the case of clotting more blood will remain within the dialyzer membrane.

Readjustment of the dosage of heparin is recommended if TAT concentrations exceed levels of 20 mg/L immediately after the start of HD [18]. In this respect, elevated concentrations of fibrinogen, as demonstrated in our study, are considered to be an increased risk factor for activation of coagulation [18]. In borderline cases, the appearance of clump formation in the afferent line of the dialyzer system, a reduction of hypercoagulability and a risk of clot formation by increasing the heparin dosage should be demonstrated in practice in further investigations. The same consideration holds when generation of fibrin during treatment with HD is indicated by increased TpP results.

More effective supplementation of anticoagulants is recommended to prevent activation of coagulation. In our opinion the various parameters just mentioned should be applied conscientiously as a means of further optimizing treatment with anticoagulant during HD.
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Deviations in coagulation activation due to treatment with different haemodialysis membranes

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ABSTRACT

Despite systemic heparinization, extracorporeal circulation will induce activation of blood coagulation. Thrombogenicity is associated with biocompatibility of dialysis membranes. Investigation of procoagulatory and fibrinolytic activity is performed prior to and during treatment with haemodialysis. In this study fluctuations of plasma coagulation factor XII, thrombin antithrombin complexes, prothrombin fragment 1+2 and thrombus precursor protein were monitored in 10 subjects during treatment with haemodialysis. Subjects were treated with both polysulphone high-flux dialyser membranes (F-60) and low-flux polymethylmethacrylate (PMMA) membranes. Immediately after start of treatment, blood in contact with artificial membrane surfaces resulted in a marked decrease in factor XII activity amounting to a mean reduction of 80% in the case of PMMA and a reduction of 40% in the case of F-60. In due course, a steady, on-going generation of thrombin antithrombin complexes was observed in several subjects, especially after treatment with F-60 membranes, amounting to increases exceeding 100% of initial values. Establishment of fibrinogen, prothrombin fragment 1+2 and thrombus precursor protein plasma concentrations yielded enhanced results for PMMA compared with the results for treatment with F-60 dialysis membranes. In order to prevent activation of clotting during several stages of haemodialysis, supplementation of anticoagulant can be established on the basis of analytical results of coagulation parameters.
INTRODUCTION

As a consequence of extracorporeal blood circulation the haemostatic balance is disturbed in subjects treated with haemodialysis [1]. Repetitive induction of acute-phase reactant response may induce a chronic micro-inflammatory state. According to recent studies, an increased acute-phase response is associated with type of dialyser membrane [2, 3]. In particular, acute phase response and concomitantly increased degree of hypercoagulability is strongly influenced by surface characteristics of the dialyser membrane and flow conditions within the circuit [4–6].

Thrombus generation in the extracorporeal circuit is demonstrated to be a multifactorial process [4]. If not effectively prevented, thrombogenicity formation will result in reduced haemodialysis efficiency because membrane area is lost [5].

Evaluation of conditions concerning biocompatibility of dialysis membranes, flow rates and appropriate application of anticoagulants is essential in order to minimize damage caused by white blood cell activation [7]. Activation of intracellular constituents in response to inflammatory stimuli is an essential step in release processes of proteolytic enzymes that are initiated by granule products such as myeloperoxidase, elastase and lactoferrin [8, 9]. Haemodialysis induced degranulation of polymorphonuclear leucocytes is considered to be a reactive process, induced by contact between blood cells and artificial dialyser membrane. A Ca\(^{2+}\)-free environment within the dialyser lumen is shown to attenuate degranulation of polymorphonuclear leucocytes [10].

Contact of blood constituents with negatively charged dialysis membrane surfaces such as polyacrylonitrile membrane AN-69 is demonstrated to result in a marked activation of coagulation [1].

Because activation of plasma coagulation factor XII is induced by mediation of a prekallikrein kininogen complex, platelet activation will stimulate this conversion by creating a procoagulant surface [11].

Heparinization not only affects haemostasis and thrombogenicity, but also other physiological systems that may become activated during haemodialysis as a result of biocompatibility [12].

Results from clinical studies with respect to thrombogenicity are widely scattered owing to varying subject-to-subject response to treatment with anticoagulants during haemodialysis.

Conversion of prothrombin into active thrombin is a major event in the final stage of the coagulation activation cascade. In the case of suspected activation, plasma concentrations of thrombin antithrombin III complexes (TAT) and prothrombin fragment 1 + 2 (F1 + 2) yield information with regard to thrombin generation.

The aim of the present longitudinal investigation was to evaluate and quantify the effects of application of alternative membranes for treatment with haemodialysis on coagulation activation, particularly the poly-methyl-methacrylate (PMMA) membrane and the polysulphone membrane F-60, both of which have been used subsequently in a selected subject group. For the purpose of appropriate comparison, additional investigations were performed in an apparently healthy subject group and in a group of predialytic uraemic subjects.
MATERIALS AND METHODS

Patients
Ten subjects with ages varying between 20 and 80 years participated in the study. Treatment with haemodialysis (HD) was indicated owing to renal failure caused by pyelonephritis, nephrolithiasis, glomerulosclerosis and membrane proliferative glomerulonephritis. In the remaining cases, the nephropathology was of unknown origin. The subjects were given treatment three times a week in periods ranging from 2 to 12 years.
The subjects were included in the study after they had given their informed consent. Criteria for exclusion included application of salicylates, Warfarin, dipyramol or any other therapy that might have affected platelet function (Persantin®).
After venipuncture, blood samples were anticoagulated with sodium citrate (0.129 mol, 1/10). Platelet-poor plasma was prepared by centrifugation of blood samples at 2500 g for 10 min at 4°C. Small aliquots of plasma were stored in plastic tubes at -70°C until analysis.
Blood samples were collected from the arterial line before dialysis (t=0) and subsequently after 5 (t=5), 30 (t=30), 60 (t=60) and 150 (t=150) min.

Reference group
Apparantly healthy subjects.
A reference group of 20 apparently healthy subjects (aged 25 – 53) was used to yield insight on the reference range.

Uraemic subjects.
A group of 17 predialytic subjects with serum urea concentrations in the range of 20 – 35 mMol/L was included in the study in order to establish the effect of uraemia on coagulation activation.

Dialysis protocol
The PMMA membrane (Toray Industries Inc., Tokyo, Japan), the polysulphone membrane F-60 (Fresenius, Bad Homburg, Germany) and the biBAG dialysis system (Fresenius, Bad Homburg, Germany) were used in the same subject group. Blood-flow rates ranged between 200 and 250 ml/min and ultrafiltration flow rates between 300 and 1000 ml/min according to the individual needs of the patient. Dialysate contains sodium at a concentration of 138 mMol/L, potassium at 2.0 mMol/L, calcium at 1.75 mMol/L, magnesium at 1.5 mMol/L, chloride at 107 mMol/L, glucose at 5.5 mMol/L and acetate at 38 mMol/L. The dialysis sessions lasted 3 – 4 h, depending on individual needs and efficacy of treatment.
Before starting HD, the extracorporeal system was rinsed with approximately 500 ml of saline. At the start of HD treatment either with the F-60 membrane or PMMA membrane, a priming dosage depending on individual body weight and amounting to 2000 to 5500 units low molecular weight heparin (LMWH) (Fragmin, Kabi, Stockholm, Sweden) was administered. After 150 min on HD treatment the patients received an additional dose of LMWH (Fragmin 500 – 1000 U). For familiarization, patients were dialysed twice already on either the PMMA or the F-60 membrane before the day of venipuncture.
Factor XII
Factor XII activity is determined by performing a modified activated partial thromboplastin time (APTT) with silica activation (Instrumentation Laboratory, Milan, Italy). Samples were diluted and added to plasma deficient in factor XII. Correction of the prolonged clotting time of the deficient plasma is proportional to the activity percentage of factor XII in the patient's plasma. Calibration is performed with application of CALplasma as supplied by the manufacturer (Instrumentation Laboratory, Milan, Italy).

TAT
The concentration of TAT complexes was determined by application of a sandwich-type enzyme-linked immunosorbent assay (Enzygnost® TAT micro, Dade Behring, Marburg, Germany)

F1+2
The concentration of prothrombin F1+2 was determined by means of a sandwich-type enzyme-linked immunosorbent assay (Enzygnost® F1+2 micro, Dade Behring, Marburg, Germany).

Thrombus precursor protein
The concentration of thrombus precursor protein polymers (TpP) was determined by application of an enzyme-linked immunoassay (ABS TpPTM Assay, American Biogenetic Sciences Inc., Columbia, USA).

Fibrinogen
Fibrinogen concentration was determined in accordance with the Clauss method by adding excess of thrombin to diluted plasma in order to convert fibrinogen to fibrin (Instrumentation Laboratory, Milan, Italy).

Statistical evaluation of results
Statistical evaluation of data was performed by applying multivariance analysis (ANOVA) and Student’s t-test for paired results (SPSS software 10.0 for Windows).

RESULTS
Investigations within a selected subjects’ group were performed in order to register kinetics along with intra-individual variations due to activation of coagulation during treatment with HD while subsequently applying F-60 and PMMA membranes, respectively. Membrane dependent results (mean value±SD) for coagulation parameters are presented in Table I. For comparison purposes, coagulation parameters which were established in a reference group of apparently healthy subjects (n=20) and a group of predialytic uraemic subjects (n=17) are listed in Table II.
TABLE I. Results for coagulation parameters (mean value, standard deviation) at several stages of haemodialysis: before (t=0) and at t=5, t=30, t=60 and t=150 min after starting HD.

| Time after starting HD (min) |          |          |          |          | Statistical significance |
|-----------------------------|----------|----------|----------|----------|--------------------------|
|                             | F60      | PMMA     |          |          |                          |
|                             | Mean     | SD       | Mean     | SD       |                          |
| Factor XII activity (%)     | 0        | 58 21    | 62 17    | NS       |                          |
|                             | 5        | 34 21    | 12 13    | **       |                          |
|                             | 30       | 36 20    | 17 13    | **       |                          |
|                             | 60       | 40 21    | 24 14    | *        |                          |
|                             | 150      | 45 18    | 36 15    | NS       |                          |
| Fibrinogen (g/L)            | 0        | 3.5 0.8  | 4.0 1.1  | *        |                          |
|                             | 5        | 3.3 0.7  | 4.1 1.2  | *        |                          |
|                             | 30       | 3.3 0.7  | 4.2 1.2  | **       |                          |
|                             | 60       | 3.5 0.7  | 4.4 1.2  | **       |                          |
|                             | 150      | 3.5 0.7  | 4.4 1.1  | **       |                          |
| TAT (μg/L)                  | 0        | 2.8 0.9  | 3.5 1.0  | **       |                          |
|                             | 5        | 2.6 0.8  | 3.3 0.8  | NS       |                          |
|                             | 30       | 3.4 1.8  | 3.2 0.9  | NS       |                          |
|                             | 60       | 4.2 2.6  | 3.6 1.4  | NS       |                          |
|                             | 150      | 6.4 5.1  | 3.8 0.9  | NS       |                          |
| TpP (mg/L)                  | 0        | 1.3 0.8  | 4.1 1.5  | **       |                          |
|                             | 5        | 1.5 0.8  | 3.8 1.6  | **       |                          |
|                             | 30       | 2.0 1.0  | 3.8 1.7  | **       |                          |
|                             | 60       | 1.6 0.7  | 4.0 1.7  | **       |                          |
|                             | 150      | 1.6 1.0  | 4.8 1.6  | **       |                          |
| F1+2 (nMol/L)               | 0        | 0.8 0.4  | 3.6 1.5  | ***      |                          |
|                             | 5        | 0.7 0.4  | 1.7 0.8  | ***      |                          |
|                             | 30       | 0.7 0.4  | 1.7 0.8  | ***      |                          |
|                             | 60       | 0.7 0.4  | 1.9 0.9  | ***      |                          |
|                             | 150      | 0.6 0.3  | 1.9 0.9  | ***      |                          |

Degree of statistical significance between F-60 and PMMA:
*p < 0.05; **p < 0.01; ***p < 0.001.
NS = difference is not statistically significant; PMMA = poly-methyl-methacrylate; F-60 = poly-sulphone F-60; HD = haemodialysis; TAT = thrombin antithrombin III complexes; TpP = thrombus precursor protein; F1+2 = prothrombin fragment 1+2.
TABLE II. Results for coagulation parameters (mean value, standard deviation) in a reference group of apparently healthy subjects (n=20) and a group of uraemic subjects (n=17).

|                               | Apparently healthy subjects | Uraemic subjects | Statistical significance |
|-------------------------------|-----------------------------|------------------|--------------------------|
|                               | Mean | SD   | Mean | SD   |                 |
| Factor XII activity (%)       | 92   | 5    | 94   | 8    | NS             |
| Fibrinogen (g/L)              | 3.5  | 0.8  | 3.2  | 1.9  | NS             |
| TAT (µg/L)                    | 1.3  | 1.8  | 4.7  | 1.1  | ***            |
| TpP (mg/L)                    | 1.1  | 0.8  |      |      |                |
| F1+2 (nMol/L)                 | 0.8  | 0.6  | 2.8  | 1.3  | ***            |

Degree of statistical significance between the two reference groups: ***p < 0.001. NS = Difference is statistically not significant; TAT = thrombin antithrombin III complexes; TpP = thrombus precursor protein; F1+2 = prothrombin fragment 1+2

Figure 1. Results for factor XII activity (%) established in 10 subjects at several stages before starting haemodialysis (HD) (1) and at t=5 (2), t=30 (3), t=60 (4) and t=150 min (5) after starting HD. The horizontal, dashed line indicates the upper level of the reference range for apparently healthy subjects.

Factor XII
As demonstrated in Figure 1, obvious decreases exceeding the lower level of the reference range were observed in 7 subjects within 5 min of starting HD treatment when using F-60 membranes and in 9 subjects when using PMMA membranes. The rate of decrease was more pronounced when PMMA membranes were used. Afterwards, at T=60 and T=150 min, steady ongoing increases were detected.

TAT and F1+2
Thrombin generation was estimated by monitoring TAT (Figure 2a) and F1+2 concentrations (Figure 2b). Determination of plasma TAT concentrations yielded markedly increased results during treatment with F-60 dialysis membranes in four subjects. Conversely, application of PMMA membranes did not result in increased generation of TAT during
treatment. However, in the latter case prothrombin F1+2 concentrations had already increased in the initial stage of HD after flushing the lines with saline. In normal circumstances patients would be dialyzed with a F-60 membrane. During treatment with PMMA, prothrombin F1+2 concentrations remained markedly increased beyond the upper limit of the reference range in three subjects. However, these patients were not the same subjects as those with increased levels of TAT at t=150 min after treatment with F-60. When compared with the reference group of apparently healthy subjects in the ureamic subjects group, statistically significant increased results were established (Table II).

**Fibrinogen and TpP**

As a result of application of PMMA membranes, fibrinogen concentrations demonstrate a tendency towards higher values if compared with application of F-60 membranes. The results of statistical evaluation of results at any time interval are listed in Table I. In the final stage of treatment with HD, fibrinogen concentrations were higher than those in the initial stage when using PMMA membranes. When using F-60 membranes, alterations in fibrinogen concentrations were not observed during HD (Figure 3a). TpP concentrations within the reference range were demonstrated in all subjects at the initial stage and during HD (Figure 3b). However, as a result of treatment with PMMA membranes, statistically significantly increased results were obtained in comparison with F-60 membranes (Table I).

![Figure 2](image.png)

**Figure 2.** Results indicating activation of coagulation established in 10 subjects at several stages before starting haemodialysis (HD) (1) and at t=5 (2), t=30 (3), t=60 (4) and t=150 min (5) after starting HD. (a): Thrombin antithrombin III complexes (TAT) (µg/L). (b): Prothrombin fragment 1+2 (F1+2) (nMol/L). The horizontal, dashed line indicates the upper level of the reference range for apparently healthy subjects.
**DISCUSSION**

Thrombotic events are common among subjects with end-stage renal disease and contribute substantially to the high cardiovascular morbidity and mortality in this population [13]. In agreement with a study elsewhere [6] we demonstrated that concentrations of TAT and F1+2 were significantly increased prior to HD. In four subjects TAT complexes were markedly further increased during treatment with F-60 membranes.

Activation of coagulation may be due to mechanical stress as a result of high pressures needed for ultrafiltration. Shear stresses result from friction between blood flow components and the capillary wall of artificial membranes. Within a capillary vessel, blood flow velocity is maximal at the centre, whereas at the bloodwall interface flow velocity is minimal and shear stress is maximal.

Haemorrhagic abnormalities associated with HD treatment implicate activation of platelets, activation of the coagulation system and modifications of the fibrinolytic system. Therefore, routine practice of HD requires systematic treatment for anticoagulation. However, use of heparin in patients with increased risk of bleeding may induce serious complications [14]. Conversely, insufficient anticoagulation treatment will give rise to increased fibrin-fibrinogen deposition in the dialyser membrane, with a subsequent reduction in dialyser efficacy.

During HD treatment activation of the plasma coagulation system occurs, owing to interrelated reactions at the artificial interface between blood cells and membrane. Thrombogenic stimuli may arise from dialyser membranes and other components of the extra-
corporeal circuit such as blood flow rate turbulence, bubble traps, shear stress induced activation of platelets due to access needles and blood roller pumps. It is important to define specific parameters that are of essential interest for evaluation of haemocompatibility [4]. Owing to lack of standardization, the findings of several clinical studies comparing the relative thrombogenicity of HD membranes are inconsistent.

As a result of our study, within 5 min of starting HD treatment a sudden decrease in Factor XII activity was demonstrated in both F-60 and PMMA membrane application. After the initial reduction a gradual increase was shown for F-60 membranes but not for PMMA membranes. Activation of the coagulation pathway induced by contact with foreign surfaces can be monitored by the establishment of Factor XII. With application of PMMA membranes, Factor XII concentrations are more markedly decreased than those with application of F-60 membranes. In a previous study, application of AN-69 membranes resulted in only a 9 – 45% decrease [1]. Reduced plasma Factor XII concentrations are associated with contact system activation [15].

Thrombogenicity is monitored by determination of circulating levels of prothrombin fragment 1+2 and TAT complexes. In four subjects, increased TAT generation during HD treatment only occurred when F-60 membranes were used. Thrombin generation and platelet activation may occur because of biomembranes or leucocyte-derived proteases [9]. Evaluation of thrombotic tendency in most cases is reliant on detection of plasma coagulation markers rather than on platelet factors.

With application of PMMA membranes, prothrombin F1+2 concentrations are already increased in the initial stage of treatment. Although patients were already dialyzed twice in order to become familiar with the alternative PMMA membrane before venipuncture took place, switching the type of dialysis membrane could still be the causal factor in the increased levels of F1+2 that are detected at t=0. With regard to initially increased F1+2 concentrations, the same phenomenon was demonstrated in a previous study using AN-69 membranes [1].

It is important to gather information concerning activation of coagulation at an early stage. In low shear stress in the extracorporeal circulation, activation of coagulation may occur without being washed away by the force of blood flow. In contrast, higher shear stress in the arterial circulation associated with increased flow tends to dilute procoagulant substances, thus preventing the formation of prothrombin, thrombin and insoluble fibrin. As a result of coagulation activation, the inner luminal capillary diameter is decreased. Ultimately, fibrin generation is increased because of reduced blood flow and blood-flow velocity [16]. Compared with F-60 membrane treatment, slightly increased fibrin concentrations were detected in the subject group that was treated with PMMA membranes. Increased fibrinolysis is hypothesized to result from the dialysis process itself, whereas no difference was observed corresponding to the type of dialysis membrane used [17].

We conclude from our results that dialysis procedures with application of both membranes consistently resulted in stimulation of procoagulatory factors. Variations of fibrin-fibrinogen degradation products between subjects indicate variable degrees of clotting activation during treatment with HD.
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Changes in platelet volume, morphology and RNA content in subjects treated with haemodialysis

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ABSTRACT

During haemodialysis treatment, blood flows from the body to the extracorporeal circuit and vice versa. In this study, pathophysiological defects in platelets indicated by alterations in RNA content and aberrations in platelet volume and morphology are detected before and during haemodialysis treatment. In subjects receiving haemodialysis treatment, qualitative interpretation of platelet characteristics with application of light microscopic evaluation reveals only 19 ± 11 % of platelets with appropriate staining density of the granule containing cytoplasm. On the contrary, a reference group of apparently healthy subjects shows 70 ± 12 % platelets with appropriate staining density of the granule-containing cytoplasm. During haemodialysis treatment, mean values for platelet volume, platelet distribution width and platelet large cell ratio demonstrate a tendency to decrease by 10 %, 11 % and 6 %, respectively, from the mean initial value to the value at $t=150$ min. Reduction of the platelet volume parameters just mentioned is hypothesized to be due to platelet degranulation as a result of platelet activation.
INTRODUCTION

Activation of coagulation occurs in subjects treated with haemodialysis (HD) as a consequence of extracorporeal blood circulation. Important factors inducing thrombogenicity include reduction of blood flow, modifications in the blood vessel wall, changes in blood composition and bioincompatibility of artificial membranes [1–3]. Acute phase reactants and concomitantly increased degree of hypercoagulability are demonstrated to be initiated by surface characteristics of the dialyser membrane; for instance, permeability and chemical composition.

Platelets (PLTs) are activated due to contact with artificial membranes during treatment with HD. PLT activation is related to composition of the protein layer coated on a dialyser membrane and heparin suppletion at the start of a dialysis session [1]. Activation of PLTs can be evaluated with application of flow cytometric detection of PLT markers. CD62p (140 kD) is an alpha and dense granule labelling membrane protein exposed on the outside of the PLT membrane after activation. The process of PLT activation results in release of granule content. The amount of Platelet Factor 4 (PF4) released from alpha granules and serotonin from dense granules in plasma is indicative of activation of PLTs [4–6]. In peripheral blood smears, the degree of PLT degranulation can be scored by microscopic evaluation.

A PLT population is considered to be a heterogeneous mixture of particles consisting of intact PLTs, shape-changed PLTs, activated PLTs and PLTs that are already more or less degranulated due to damage as a result of previous activation. During extracorporeal circulation, blood flows from the body to the extracorporeal circuit and vice versa. As a consequence of activation, a heterogeneous population with young, reticulated PLTs, and old, smaller PLTs is present [7]. Shape anomalies occur when PLTs lose their discoid form upon reorganization of the cytoskeleton. A reversible shape modification is likely to be associated with changes in cellular forward and right-angle light scatter intensity [8]. Shedding of thrombogenic microparticles from PLT membranes is thought to be indicative of PLT activation. Microparticles expose an attractive surface for adhesion of activated plasma coagulation factors that can promote thrombin generation [9].

Levels of PLT counts in peripheral blood reflect the result of a balance between output of thrombopoiesis and rate of PLT removal. In the case of disorders such as inflammation, discrimination between bone marrow suppression and enhanced rate of PLT destruction is difficult. By applying flow cytometric analysis, the population of immature PLTs (IPF) or so-called reticulated PLTs is discriminated on the basis of increased PLT RNA content. Morphologically, immature reticulated PLTs are characterized by similar volumes, but increased granule density compared with mature PLTs [10]. IPF is expressed as a percentage of the total optical PLT count, or may be quantified as the absolute reticulated PLT count. Conclusive studies concerning disturbances of the thrombopoietic status of subjects with uraemia are not yet available. A result of previous studies in the case of HD patients is that activation of PLTs does not coincide with statistically significant changes in mean platelet volume (MPV) and platelet distribution width (PDW) [1,11,12].

We hypothesize that decreases in MPV and PDW will occur during HD treatment as a result of disturbance of the ratio of reticulated and degranulated PLTs in peripheral blood. Before the start of HD treatment, flow cytometric results (IPF, PLT-x), in conjunction with
PLT counts, together with MPV, PDW and PLT large cell ratio (p-LCR), are compared with a reference group of apparently healthy subjects. In this study, longitudinal investigations during HD treatment are performed with regard to deviations in PLT volume and RNA content in order to elucidate the relationship between PLT morphology and PLT activation.

PATIENTS AND METHODS

A group of 20 subjects (age 28–82 years) with chronic renal failure and who are treated with HD three times a week participated in the study. The aetiology of renal insufficiency includes hypertensive nephrosclerosis, chronic pyelonephritis and membranous nephropathy. Subjects with active inflammation, thrombocytopenia, autoimmune disease or malignancy, and/or using specific categories of drugs (immunosuppressives, calcium antagonists, serotonin receptor antagonists, coumarin derivatives or salicylates), were excluded from participation.

For treatment with HD, a low flux polysulphone® F8 membrane (Fresenius, Bad Homburg, Germany) is used with anticoagulant Fragmin® (intravenously 2000–5000 U bolus injection).

The patients received 10–150 µg/week erythropoietin-α (Amgen Europe b.v., Breda, The Netherlands) subcutaneously. According to the guidelines of the Dutch Dialysis Society, patients receive intravenously 0–100 mg/2 weeks iron sucrose (Venofer®; American Reagent Inc. Shirley, N.Y. 11967, USA).

Bicarbonate dialysate (Fresenius Medical Care, Bad Homburg, Germany) was applied with a dialysate flow of 500 mL/min. Depending on individual need and efficacy of treatment, ultrafiltration flow rates varied between 300 and 1000 mL/min. Blood flow rates were kept constant at 250–300 mL/min, resulting in HD sessions of 3–4 h. Blood samples were collected from the arterial line before starting HD (t=0) and subsequently from the efferent line after 1, (t=1), 5 (t=5), 30 (t=30), 60 (t=60) and 150 (t=150) min.

Reference group
A reference group of 20 healthy subjects (laboratory technicians, aged between 20 and 50 years) was selected in order to establish reference ranges for parameters reflecting morphological aspects of PLTs. The subjects were not using any medication that would interfere with PLT activation.

Analytical methods
Blood samples were drawn into K₂EDTA tubes (Vacutainer®, Becton Dickinson, Plymouth, UK) and analysed within 2 h after collection.

1. PLT, PDW, MPV, p-LCR, IPF and PLT-x
PLT counts, PDW, MPV, p-LCR and IPF are measured using a Sysmex XE-2100 haematology analyser with a dedicated IPF-Master software package (Sysmex Corporation, Kobe, Japan). A fluorescent dye reagent containing polymethine and oxazine is used with the flow cytometric IPF test methodology. After injecting the dye through the cell membrane,
RNA in reticulated red blood cells and reticulated PLTs is stained. The stained cells pass through a semiconductor diode laser beam and the resulting forward scatter light intensity (measure for cell volume) and fluorescence intensity (measure for RNA content) are measured. IPF count is calculated from the combination of intensity of the fluorescence signal and the forward scattered light signal from the XE-2100 reticulocyte measurement channel by application of a Sysmex algorithm. PLT-x indicates the amount of RNA present in reticulated PLTs. The mean value of fluorescence intensity corresponding with the mean RNA content is expressed by channel numbers.

2. PLT morphology
Two peripheral blood slide smears are prepared for evaluation of PLT morphology aberrations and are stained according to May-Grünwald-Giemsa methodology on a Sysmex SP-100 analyzer (Sysmex Corporation, Kobe, Japan). The slides are microscopically screened for qualitative evaluation of morphological aspects of PLTs with application of a CellaVision™ DM96 analyzer (CellaVision AB, Lund, Sweden). Using the CellaVision™ DM96 analyzer it is possible to reduce deviations between observations of biomedical scientists. Granule-containing cytoplasm, indicated as the granulomere, is stained light purple or pink. PLTs contain several kinds of granules, of which α-granules correspond with azurophilic granules viewed in light microscopy. After discharge of granule content, activated PLTs are grey [13]. Modifications of qualitative aspects of PLTs are evaluated by classification of the PLT content in four categories corresponding with staining density of granule-containing cytoplasm amounting to <25 %, 25–50 %, 50–75 % or >75 %, respectively. Staining density of >75 % of the granule-containing cytoplasm in >50 % of PLTs is considered to be appropriate.

Statistical evaluation
Statistical evaluation of data is performed with application of SPSS software v. 14.0 for Windows. Data are expressed as mean values±SD. Statistical significance of deviations between mean values during HD treatment is evaluated using Student’s t-test for paired data. The statistical significance of differences between mean values of the group of HD subjects and the reference group is calculated by applying Student’s t-test two-tailed for unpaired data. A p-value<0.05 is considered to be statistically significant.

Results for PLT counts, PDW, MPV and IPF counts at t=1, t=5, t=30, t=60 and t=150 min are corrected for changes in haematocrit (Ht). For example: corrected value \( t=30 \) = \( \frac{Ht_{t=0}}{Ht_{t=30}} \times \text{value}_{t=30} \).
**TABLE I.** Evaluation of platelet (PLT) parameters, including mean value, standard deviation (SD), minimum-maximum values established in 20 subjects at several stages of haemodialysis (HD). This longitudinal study concerns results before HD (t=0) and at t=1, t=30 and t=150 min, respectively, after starting HD treatment.

| Time after starting HD (min) | PLT (10^9/L) | MPV (fL) | PDW (fL) | p-LCR (%) | IPF (10^9/L) | PLT-x (ch) | PLTs with >75 % staining intensity |
|-----------------------------|-------------|---------|-----------|-----------|-------------|-----------|-------------------------------|  
|                             | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (1) | Mean value  | SD      | Minimum-maximum | Statistical significance (2) | Mean value  | SD      | Minimum-maximum | Statistical significance (2) | Mean value  | SD      | Minimum-maximum | Statistical significance (2) | Mean value  | SD      | Minimum-maximum | Statistical significance (2) |
|                             | 0           | 198     | 43        | 117–229   | p = 0.006   | 0         | 19.0    | 14.4–20.9    | p = 0.005   | 0           | 18.0    | 1.8        | 1.8–12.3    | p = 0.000   | 0           | 18.0    | 14.4–20.9    | p = 0.005   | 0           | 19.0    | 14.4–20.9    | p = 0.005   | 0           | 18.0    | 14.4–20.9    | p = 0.005   | 0           | 18.0    | 14.4–20.9    | p = 0.005   | 0           | 18.0    | 14.4–20.9    | p = 0.005   |
|                             | 1           | 173     | 36        | 125–230   | p = 0.013   | 1         | 5.2     | 2.6        | 1.9–9.9    | p = 0.019   | 1           | 18.2    | 1.9        | 14.2–21.2   | NS         | 1           | 18.2    | 1.9        | 14.2–21.2   | NS         | 1           | 18.2    | 1.9        | 14.2–21.2   | NS         | 1           | 18.2    | 1.9        | 14.2–21.2   | NS         |
|                             | 30          | 185     | 45        | 82–285    | p = 0.000   | 30        | 6.3     | 3.5        | 2.4–15.0   | NS         | 30          | 18.8    | 1.8        | 16.0–21.8   | p = 0.000   | 30          | 18.8    | 1.8        | 16.0–21.8   | p = 0.000   | 30          | 18.8    | 1.8        | 16.0–21.8   | p = 0.000   | 30          | 18.8    | 1.8        | 16.0–21.8   | p = 0.000   |
|                             | 150         | 182     | 42        | 73–264    | p = 0.000   | 150       | 27.2    | 7.8        | 13.8–36.7 | p = 0.000   | 150         | 27.2    | 7.8        | 13.8–36.7 | p = 0.000   | 150         | 27.2    | 7.8        | 13.8–36.7 | p = 0.000   | 150         | 27.2    | 7.8        | 13.8–36.7 | p = 0.000   | 150         | 27.2    | 7.8        | 13.8–36.7 | p = 0.000   |

Results of statistical evaluation are indicated: (1) Statistical significance of deviations between results at t=0 and t=1, t=30 and t=150 min after starting HD. (2) Statistical significance of deviations between results at t=0 and the group of reference subjects (n=20). NS=deviation not statistically significant.
RESULTS

Investigations in the selected subjects’ group before and during HD treatment are performed for monitoring kinetics along with intra-individual variations due to activation of PLTs during HD treatment. Results for parameters reflecting PLT characteristics (mean value ± SD) are presented in Table I. For purposes of comparison, PLT parameters established in a reference group of apparently healthy subjects are listed in Table II.

PLT, PDW, MPV and p-LCR
As demonstrated in Table I, PLT counts in subjects with HD treatment are in the lower range of the reference interval. If compared with initial values, during HD treatment a statistically significant decrease in PLT count amounting to 8 % at \( t=150 \) min occurs (\( p=0.000 \)). Changes in PLT volumes are estimated by monitoring MPV, PDW and p-LCR (Figure 2). Longitudinal evaluation of MPV and PDW yielded markedly decreased results during HD treatment. MPV values decreased from 10.5 ± 0.8 fl at \( t=0 \) to 9.5 ± 0.9 fl at \( t=150 \) min, a reduction of approximately 10 %. PDW results decreased from 12.3 ± 1.9 fl at \( t=0 \) to 11.0 ± 1.6 fl at \( t=150 \) min, a reduction of 11 %.

Results for p-LCR showed a decrease amounting to 6 % from 29.0 ± 7.4 % at \( t=0 \) to 27.2 ± 7.8 % at \( t=150 \) min (\( p=0.000 \)). When results of the group of HD subjects before starting HD treatment are compared with results of the reference group of apparently healthy subjects, no statistically significant deviations for MPV, PDW and p-LCR are established.

IPF
IPF counts in subjects with HD treatment are situated in the lower range of the reference interval (Figure 3). At \( t=1 \) min after starting HD treatment, 250–300 mL of blood has already passed along the dialysis membrane. Immediately after starting extracorporeal

| Table II. Platelet (PLT) parameters concerning mean value, standard deviation (SD), minimum-maximum values established in a group of healthy subjects (\( n=20 \)). |
|---------------------------------|---------|---------|-----------------|
| PLT (10\(^9\)/L)               | 238     | 47      | 150–337         |
| MPV (fl)                       | 10.1    | 0.6     | 8.9–11.6        |
| PDW (fl)                       | 11.7    | 1.3     | 9.5–14.1        |
| p-LCR (%)                      | 25.5    | 5.4     | 15.2–37.3       |
| IPF (10\(^9\)/L)               | 10.6    | 5.5     | 4.0–23.3        |
| PLT-x (ch)                     | 19.7    | 2.0     | 17.3–23.4       |
| PLTs with >75 % staining intensity of granule containing cytoplasm (%) | 70      | 12      | 44–86           |
blood circulation, a statistically significant decrease of IPF counts is observed from $6.7 \pm 2.8 \times 10^9/\text{L}$ at $t=0$ to $5.2 \pm 2.6 \times 10^9/\text{L}$. The decrease in IPF counts amounts to 22%, whereas total PLT counts reveal a smaller decrease of 13% within the first minute. Afterwards, a steadily ongoing increase is detected. At $t=150$ min, a statistically significant decrease in IPF count of 9% from the initial level is demonstrated. Mean fluorescence intensity of PLTs expressed as PLT-x in the group of subjects with HD treatment is statistically significantly decreased compared with the reference group of apparently healthy subjects (Table II).

**PLT morphology**
In subjects with chronic HD treatment, staining density of the granule-containing cytoplasm is reduced to a minimum score compared to that of the group of reference subjects (Figure 4). In HD subjects, only $19 \pm 10\%$ of the PLTs yield an appropriate staining density. In the group of apparently healthy subjects, $70 \pm 12\%$ of the PLTs reveal appropriate staining.

During HD treatment, after $t=1$ min an ongoing decrease of PLTs with an appropriate staining density is detected until $t=30$ min. From $t=60$ min an increase of PLTs with an appropriate staining density is observed amounting to $29 \pm 16\%$ at $t=150$ min ($p=0.001$) (Figure 4).
DISCUSSION

PLT activation in subjects with HD treatment may result in increased amounts of less densely granulated PLTs in the blood circulation. Activation of PLTs and activation of coagulation in an extracorporeal circuit is known to be a multifactorial process. If not effectively managed, thrombogenicity may result in HD inefficacy, because the membrane area does not function appropriately. It has been demonstrated that cuprophan and polyacrylonitrile membranes induce a higher degree of intradialytic PLT activation compared
with polysulphone and cellulose-triacetate membranes [3,14–16]. In addition, the anticoagulation mode has an important role in the efficacy of HD treatment [5,17,18]. Results of the present study with application of low-flux polysulphon F8 membranes demonstrate similar deviations in activation of PLTs when compared with high-flux polysulphon F60 membranes [5,6].

PLT adhesion is induced by interaction between PLTs, adhesive proteins and dialyser membrane. PLT aggregation is enhanced by increased plasma levels of fibrinogen and Von Willebrand factor [9]. The results in our study, concerning decreased PLT counts in patients on chronic HD treatment compared with apparently healthy controls, concur with those of other studies [19,20]. An obvious further decrease in PLT count is detected amounting to 13 % after the first passage of blood along the dialysis membrane at $t=1$ min after the start of HD treatment. Afterwards, a steady state occurs with decreased levels of PLT counts with a reduction of 8 % from the initial value at $t=0$. 

**Figure 3.** Results (mean±SD) for IPF count ($10^9/L$) established in a longitudinal study for subjects before the start of haemodialysis (HD) treatment and at stages $t=1$, $t=5$, $t=30$, $t=60$ and $t=150$ min after starting HD (n=20). If compared with results at $t=0$, statistically significant decreases have been detected at $t=1$ and $t=150$. For comparison, results referring to a group of 20 apparently healthy subjects (REF) are added. At $t=0$, a statistically significant reduction ($p = 0.006$) has been demonstrated. The horizontal dashed lines indicate the upper and lower level of the reference range for apparently healthy subjects.

**Figure 4.** Results (mean±SD) for the percentage of PLTs with >75 % staining intensity of granule containing cytoplasm (%) established in subjects before the start of haemodialysis (HD) treatment and at stages $t=1$, $t=5$, $t=30$, $t=60$ and $t=150$ min after starting HD (n=20). If compared with results at $t=0$, slightly increased values are demonstrated at $t=1$ and $t=150$. Increases are detected to be statistically significant. Results with regard to a group of 20 apparently healthy subjects (REF) are also added. At $t=0$ a statistically significant reduction ($p=0.000$) has been demonstrated. The horizontal dashed line indicates the lower level of the reference range for apparently healthy subjects.
Establishment of IPF count is a useful tool for elucidating aberrations in respect of PLT pathophysiology [10]. The amount of reticulated PLTs is considered to be a marker of marrow megakaryopoiesis activity [21,22]. As a result of the present study, IPF counts have been demonstrated to be statistically significantly decreased in patients receiving chronic HD treatment compared with apparently healthy subjects. PLT interaction with the dialysis membrane will result in adhesion of PLTs on the membrane surface and release of PLT-derived factors. Adherence of PLTs and leukocytes to the dialysis membrane probably occurs during the first minutes of HD treatment. In the present study, IPF counts reveal an immediate decrease of 22 % at t=1 min. Subsequently, a slight recovery of IPF count is demonstrated. Finally, at t=150 min a decrease in IPF count amounting to 9 % from the initial level is observed. We hypothesize that young reticulated PLTs, because of a high quality of viability during the first passage of blood through sterile lines of the extracorporeal circuit, readily adhere to the dialysis membrane. The slight decrease in p-LCR results at t=1 min, as well as the observation of steadily decreasing MPV and PDW results during HD treatment, also supports the hypothesis just mentioned. A decrease in serotonin concentration in PLTs and an increase in plasma serotonin concentrations revealed an indication for increasing PLT activation during HD treatment [4,6]. Reticulated PLTs are considered to be more viable compared with degranulated PLTs [23]. A rapid decrease in RNA-rich IPF count during HD treatment is hypothesized to be due to elimination of young, viable PLTs. Preferred removal of young active PLTs is presumed to exaggerate thrombocytopathy, which may be present in uraemic patients [24]. When PLT turnover is increased, increasing amounts of young PLTs with fairly large volumes are produced. The absence of large PLTs in the case of subjects with HD treatment is indicative of a qualitative defect of PLT production. PLTs contain azurophilic granules that are finely dispersed throughout the cytoplasm or are concentrated near the centre. PLTs contain various species of granules. The azurophilic granules are equivalent to α-granules and may be visualized by light microscopy [13]. When PLTs are being activated, CD62p is expressed at the outside of the PLT surface membrane. Various products are released from PLT granules; among others, PF4 from alpha granules and serotonin from dense granules. Cytoplasm of PLTs that lack α-granules appear grey or pale blue, implying that PLTs contain few stained azurophilic granules [13]. Degranulated PLTs still demonstrate increased exposition of p-selectin and procoagulant phospholipids on their surface and continue to circulate [13, 25]. As a result of our study, PLTs with rather small volumes in the case of subjects with HD treatment are detected. With respect to a 10-day life-span of PLTs and HD treatment three times a week, the pale blue stained granule containing cytoplasm and decreased mean fluorescence intensity reflecting RNA content elucidate that peripheral PLT populations of HD subjects consist of about 30 % of PLTs which remained briefly in the extracorporeal circuit. Secondary, increased urea concentrations in HD patients may yield an additional factor exaggerating the pale blue aspect of cytoplasm. A group of 20 uraemic subjects with a glomerular filtration rate ranging from 3 to 74 mL/min demonstrated 49 ± 14 % of PLTs with appropriate staining, whereas the reference group of apparently healthy subjects reveal a score of 70 ± 12 % (pers. comm.). The degranulation phenomenon may result from exhaustion due to exposition of PLTs to increased urea concentrations over a short period of time.
As a result of adherence of PLTs to the dialysis membrane at $t=1$ min and simultaneously increased degree of activation of PLTs till $t=30$ min of HD treatment, native and not yet activated PLTs are released into the circulation [5, 6, 26]. As a consequence, an increased number of PLTs with appropriate staining density or modal fluorescence intensity is demonstrated to be present. Young PLTs are characterized with a high content of azurophilic granules or RNA content. We hypothesize that a balance arises between adherence and retention of PLTs to the dialysis membrane and release of PLTs into peripheral blood while the HD treatment process continues.

**CONCLUSIONS**

Reduced PLT and IPF counts are indicative of suppressed activity of megakaryopoiesis or destruction as a result of chronic HD treatment. Results of this study demonstrate an obvious decrease in MPV, PDW and p-LCR during HD treatment. Predominantly, adherence of young reticulated PLTs to the dialyser membrane is hypothesized to occur. As a consequence of repeated PLT activation in HD subjects, increased amounts of degranulated PLTs with smaller volumes are detected in the blood circulation. Increased urea concentrations may reveal an additional cause for occurrence of PLTs with reduced density of granules in the cytoplasm. In subjects with HD treatment, suppressed activity of megakaryopoiesis is accompanied by PLT volume reduction as a result of activation.
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The Role of the Extracorporeal Circuit in the Trapping and Degranulation of Platelets

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ABSTRACT

Background: Although platelet (PLT) activation and degranulation are well-known phenomena during hemodialysis (HD), controversies still exist about their nature and origin. Methods: PLT characteristics [PLT numbers, mean PLT volume (MPV), PLT distribution width (PDW), PLT large cell ratio (p-LCR), immature PLT fraction] and activation status [CD62p expression, platelet factor 4 (PF4) and β-thromboglobulin (BTG) plasma levels] were estimated in 19 patients before and during HD. Blood was sampled from both the afferent and efferent lines. Additionally, the influence of low-molecular-weight heparin (LMWH) on PF4 and BTG concentrations was analyzed.

Results: CD62p expression increased in the extracorporeal circuit (ECC) in the first 30 min. Simultaneously, PLT numbers dropped markedly within the ECC. MPV, PDW and p-LCR decreased over time. Like CD62p expression, BTG reached peak values at t30, was exclusively released within the ECC and was not influenced by the application of LMWH. In contrast, PF4 was significantly released outside the ECC in response to LMWH.

Conclusions: PLTs are predominantly activated within the ECC and not on a remote distance. PLTs stick to the ECC, particularly after first passage. BTG is an appropriate marker for HD-induced PLT degranulation, whereas PF4 originates from both activated PLTs and LMWH-induced detachment from the endothelium. PLTs are not exhausted due to the repetitive stimulation of clinical HD. Hence, dialysis modalities with longer duration or greater frequency may be associated with a less beneficial PLT activation profile, which may counteract their clinical benefits.
INTERRODUCTION

During hemodialysis (HD) various side effects occur, including the activation of protein systems in the blood and the stimulation of circulating cellular elements. The sum of these undesirable side effects has been termed bioincompatibility [1]. With respect to platelets (PLTs), during HD both an increase in the expression of cell surface molecules and the release of intragranular substances has been well documented. Recently, we showed that the upregulation of the PLT surface marker P-selectin (CD62p) is an early process, occurring over the entire length of the extracorporeal circuit (ECC) [2]. Of the many PLT granule products that may increase during the course of HD, platelet factor 4 (PF4) and β-thromboglobulin (BTG) are well documented [3]. Activated PLTs adhere to various blood cell elements, including other PLTs, neutrophils, lymphocytes [4], monocytes and erythrocytes [5]. As a result of these cell-cell interactions, a mixture of microaggregates is formed, with specific pathophysiological effects. Flow cytometric analysis showed production of reactive oxygen species after the intradialytic formation of platelet-neutrophil aggregates [6]. In vitro, it was shown that PF4 binds directly to ox-LDL and increases ox-LDL binding to vascular cells and macrophages, thus contributing to the development of atherosclerosis [7]. In HD patients, PLT activation correlated both with bleeding and thrombotic diathesis [8], and vascular access failure [9]. In the clinical situation, PLT reactivity corresponded positively with the subsequent incidence of cardiovascular morbidity and mortality, both in nonrenal patients [10] and in patients with end-stage renal disease [11]. Although PLT activation and degranulation are well established phenomena during HD [12], controversies remain about the nature of PLT activation, intermittent or continuously [5], and the origin of PF4 and BTG, which are generally considered as markers of HD-induced PLT degranulation [2]. Therefore, 19 patients were included in the present analysis. PLT characteristics and activation status were estimated immediately before and during a single HD session. Not only the expression of the cell surface marker CD62p was measured, but also the plasma levels of PF4 and BTG. In addition, a variety of PLT indices, such as PLT numbers, mean PLT volume (MPV), PLT distribution width (PDW), PLT large cell ratio (p-LCR), and immature PLT fraction (IPF) was estimated. To establish the role of the ECC, blood was sampled both from the afferent (before the blood pump and analyzer) and efferent (after the dialyzer) lines at various time points. In an additional study, the influence of low-molecular weight heparin (LMWH) on the plasma concentrations of both PF4 and BTG was analyzed to determine the involvement of this anticoagulant in PLT degranulation.

SUBJECTS AND METHODS

Patients
Nineteen stable patients (11 males and 8 females, median age 63 years, range 28–82) undergoing regular HD treatment for at least 2 months (median 25 months, range 7–83 months) were included. Criteria for exclusion were age under 18 years, severe incompliance and a life expectancy less than 3 months due to nonrenal disease. In the present study,
patients with clinical signs of infection, autoimmune disease or malignancy as well as the use of medication that might interfere with PLT function were excluded. The etiology of renal insufficiency was hypertensive nephrosclerosis in 8 patients, diabetic nephropathy in 5 and adult dominant polycystic kidney disease in 3 patients. The other 3 patients had one of the following diagnoses: IgA nephropathy, membranous nephropathy and tubulointerstitial nephritis. Written informed consent was obtained in all cases. The protocol was approved by the local medical ethics committee.

**Study Design and Blood Sampling**

All parameters were measured over time, i.e. before HD by sampling from the fistula (t0) and after 1 (t1), 5 (t5), 30 (t30), 60 (t60) and 150 (t150) min from the efferent line. Furthermore, samples were taken from the afferent lines after 5 min (t5aff) and 30 min (t30aff). The calculated differences over the ECC (Δ, efferent value – afferent value) at t1, t5 and t30 were designated Δt1 (first passage), Δt5 and Δt30, respectively. Results were corrected for changes in plasma volume, based on hematocrit (Ht) measurements (corrected valuetx = value0 x [Ht0 / (1 – Ht0)] x [(1 – Htx) / Htx]). PLT numbers were corrected for changes in plasma volume based on a different formula: corrected valuetx = value0 x [Ht0 / Htx]. All measurements were performed in duplicate.

**Additional Study**

The influence of LMWH on BTG release was studied in 5 patients during and just before another single HD session. Conforming to our previous study [2], LMWH (dalteparin, median 5000 IU, range 2500–7500) was administered in the AV fistula 10 min before the actual start of HD. Blood was drawn directly from the AV fistula 10 min before dialysis, just before dalteparin injection (t–10). Ten minutes after dalteparin injection, and just before starting HD, a second blood sampling was performed (t0). After the start of HD, samples were taken from the afferent line after 5 (t5aff) and from the efferent line after 1 (t1) and 5 (t5eff) min. All results (CD62p, BTG and PF4) were corrected for changes in plasma volume, based on Ht measurements.

**HD Procedure and Materials**

Only first use low-flux polysulfone dialyzers were utilized (F8 HPS; Fresenius Medical Care, Bad Homburg, Germany; ultrafiltration factor 18 ml/h x mm Hg, surface area 1.8 m², steam-sterilized) on Fresenius 4008 and 5008 series dialysis machines. Bicarbonate dialysate was used with a dialysate flow of 500 ml/min, whereas dialysate temperature was kept at 36° C. For dialysate preparation, tap water, purified by reverse osmosis, was used for the dilution of a concentrated bicarbonate solution to the following concentrations (mmol/l): 138 Na⁺, 2.0 K⁺, 1.50 Ca²⁺, 0.50 Mg²⁺, 109 Cl⁻, 2.5 CH₃COO⁻ and 32.5 HCO⁻₃ (SK-F 216/1; Fresenius Medical Care). All dialyzers were prerinsed with 1000 ml 0.9% NaCl. Individual doses of dalteparin, which is the standard type of anticoagulation in our center, were based on body weight (50 IU/kg) and duration of dialysis and given as a bolus injection at the beginning of the dialysis session (median 5000 IU, range 2500–7500).

**Analytical Methods**

**PLT Surface Markers.** The PLT surface markers CD62p (P-selectin; clone CLB Thromb
Blood samples were drawn into K2EDTA (ethylenediaminetetraacetic acid) tubes and within 2 h after collection incubated with a glycoprotein-specific fluorochrome-labeled monoclonal antibody. A flow cytometer (Epics XL; Beckman Coulter) was used to determine the percentage of PLTs with CD62p surface expression. CD41 served as a PLT-specific label.

**BTG, PF4.** Blood samples were drawn into CTAD tubes (Vacutainer® CTAD; Becton Dickinson, Plymouth, UK), cooled on ice and centrifuged for 20 min at 2500 g. Plasma samples were stored at −70°C until measurement. BTG and PF4 were determined using commercially available sandwich ELISA kits (Asserachrom b-TG® and Asserachrom PF4®; Diagnostica Stago, Asnières, France).

**PLT, PDW, MPV, p-LCR, and IPF.** Blood samples were collected in K2EDTA tubes (Becton Dickinson). PLT counts, PDW, MPV, p-LCR and IPF are measured using a Sysmex XE-2100 hematology analyzer with a dedicated IPF-Master software package (Sysmex Corp., Kobe, Japan). A fluorescent dye reagent containing polymethine and oxazine is used with the flow cytometric IPF test methodology. After injecting the dye through the cell membrane, RNA in reticulated red blood cells and reticulated PLTs is stained. The stained cells pass through a semiconductor diode laser beam and the resulting forward scatter intensity (measure for cell volume) and fluorescence (measure for RNA content) are measured. IPF

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**Figure 1.** PLT CD62p expression (% PLT, mean ± SD) during HD. Concentrations in both the afferent and the efferent lines are depicted in the line plot. Changes in concentrations in the efferent line from t0 (fistula) over time: * p < 0.001. The difference between the efferent line at t1 and the afferent line at t5 was significant (p < 0.001). Gray bars represent the absolute changes in PLT CD62p expression (% PLT, mean ± SD) over the ECC during HD: ° p < 0.001.
count is calculated from the combination of intensity of the fluorescence signal and the forward scattered light signal from the XE-2100 reticulocyte measurement channel by application of a Sysmex algorithm.

Statistical Analysis
All analyses were performed with the SPSS 15.0 software system. Data were tested for normality. A general linear model for repeated measures was applied to specify the effects of treatment time (based on measurements from the fistula at t0 and from the efferent line thereafter) and sampling point (efferent vs. afferent lines) on the course of PLT activation during a single session of HD. Paired-samples t tests were used as post-hoc analysis. Correlations between BTG, PLT numbers and CD62p expression were calculated and expressed as Pearson’s coefficients. Data are expressed as mean ± standard deviation. Differences were considered significant at p < 0.05.
RESULTS

CD62p
Changes over Time: t0 (Fistula) → t150. As shown in the line plot (Figure 1), CD62p expression showed an increase over time (p < 0.001) reaching a maximum at t30: from 24 ± 13% at t0 to 45 ± 22% at t30. Thereafter, CD62p expression declined gradually.
Changes over the ECC (∆t1, ∆t5 and ∆t30). As illustrated by the gray bars (Figure 1), CD62p expression increased similarly over the ECC after first passage, t5 and t30 (effect of sampling point: p < 0.001).

PLT Numbers
Changes over Time: t0 (Fistula) → t150. As shown in the line plot (Figure 2), PLT numbers decreased over time: from 212 ± 48 x 10^9/l at t0 to 194 ± 43 x 10^9/l at t30 (efferent line) and 206 ± 48 x 10^9/l at t150 (p < 0.001). At t60, a modest rebound was observed.
Changes over the ECC (∆t1, ∆t5 and ∆t30). As demonstrated by the bars (Figure 2), PLT numbers dropped considerably over the ECC only at first passage: ∆t1 –31 ± 31 x 10^9/l (effect of sampling point: p = 0.002, effect of time: p = 0.002).

Figure 3. BTG (IU/ml, mean ± SD) concentrations during HD. Concentrations in both the afferent and efferent lines are depicted in the line plot. Changes in concentrations in the efferent line from t0 (fistula) over time: * p < 0.001. Gray bars represent the absolute changes in BTG (IU/ml, mean ± SD) concentrations over the ECC during HD: ° p < 0.001.
**Figure 4.** PF4 concentrations (IU/ml, mean ± SD) during HD. PF4 concentrations in both the afferent and efferent lines are displayed in the line plot. Changes in concentrations in the efferent line from t0 (fistula) over time: * p < 0.001. The difference between the afferent line at t1 and the efferent line at t1 was highly significant (p = 0.001). Gray bars represent the absolute changes in PF4 concentrations (IU/ml, mean ± SD) over the ECC during HD: ° p < 0.001. Effect of time: • p = 0.001.

**β-Thromboglobulin**

Changes over Time: t0 (fistula) → t150. As shown in the line plot (Figure 3), during HD a highly significant increase was observed reaching maximum values at t30: from 180 ± 45 IU/ml at t0 to 364 ± 138 IU/ml at t30 (p < 0.001). At t150, BTG levels were still considerably elevated if compared to baseline. BTG levels at t5 in the afferent line were fairly similar to those in the efferent line at t1 (dotted part, p = NS), suggesting no substantial release from outside the ECC.

Changes over the ECC (Δt1, Δt5 and Δt30). As illustrated in the bar chart (Figure 3), marked increments across the ECC were observed after first passage, t5 and t30 (effect of sampling point: p < 0.001), and were similar over time.

**Platelet Factor 4**

Changes over Time: t0 (Fistula) → t150. As shown in the line plot (Figure 4), PF4 levels increased over time (p < 0.001) and maximum values were observed at t5: from 13 ± 8 IU/ml at t0 to 114 ± 31 IU/ml at t5. Although PF4 levels declined gradually thereafter, at t150 PF4 levels were still elevated if compared to baseline (39 ± 21 IU/ml). Interestingly, the considerable difference in PF4 observed between the efferent line at t1 and the arterial
line at t5 (dotted part, p = 0.001) implies substantial release from outside the ECC shortly after the start of HD.

Changes over the ECC (Δt1, Δt5 and Δt30). As demonstrated by the gray bars (Figure 4), marked increments across the ECC were observed at t1, t5 and t30 (effect of sampling point: p < 0.001). Interestingly Δt1 was significantly higher than Δt5 and Δt30 (effect of time: p = 0.001).

**MPV, PDW, and p-LCR**

**Changes over Time: t0 (Fistula) → t150.** As shown in Table 1, from t60 onwards MPV, PDW and p-LCR declined, reaching their nadirs at t150 (p < 0.001 for each parameter).

**Changes over the ECC (Δt1, Δt5 and Δt30).** Neither parameter showed changes over the ECC.

**Immature PLT Fraction**

**Changes over Time: t0 (Fistula) → t150.** Neither the absolute value (table 1) nor the percentage of immature PLTs changed over time.

**Changes over the ECC (Δt1, Δt5 and Δt30).** Immature PLTs decreased over the ECC both at first passage and at t5 (effect of sampling point: p = 0.005). Considering the change of immature PLTs as a percentage of the total number of PLTs, only at t5 a slight decrease was observed over the ECC (p = 0.037).

**Correlations between BTG, CD62p and PLT Counts**

There was a correlation between the increase in CD62p expression and the release of BTG over the ECC at all time points measured (Δt1 CD62p vs. Δt1 BTG: r² 0.28, p = 0.020, Δt5 CD62p vs. Δt5 BTG: r² 0.41, p = 0.003 and Δt30 CD62p vs. Δt30 BTG: r² 0.19, p = 0.063), suggesting that BTG is a proper marker for PLT activation in clinical HD. The drop in PLT counts at t1 correlated with the increase in CD62p expression over the ECC at t5 (r² –0.26, p = 0.027) and t30 (r² –0.24, p = 0.035).

**Additional Study**

After dalteparin injection (t –10) BTG levels remained stable from 165 ± 51 IU/ml at t –10 to 164 ± 48 IU/ml at t0 (before pump start) and increased thereafter to 185 ± 69 IU/ml at

| TABLE I. | MPV, PDW, p-LCR and IPF during HD |
|----------|----------------------------------|
|          | T0     | T60eff | T150eff | Δt1   | Δt5    |
| MPV, fl  | 10.4±0.8 | 10.1±0.8* | 10.1±0.9* | −0.0±0.2 | 0.1±0.3 |
| PDW, fl  | 11.9±1.9 | 11.7±1.7 | 11.5±1.8* | 0.1±0.4 | 0.2±0.6 |
| p-LCR, % | 28±7    | 26±7*   | 25±7*   | 0±1   | 1±3   |
| IPF, x10⁹/l | 6±3    | 6±3     | 6±3     | −1±1** | −1±1** |

Effect of time: * p < 0.001; effect of sampling point: ** p = 0.005.
t1 and 254 ± 122 IU/ml at t5eff (p = 0.009, Figure 5). In line with the results of our previous study [2], CD62p expression remained stable after dalteparin injection and increased only after the start of dialysis (data not shown), whereas PF4 levels increased immediately after dalteparin injection from 10 ± 6 IU/ml at t–10 to 90 ± 34 IU/ml at t0 (p = 0.009) just before pump start.

**DISCUSSION**

PLT activation and degranulation are well-known phenomena in HD patients. However, the exact mechanism of these processes and the specific contribution of the ECC herein are incompletely understood. To elucidate these issues 19 chronic HD patients were included in the present analysis. Blood samples were drawn at various time and sampling points during treatment. Calculated differences between the efferent and afferent sampling points at t0/1 (first passage), t5 and t30 were designated ∆t1, ∆t5 and ∆t30, respectively. In line with previous observations [2], CD62p expression increased over time, followed by a progressive decline after 30 min. The increase over the ECC after first passage (∆t1) was comparable to both ∆t5 and ∆t30, indicating that PLTs are predominantly activated within the ECC and not on a remote distance. This view is further substantiated by the observation that at all time points measured, CD62p expression in the efferent line was higher than in the afferent line.

**Figure 3.** Additional experiment: PF4 (IU/ml, mean ± SD) and BTG (IU/ml, mean ± SD) concentrations before and after dalteparin injection (t –10: 10 min before dialysis) and before and after start of HD (t0): • p < 0.05 versus t0, + p = 0.009 versus t –10.
With respect to PLT numbers, a marked drop occurred after first passage, followed by an incomplete rebound at t5. As the drop over the ECC at first passage amounted to approximately 15%, the combined data of CD62p and PLT numbers suggest that a considerable number of activated PLTs stick to the ECC, most probably the dialyzer membrane [3] directly after the start of HD. Interestingly, in this respect, PLT behavior is different from that of polymorphonuclear cells, as these cells attach mainly to the lung vasculature after activation in the ECC and only to a limited extent to the dialyzer [13]. Whether the increase at t5 is due to mobilization of fresh PLTs from pools and/or detachment from the ECC is not readily apparent from this study.

With respect to PLT degranulation, BTG levels reached peak values at t30 and decreased gradually over time thereafter. The increase over the ECC was highly significant, without marked differences between Δt1, Δt5 and Δt30. There was in fact a significant correlation between ΔCD62p and ΔBTG at t1 and t5. As BTG levels were lower in the afferent line at all time points, these data suggest that BTG is almost exclusively released within the ECC. With respect to PF4, a quite different profile was observed. After an initial increase over the ECC, at t5 a sharp increase was observed in the afferent line, suggesting considerable release outside the ECC. Indeed, we have recently shown that the increase in PF4 during HD is mainly caused by LMWH-induced release from outside the ECC, most likely due to detachment from the endothelium [2]. Interestingly, the additional experiment in the present study showed that BTG release was not attributable to LMWH-induced release from outside the ECC. Our current and previous data taken together, it seems reasonable to conclude that PF4 is mainly released from the endothelium after the administration of LMWH and to a more limited extent from PLTs that are activated during first passage through the ECC. With respect to PF4 and BTG, only the latter substance is an appropriate parameter for the assessment of PLT degranulation during clinical HD. Moreover, its abundant release indicates that PLTs are not exhausted due to the repetitive stimulation of HD treatment, as was suggested before for PLTs [14, 15] and polymorphonuclear cells [16].

With respect to the PLT indices MPV, PDW and p- LCR, all values decreased over time, whereas significant changes over the ECC were absent. Previously, it was suggested that PLT volume decreases due to degranulation [14]. In that analysis, however, neither volume parameters nor degranulation products were measured over the ECC. In the present study, BTG was released continuously over the ECC, whereas volume parameters remained unaltered. Therefore, it is less likely that degranulation itself is responsible for the changes in volume parameters over time. Hence, we propose an alternative mechanism which might explain the observed changes. It has been well established that during the course of HD artificial membranes are progressively covered with protein depositions [17]. In the present study, PLTs adhered to the ECC, but mainly after first passage, on native material, before the deposition of proteins. As shown in this study, activated PLTs still released their granule content thereafter, but did hardly stick any further to the dialyzer membrane, most probably as a result of the protecting protein layer. Thus, with the exception of first passage, activated and degranulated PLTs leave the ECC during the entire course of HD and enter the circulation of the patients [5]. Subsequently, these activated PLTs are trapped and removed by the reticuloendothelial system of the body. As younger and larger PLTs are more prone to activation, during HD volume parameters in the circulation may decrease over time due to a proportional increase in older and smaller PLTs.
With respect to IPF, marked changes did not occur. However, at t5 the decline of this parameter was proportionately greater than the decrease in PLT, suggesting that in the first minutes of HD especially the younger and larger PLTs with a relatively high RNA content stick to the ECC.

The findings of our study may have important practical consequences. Cardiovascular disease is the most important cause of death in patients with ESRD. Besides traditional risk factors and the accumulation of middle and large-molecular-weight uremic toxins [18], HD-related risk factors, such as the bioincompatibility of the ECC, may contribute to the development of cardiovascular disease. Interestingly, in this respect, PLT activation is thought to play a causal role in atherogenesis, especially in the early stages [19]. As mentioned above, PLTs are not exhausted due to the repetitive stimulation of repeated HD. Moreover, during HD activated PLTs and their degranulation products leave the ECC and enter the circulation. Therefore, it is conceivable that modalities of longer duration or greater frequency, such as nocturnal HD and daily HD, are associated with a less beneficial PLT activation profile. Hemodiafiltration, which is characterized by a higher transmembrane pressure and considerable hemoconcentration, may also negatively influence PLT activation. Hence, it is conceivable that the beneficial effects of these modalities on hemodynamics [20] and/or bone mineral disease [21] are counterbalanced by an adverse profile of various biocompatibility parameters. Unfortunately, little or no information is available on these issues in either of these modalities.

To summarize, as demonstrated by the expression of CD62p, PLTs are activated over the ECC during the first 30 min of HD. Similar to leukocytes, a sharp drop in numbers is observed shortly after the start of treatment. However, whereas the drop in leukocytes is caused mainly by the attachment of activated polymophonuclear cells to the lung vasculature, activated PLTs stick to the ECC, most probably to the membrane of the dialyzer. PLT degranulation, as measured by an increase in BTG, parallels CD62p expression, whereas the increase in PF4 levels is caused by release from both inside and outside the ECC. From these data it can be concluded that PLTs are not exhausted, possibly due to the short half-life time, especially in uremic conditions. As volume parameters declined gradually over time, it is conceivable that especially younger, larger and more viable PLTs are activated during treatment and entrapped in the reticuloendothelial system of the body. Whether the potentially negative effects of fashionable modalities, such as nocturnal HD, daily HD and hemodiafiltration, on PLT activation and degranulation counterbalance their supposed beneficial effects on clinical endpoints is currently unknown.
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PLATELET DEPLETION, PLATELET ACTIVATION AND COAGULATION DURING TREATMENT WITH HEMODIALYSIS

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ABSTRACT

Bioincompatibility is the total of side effects during hemodialysis (HD) including, amongst others, changes in platelet (PLT) level. Deviations in PLT count, immature PLT count, PLT morphology, CD62p expression, Platelet Factor 4 (PF4), β-Thromboglobulin (β-TG), serotonin, Thrombin-Antithrombin III (TAT) and Prothrombin Fragment 1+2 (F1+2) are monitored before and during treatment with HD in order to elucidate the interaction between modifications in PLT morphology, PLT activation and markers concerning activation of coagulation. Different patterns with time indicate that there is no correlation between an increased amount of depleted PLTs and increased amounts of PLT activation markers such as CD62p, PF4, β-TG and serotonin. A statistically significant correlation between increased PLT activation markers and markers for increased activation of coagulation such as TAT and F1+2 has not been established. Only a weak correlation is demonstrated between the increase of markers for activation of coagulation and the decrease in PLT counts, immature PLT counts and depleted PLTs during HD treatment. The change in the extracorporeal circuit during HD is probably a more critical factor in the mechanism leading to activation of the coagulation pathway than the modifications in PLT morphology.
INTRODUCTION

During treatment with hemodialysis (HD) undesirable interactions occur between the extracorporeal circuit (ECC) and the human body. The total of interactions has been called bioincompatibility [1]. In addition to components of the ECC, including blood lines, roller pump and dialyser, the mode of anticoagulation plays an essential role [2, 3]. Intravascular coagulation and platelet (PLT) activation is most crucial in the case of extracorporeal blood circulation [4, 5]. Coagulation can cause clogging of the capillaries within the artificial dialyser and thus result in a decrease in the efficacy of HD treatment.

PLT interaction in the ECC results in adhesion and retention of PLTs on the artificial membrane and subsequently release of platelet-derived growth factors [6]. Increased expression of surface marker p-selectin (CD62p) indicate PLT activation. PLT degranulation is indicated by release of intracellular products, such as serotonin from dense granules and platelet factor 4 (PF4) and β-thromboglobulin (β-TG) from α-granules [7–9]. Due to the ongoing PLT activation in subjects on maintenance HD, PLT characteristics in peripheral blood show increased amounts of depleted PLTs with a smaller volume and a shortened life span [10, 11]. A rather heterogeneous population of PLTs will remain, consisting of intact larger PLTs, shape-changed PLTs and PLTs which are already more or less depleted due to damage as a result of bioincompatibility.

Activation of coagulation is a multifactorial event initiated with interaction of PLTs, Von Willebrand Factor and vessels. Thrombin generation is induced by Tissue Factor from endothelium and Factor VIIa [12]. In case of coagulation activation, plasma concentrations of thrombin antithrombin III complexes (TAT) and prothrombin fragment 1 + 2 (F1+2) will yield an indication with respect to thrombin generation [13,14]. Within the ECC, intact endothelium is lacking and activation of PLTs and markers concerning activation of coagulation will be induced by mechanical events. Moreover, immediately after starting HD, bio-artificial materials of ECC are contaminated with circulating proteins. It is yet unknown whether PLT activation and activation of coagulation in HD are interrelated.

Important factors resulting in increased thrombogenicity in case of HD treatment include reduction of blood flow, alterations in the blood vessel wall, changes in blood composition and biocompatibility of artificial membranes, respectively. If not effectively managed, thrombogenicity will result in reduced hemodialysis efficacy, because the membrane does not function appropriately. In the current study PLT counts and morphological PLT aberrations are considered simultaneously with evaluation of markers for PLT activation and markers for activation of coagulation during HD treatment in order to elucidate a correlation and to determinate if remaining exhausted PLTs during and after HD treatment is the critical factor in the mechanism leading to activation of the coagulation pathway.
PATIENTS AND METHODS

Patients
A group of 20 chronic HD subjects (age 28 – 82 years) from the dialysis unit of the Medical Center Alkmaar participated in the study, only including patients who had been on regular HD treatment for at least 1 year. The study protocol was approved by the local ethics committee. Written informed consent was obtained from participants. The etiology of chronic renal insufficiency was hypertensive nephrosclerosis \((n=8)\), diabetic nephropathy \((n=5)\), adult dominant polycystic kidney disease \((n=3)\), IgA nephropathy \((n=1)\), tubulo-interstitial nephritis \((n=1)\), chronic pyelonephritis \((n=1)\) and membranous nephropathy \((n=1)\). Subjects with an age of <18 years, a life expectancy <3 months, active inflammation, thrombocytopenia, autoimmune disease or malignancy as well as supplementation of drugs interfering with PLT function or anticoagulation (immunosuppressive drugs, calcium antagonists, serotonin receptor antagonists, coumarin derivatives and aspirin) were excluded from participation.

HD treatment
Low flux polysulfone® F8 dialyser membranes (Fresenius Medical Care, Bad Homburg, Germany; ultrafiltration (UF) factor 18 mL/mmHg/h, surface area 1.8 m², steam sterilized) were applied. According to individual needs, blood flow rate (250–300 mL/min) was kept constant and ultrafiltration flow rate varied between 300 and 1000 mL/min. For dialysate preparation, tap water, purified by reverse osmosis, was used for dilution of a concentrated bicarbonate solution to appropriate concentrations (mMol/L): \(138\) Na⁺, \(2.0\) K⁺, \(1.50\) Ca²⁺, \(0.50\) Mg²⁺, \(109\) Cl⁻, \(2.5\) CH₃COO⁻ and \(32.5\) HCO₃⁻ (SK-F 216/1; Fresenius Medical Care, Bad Homburg, Germany). Dialysate flow rate amounted to 500 mL/min. Dialysers were pre-rinsed with 1000 mL 0.9% NaCl. Individual doses of Fragmin® were calculated based on body weight (50 IU/kg) and supplied intravenously as a bolus injection when starting a dialysis session (mean 4750 ± 1419 IU).

As a result of PLT activation during HD treatment micro PLT aggregates and thrombin generation occur. In practice, the presence of macro fibrin deposits in air-trap devices is observed.

Group of reference subjects
For the purpose of comparison and clinical interpretation with regard to pathophysiology, additional investigations are performed in an apparently healthy subjects’ group. A group of 20 subjects (laboratory technicians, age 20 – 50 years) was selected in order to establish reference range intervals for parameters reflecting activation of PLTs and coagulation. Subjects did not use any drugs interfering with PLT activation or coagulation.

Blood sampling
During one dialysis session blood samples were drawn from the arterial line before starting HD \((t = 0)\) and subsequently from the efferent line after 5 \((t = 5)\), 30 \((t = 30)\) and 150 \((t = 150)\) minutes.
Analytical methods

Blood samples for PLT count, immature PLT count, CD62p and establishment of serotonin in platelet rich plasma (PRP) and platelet poor plasma (PPP) were collected into K<sub>2</sub>EDTA-tubes (Vacutainer®, Becton Dickinson, Plymouth, UK). For determination of PF4 and β-TG blood samples were collected into CTAD tubes (Vacutainer®, Becton Dickinson, Plymouth, UK). Sodium citrate tubes (0.109 Mol, Vacutainer®, Becton Dickinson, Plymouth, UK) were applied to establish TAT and F<sub>1</sub>+2 concentrations. CTAD and sodium citrate anticoagulated blood samples were cooled on ice in order to prevent in vitro activation of PLTs. Blood samples were analysed immediately after sampling or aliquoted for storage at -70° C within 2 h after collection.

PLT count, Immature PLT count (IPF) and PLT morphology

PLT counts and Immature PLT counts were measured using a Sysmex XE-2100 Hematology analyser with a dedicated IPF-Master software package (Sysmex Corporation, Kobe, Japan). Duplicated peripheral blood slide smears were prepared for evaluation of PLT morphology aberrations. Slide smears were stained according to May-Grünwald-Giemsa methodology on a Sysmex SP-100 analyser (Sysmex Corporation, Kobe, Japan). Slides were microscopically screened for the presence of PLT aggregates and qualitative evaluation of morphological PLT aberrations with application of a CellaVision<sup>TM</sup> DM96 analyser (CellaVision AB, Lund, Sweden). As previously established, a staining density >75% of the granule containing cytoplasm in >50% of PLTs was considered to be the lower limit of the reference range [10]. Depleted PLTs were defined as PLTs with a staining density amounting to <25% of granule containing cytoplasm. The upper limit of the reference interval for depleted PLTs was determined at <20% of PLTs.

CD62p, PF4, β-TG and serotonin

PLT activation marker CD62p was measured by application of a direct labelling procedure with subsequent detection by flowcytometric analysis. Within 2 h after blood collection, blood samples were incubated with a glycoprotein specific fluorochrome-labeled antibody. A double labeling procedure was performed with CD41 as the cell defining marker. Flowcytometric analysis with a Coulter EPICS-XL (Beckman-Coulter, Fullerton, USA) was applied in order to establish the amount of antigen presentation on the PLT surface membrane.

Blood samples for determination of PF4 and β-TG plasma concentrations were centrifuged at 2–8° C for 20 min at 2500 g in order to separate plasma from the cellular fraction. Plasma aliquots were stored at −70° C until analysis. PF4 and β-TG were assayed with ELISA (Roche PF4 and Roche β-TG, Roche, Asnières, France). For quantification of serotonin concentrations in PRP and PPP blood specimens were centrifuged at 200 g and 4000 g respectively. Until analysis PRP and PPP samples were stored at −70° C. Serotonin concentrations were established in PRP and PPP aliquots by application of a commercially available ELISA testkit (DSL-serotonin, Diagnostic Systems Laboratories GmbH, Sinsheim, Germany).
TAT and F1 + 2
Blood samples for determination of concentrations of TAT complexes and F1 + 2 were centrifuged at 2500 g in order to separate plasma from the cellular fraction. Plasma aliquots were stored at –70° C until analysis. TAT and F1 + 2 concentrations were assayed with ELISA (Enzygnost® TAT micro and Enzygnost® F1 + 2 monoclonal, Siemens Healthcare Diagnostics Inc., Marburg, Germany).

Statistical evaluation
Statistical evaluation of analytical data was performed with SPSS software 14.0 for Windows. A general linear model for repeated measures was applied in order to evaluate the kinetic effects of treatment with time, based on comparison with measurements at t = 0. All parameters were normally distributed. Paired-sample t-tests were performed as post-hoc analysis. Statistical significance of deviations between mean values of the group of HD subjects and the reference group of apparently healthy subjects was calculated by application of the 2-tailed Student t-test for unpaired data. A p-value <0.05 was considered to be statistically significant. Correlations between markers for PLT activation and markers for activation of coagulation were calculated and expressed as Pearson’s coefficients, with significance at the 0.05 level (2-tailed). Results for PLT counts, immature PLT counts, plasma concentrations of PF4, β-TG, serotonin, TAT and F1 + 2 at t = 5, t = 30 and t = 150 minutes were corrected for changes in hematocrit (Ht).
For example: corrected value $t = 30 = \frac{Ht_{t = 0}}{Ht_{t = 30}} \times$ value $t = 30$. 
RESULTS

Investigations in subjects before and during HD treatment are performed for monitoring kinetics together with the establishment of intra-individual variations due to activation. In practice, visible clotting in the air-trap device or blocked dialyser membranes, due to macro fibrin deposits were not demonstrated. Therefore, early termination of a HD session did not occur. Results for parameters reflecting PLT counts, aberrations in PLT morphology, PLT activation and PLT degranulation in the case of HD subjects and apparently healthy subjects are demonstrated in Figures 1 and 2 respectively. Results for parameters concerning activation of coagulation in the group of HD subjects and the group of apparently healthy subjects are demonstrated in Figure 3. Results in the text are listed as mean ± SD.

Figure 1. Box plots representing PLT count (A), immature PLT count (B) and the percentage of PLTs with <25% (C) and >75% (D), with the staining intensity of granule containing cytoplasm established in subjects before the start of hemodialysis treatment (t = 0) and at stages t = 5, t = 30 and t = 150 min after starting HD (n = 20). For comparison, results for a group of 20 apparently healthy subjects (REF) are depicted. The box extends from the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to the largest and smallest observed values within 1.5 box lengths. Outlying and extreme values corresponding with values between 1.5 and 3 times the box length or >3 times the box length, respectively, are designated as (o) and (*). The horizontal dashed lines indicate the upper and lower level of the reference range for apparently healthy subjects.
**PLT count, Immature PLT count, PLT morphology**

From the results demonstrated in Figure 1A and 1B it can be observed that initial PLT counts and immature PLT counts in subjects with HD treatment (mean ± SD: 198 ± 43 \(10^9/L\) and 6.7 ± 2.8 \(10^9/L\) respectively) are situated in the lower quartile range of the reference interval. Compared with results for the reference group statistically significant decreases at t = 0 for PLT counts (\(p = 0.006\)) and immature PLT counts (\(p = 0.006\)) are established. Due to HD treatment statistically significant decreases in PLT counts (\(p = 0.000\)) and immature PLT counts (\(p = 0.031\)) to 182 ± 42 \(10^9/L\) and 6.1 ± \(10^9/L\) are established at t = 150 min. Microscopic evaluation of the stained blood slides did not show PLT aggregates. In subjects with chronic HD treatment appropriate staining density of the granule containing cytoplasm decreased to a minimum score (Figure 1D). Only 19 ± 11% of the PLTs yielded >75% staining density. On the contrary, in the group of reference subjects, 70 ± 12% of the PLTs are established to reveal appropriate staining density (\(p = 0.000\)). At t = 150 min an increase of PLTs occurs with appropriate granule staining density amounting to 29 ± 16% (\(p = 0.001\)).

PLTs with a staining density amounting to <25% of the granule containing cytoplasm has been defined as depleted. At t = 0, 36 ± 9% of the PLTs were depleted (Figure 1C). In the reference subjects’ group only 9 ± 6% of the PLTs were shown to be depleted (\(p = 0.000\)). At t = 150 minutes a decrease of depleted PLTs occurs amounting to 29 ± 11% (\(p = 0.007\)).

**CD62p, PF4, β-TG, serotonin**

In subjects with HD treatment results for CD62p expression (Figure 2A) indicate an immediate statistically significant increase from 22 ± 8% at t = 0 to 37 ± 18% at t = 5 min (\(p = 0.000\)). After reaching peak levels at t = 30 minutes, results decrease to a base level at t = 150 minutes (26 ± 12%, NS). Results for CD62p expression before starting HD treatment are within the reference range and do not demonstrate statistically significant deviations if compared with the reference group.

Results for PF4 concentrations (Figure 2B) demonstrate an immediate increase from 10 ± 6 kIU/L at t = 0 to 98 ± 42 kIU/L at t = 5 min (\(p = 0.000\)). After t = 5 min a steadily ongoing decrease is observed to 44 ± 32 kIU/L at t = 150 min (\(p = 0.000\)). Although results for PF4 in the group of HD subjects before starting HD treatment are within the limits of the reference range interval statistically significant deviations are observed (\(p = 0.029\)) when results are compared with results of apparently healthy subjects (16 ± 9 kIU/L).

Compared with results for the reference group β-TG concentrations before starting HD treatment (Figure 2C) are statistically significantly increased (\(p = 0.000\)). Results for β-TG concentrations an increase from 188 ± 61 kIU/L at t = 0 to 335 ± 108 kIU/L at t = 30 min (\(p = 0.000\)). After t = 30 min no further release of β-TG was observed.

Before starting HD treatment, concentrations of serotonin in PRP are within the reference range (Figure 2D). Concentrations of serotonin in PRP amounted to 3.2 ± 1.9 nMol/10^9 PLTs before starting HD treatment and 2.2 ± 1.3 nMol/10^9 PLTs at t = 150 min (\(p = 0.007\)). Although during HD treatment serotonin content in PRP is steadily decreasing, additional release of serotonin in PPP could not be detected (Figure 2E). Initial concentrations of serotonin in PPP are increased (110 ± 74 nMol/L) and statistically significantly different if compared with the reference subjects’ group (9 ± 6 nMol/L, \(p = 0.000\)).
Figure 2. Box plots for evaluation of CD62p (A), PF4 (B), β-TG (C) and serotonin concentrations in PRP (D) and PPP (E) established in subjects before the start of hemodialysis treatment (t = 0) and at stages t = 5, t = 30 and t = 150 min after starting HD (n = 20). For comparison, results for a group of 20 apparently healthy subjects (REF) are depicted. The box extends from the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to the largest and smallest observed values within 1.5 box lengths. Outlying and extreme values corresponding with values between 1.5 and 3 times the box length or >3 times the box length, respectively, are designated as (o) and (*). The horizontal dashed lines indicate the upper and lower level of the reference range for apparently healthy subjects.
TAT, F1 + 2
TAT plasma concentrations (Figure 3A) show statistically significant increasing results from 2.9 ± 0.6 μg/L at t = 0 to 6.2 ± 2.5 μg/L at t = 150 min (p = 0.000). Compared to results from the reference subjects’ group F1 + 2, concentrations are already increased when starting HD treatment (278 ± 126 pMol/L, p = 0.013). During HD treatment a steadily ongoing further increase of F1 + 2 concentrations was established (Figure 3B).

Correlation between aberrations in PLT morphology, PLT activation, PLT degranulation and activation of coagulation
An association between PLT count, immature PLT count and aberrations in PLT morphology was not established. During HD, statistically significant correlations between CD62p and PF4 were obtained at t = 5 (r = 0.68, p = 0.001), t = 30 (r = 0.70, p = 0.001) and t = 150 min (r = 0.61, p = 0.005). Only at t = 30 min was a correlation between CD62p and β-TG (r = 0.47, 0.036) detected. Correlation between PF4 and β-TG was established at t = 5 minutes (r = 0.57, p = 0.008) and t = 30 minutes (r = 0.69, p = 0.001).
At t = 0 a correlation of r = 0.64 (p = 0.004) was obtained between the coagulation markers TAT and F1 + 2. After starting HD the correlation between TAT and F1 + 2 disappeared. Depleted PLTs correlate with CD62p at t = 5 (r = 0.55, p = 0.011), t = 30 (r = 0.55, p = 0.011) and t = 150 minutes (r = 0.48, p = 0.033). PF4, CD62p, β-TG and serotonin concentrations during HD do not correlate with the amount of PLTs revealing appropriate staining density, TAT and F1 + 2. Correlations between immature PLT count and TAT are
calculated during HD at \( t = 5 \) \((r = 0.53, p = 0.015)\), \( t = 30 \) \((r = 0.72, p = 0.000)\) and \( t = 150 \) minutes \((r = 0.68, p = 0.001)\). A significant correlation between the percentage of PLTs with appropriate staining density and \( F1 + 2 \) is calculated at \( t = 0 \) \((r = 0.62, p = 0.004)\) and \( t = 5 \) minutes \((r = 0.46, p = 0.041)\).

**DISCUSSION**

In agreement with previous reports, results from the present study demonstrate that PLT counts and IPF counts in chronic HD subjects are lower than in healthy subjects, whereas an even further gradual decrease is observed after the start of HD [15].

As demonstrated in the present study, PLTs in HD subjects are depleted and remain depleted during HD treatment. Increased concentrations of \( F1 + 2 \) and \( \beta-TG \) are already present before starting HD treatment. Within the first 5 min of HD treatment, PLTs are activated and already degranulated, while thrombin formation is initiated. An interdependency between a decrease in PLT counts and aberrations in PLT morphology has not been established. An association between the PLT activation markers CD62p, PF4 and \( \beta-TG \) has not been clearly demonstrated. Rather surprisingly, an analogous tendency with regard to markers of PLT activation and activation of coagulation fail. Particular pathophysiological aspects of the HD procedure itself, such as administration of LMWH and deposition of a protein layer onto the dialyser membrane, may partly explain the results. Moreover, loss of renal catabolic and excretory functions will yield additional variation.

Pathophysiological mechanisms inducing activation of coagulation are based on Virchow’s triad including modifications in vessel wall, blood flow and composition of blood components [16–18]. In the case of the extracorporeal circulation, the endothelial surface with anticoagulatory and vaso-dilating capacities, is restituted by blood lines and a dialyser membrane consisting of bioincompatible material. Thereby, blood components may be damaged by mechanical compression in the roller pump segment. Ultrafiltration of excessive fluid occurs by application of a negative pressure between the blood compartment and dialysate compartment. Under extremely unphysiological conditions, artificial membranes are instantaneously contaminated by deposition of a protein layer [19–21]. Proteomic analysis indicates that many proteins, such as complement components, antithrombin-III, fibrinogen and \( \beta-2 \)-glycoprotein-1 are involved in blood-dialyser interactions [21]. Unphysiological conditions within the ECC, amplified by pre-dialysis increased uremia related factors, will induce activation of coagulation during HD treatment. Probably, the degree of activation of coagulation, as indicated by increasing TAT en \( F1+2 \) concentrations, does not reflect accurately real time intradialyser clotting performance.

Analogous considerations were applied to PLT activation and degranulation. Contact of already depleted PLTs with the artificial membrane resulted in activation, as indicated by the increase in CD62p expression and release of PLT granule products. Besides adherence in the ECC, shear stress-activated PLTs will demonstrate a tendency for aggregation [8,22]. Probably, adherence of PLTs to artificial membranes is rather weak. It is hypothesized that PLTs are released during HD from storage pools [7, 10]. Therefore, a heterogeneous mixture of intact larger PLTs, detached shape-changed PLTs and more or less degranulated PLTs, with a low PLT volume was observed [7, 10, 11].
The phenomenon of PLT degranulation can be elucidated to some extent. PF4 release mainly results of LMWH-induced detachment from endothelium and to a limited extent from α-granules in PLTs [8]. In the study of Gritters et al., the influence of LMWH on CD62p expression and PF4 release during and just before a single HD session was demonstrated. To demonstrate or exclude release from platelets, blood from healthy volunteers was incubated with heparin in clinical doses. From that experiment it appeared that PF4 was not released as long as clotting did not occur [8].

Concentrations of PLT serotonin slightly decrease during HD treatment. Serotonin is metabolised in 5-hydroxy-indol-acetic acid (5-HIAA, MW 191 g/Mol) in endothelial and proximal tubular cells [23]. In the case of decreased glomerular filtration rate, elimination of serotonin is reduced, resulting in increased levels of plasma serotonin [23]. Probably due to the state of hyponatremia, the balance in serotonin transport is disturbed [24]. However, in our patient group, sodium concentrations before HD treatment were 139 ± 3 mMol/L (range 134–144 mMol/L). During HD, the 5-HIAA molecule easily passes through the pores of the dialyser membrane resulting in a decrease of plasma serotonin concentration. β-TG is metabolized and excreted by the kidney. In the case of reduced kidney function, the β-TG normal half life time of approximately 100 min is considerably increased [25]. Gritters et al. have demonstrated that β-TG, which is like PF4 stored in the α-granules of PLTs, is almost exclusively released within the extracorporeal circuit (ECC) and is not influenced by the application of LMWH [3]. In contrast, PF4 is significantly released outside the ECC in response to LMWH [3]. Within this respect, β-TG may be regarded as a reliable indicator of HD-induced PLT degranulation. With respect to degranulation during HD and hemodiafiltration (HDF), rather dissimilar results for PF4 and β-TG are obtained [26]. During HD, both PF4 and β-TG increase over time, whereas during HDF, PF4 increases but β-TG does not change, neither in the ECC, nor over time. As the molecular weights of PF4 and β-TG are 27kD and 36 kD, respectively, it seems plausible that these substances are removed by convective transport during HDF, which is obviously not the case in low-flux HD [26].

To summarize, the results of our study demonstrate that depleted PLTs are going to be activated. When starting HD treatment, activation of coagulation is initiated. In our opinion, highly unphysiological conditions within the ECC results in the attachment of proteins and PLTs and is responsible for the mechanism that leads to activation of coagulation during HD treatment.
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Electron microscopic observation in case of platelet activation in a chronic haemodialysis subject

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ABSTRACT

During haemodialysis (HD), platelets (PLTs) are activated and release granule contents. As HD treatment occurs three times a week, it has been demonstrated that PLTs are exhausted due to the repetitive character of the treatment. To identify PLT depletion morphologically, PLT evaluation was performed by light microscopy and electron microscopy (EM) in a chronic HD subject and a healthy reference subject. Blood samples were taken before the start of HD treatment for measurement of PLT count, PLT volume and size parameters. Blood smears were screened by light microscopy for qualitative evaluation of PLT granule containing cytoplasm, as indicated by its staining density. Morphological PLT parameters of surface area and size of dense bodies were assessed by EM. Data were compared with results of a group of 20 chronic HD subjects and a group of 20 healthy reference subjects. With respect to the percentage of PLTs with appropriate staining density (>75%), light microscopic evaluation showed that this value (9%) was within the range of a group of chronic HD subjects, but considerably below the reference range (70%). EM evaluation revealed an average PLT surface area and dense bodies area of respectively 42% and 31%, if the healthy reference subject was set on 100%. PLTs from a chronic HD subject are considerably smaller and substantially less granular than PLTs from a healthy reference subject. These findings support the hypothesis of PLT depletion in chronic HD subjects due to frequent PLT activation and/or increased urea concentrations.
INTRODUCTION

As a consequence of extracorporeal blood circulation activation of PLTs occurs in subjects treated with haemodialysis (HD). PLTs are activated due to contact with artificial membranes during HD treatment. The process of PLT activation results in exposure of CD62p, an alpha and dense granule labelling membrane protein, on the outside of the PLT membrane and release of PLT granule content. Concentrations of Platelet Factor 4 (PF4), b-thromboglobulin (b-TG) and serotonin in plasma are indicative for the degree of activation of PLTs.1-3

As HD-induced PLT activation and degranulation occur three times a week, it has been suggested that PLTs from chronic HD subjects are continuously exhausted due to the repetitive character of the treatment. Recently we demonstrated in a light microscopic study that the staining density of the granule containing cytoplasm of PLTs was considerably reduced.4,5 Whereas 70% of PLTs revealed appropriate granule staining density in healthy subjects, in HD subjects this proportion amounted only to 19% on average. To observe the morphological aspects of PLT depletion in more detail, both an EM and a light microscopic evaluation of PLTs is performed. Results are compared with a healthy reference subject. In addition, blood samples are taken for haemocytometric analysis of PLTs and compared with recent results in groups of chronic HD subjects and healthy reference subjects.4,5

MATERIALS AND METHODS

HD subject and healthy reference subject
Particularly for EM, one representative HD subject of a group of 20 chronic HD subjects was selected.4,5 The patient (woman, age 57) underwent 44 months for three times a week HD treatment with a low flux polysulphone® F8 membrane (Fresenius, Bad Homburg, Germany). As anticoagulant Fragmin® (intravenously 3500 U bolus injection) was used. The original kidney disease was due to hypertension. She did not use coumarines, acetylsalicylic acid, clopidogel, non-steroidal anti-inflammatory drugs or related compounds. Blood samples were collected from the arterial line before starting HD (t=0).

The representative healthy subject of a group of 20 healthy reference subjects, was a healthy 45 years old female laboratory technician without a medical history and without any medication.4,5

PLT counts, PDW, MPV, and p-LCR
Blood samples were collected in K₂EDTA tubes (Vacutainer, Becton Dickinson, Plymouth, UK). PLT count, PLT distribution width (PDW), mean PLT volume (MPV) and PLT large cell ratio (p-LCR) were measured using a Sysmex XE-2100 haematology analyser with a dedicated IPF-Master software package (Sysmex Corporation, Kobe, Japan).

PLT morphology
Two peripheral blood smears were prepared for evaluation of PLT morphology and stained according to May-Grünwald-Giemsa methodology on a Sysmex SP-100 analyser.
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(Sysmex Corporation, Kobe, Japan). The slides were light microscopically screened for qualitative evaluation of morphological aspects of PLTs with application of a Cella Vision™ DM96 analyser (Cella Vision AB, Lund Sweden). Using the Cella Vision™ DM96 it is possible to reduce deviations between observations of biomedical scientists. Granule-containing cytoplasm, indicated as a granulomere, is stained light purple or pink. After discharge of granule content, activated PLTs are faintly stained grey. Qualitative aspects were evaluated by classification of the PLT content in four categories corresponding to a staining density of granule-containing cytoplasm amounting to <25%, 25-50%, 50-75% or >75%, respectively. Staining density of >75% of the granule-containing cytoplasm in >50% of PLTs was considered to be appropriate (Figure 1).

Electron microscopy
PLTs were fixed in 2% glutaraldehyde in 0.1M sodiumcacodylate buffer PH 7.4. After postfixation in 1% osmiumtetroxide in sodiumcacodylate buffer, PLTs were pelleted in 1% agar and subsequently dehydrated in ethanol and embedded in Agar 100 Resin (Agar Scientific, Stansted, UK). Ultrathin sections were stained with uranylacetate and leadcitrate and examined with application of a transmission electron microscope (TEM), model Philips CM100 Bio Twin (Philips/FEI Corporation, Eindhoven, The Netherlands).

Image analysis
Electron micrographs were imported as Tag Image File Format (TIFF) and analyzed with application of dedicated Slidebook™ image analysis software (Slidebook version 4.2; Intelligent Imaging Innovations (3I), Denver, CO, USA). Platelet morphology parameters (e.g. cross sectional surface area, xy-shape and number and size of dense bodies per platelet) were derived from so called digital image masks, binary overlays which were created in an operator-independent fashion based on fixed threshold settings. Subsequently, individual mask objects were analyzed and quantified. In each subject, 20 regions of interest were analyzed yielding > 250 PLTs per individual.

RESULTS

Haemocytometry
As can be seen from Table I, the haemocytometric data of the selected HD subject, reflecting PLT volume and size characteristics, are within the range of the group of 20 chronic HD subjects and almost comparable to the selected healthy reference subject / reference ranges.

Light microscopic evaluation
Light microscopic evaluation of PLTs with appropriate (>75%) and with decreased (<25%) staining density of the granule-containing cytoplasm is shown in Figure 1. In the subject with chronic HD treatment appropriate staining density of the granule containing cytoplasm is decreased to a minimum of 8% if compared with the reference ranges for healthy controls (mean 70%, range 44-86%), but within the outer limits of the group of 20 HD subjects (mean 19%, range 4-41%) (Table 1).
**Electron microscopic evaluation**

EM photographs of PLTs of the HD subject and healthy reference subject are shown in Figure 2. Evaluation revealed an average PLT surface area only of 242.481 pixels in the HD subject (Figure 2A) and 576.533 pixels in the healthy reference subject (Figure 2B). The proportional area of dense bodies per PLT, set at 100% in the healthy reference subject, was 31% in the HD patient (Table 1).

**DISCUSSION**

In the present study, several PLT characteristics are assessed in a representative HD subject of a group of 20 chronic HD subjects, who did not use any drugs interfering with PLT activation. It is demonstrated, that the PLT surface area, as assessed by EM, was considerably smaller in case of the HD subject (42%) than in the healthy reference subject. In comparison with the healthy reference subject, the area of dense bodies per PLT amounted to 31%. The staining density of PLTs, reflecting the amount of granule-containing cytoplasm as assessed by application of light microscopy in the HD subject, is obviously below the result of the reference subject. These data suggest either a failure in the synthesis and storage of granule contents, and/or preceding PLT degranulation. Indeed, as previously demonstrated by our group and others, PLT granule products like PF4, β-TG and serotonin are released during HD. As these phenomena occur three times a week, month after months, it has been suggested that PLTs from chronic HD subjects are depleted and, in combination with the uremic environment, persistently deficient.

![Figure 1](image)

**Figure 1.** Light microscopic evaluation of PLTs with appropriate (>75%, left) and with decreased (<25%, right) staining density of the granule-containing cytoplasm. Magnification: 600x.
Figure 2. (A) Digital mask of electron microscopic PLT evaluation of a HD subject and (B) healthy reference subject. Masks are shown in blue. Magnification: 2500x.

|                     | HD subject | HD subjects’ group (mean, range) N=20 | Healthy reference subject | Healthy reference group (mean, range) N=20 |
|---------------------|------------|--------------------------------------|---------------------------|------------------------------------------|
| PLT \((10^9/L)\)    | 206        | 198 (117-229)                        | 254                       | 238 (150-337)                           |
| PDW \((fL)\)        | 11.3       | 9.5 (7.7-11.4)                       | 12.1                      | 11.7 (9.5-14.1)                         |
| MPV \((fL)\)        | 10.0       | 10.5 (9.1-11.8)                      | 10.3                      | 10.1 (8.9-11.6)                         |
| P-LCR \(\%\)       | 23.4       | 29.0 (16.9-40.1)                     | 27.2                      | 25.2 (15.2-37.3)                        |

Light microscopic evaluation:

| % PLTs with > 75% staining density |
|-----------------------------------|
| 8                                 |
| 19 (4-41)                         |
| 72 (44-86)                        |

EM evaluation:

| average PLT surface area ( # pixels) |
|--------------------------------------|
| 242.481                              |
| 576.533                              |

| area of dense bodies per PLT (\%)    |
|--------------------------------------|
| 31                                   |
| 100                                  |
Size and volume parameters MPV, PDW and p-LCR of the HD subject are within the range of a group of chronic HD subjects. From the current study it is not immediately apparent why the decline in PLT volume, as derived from digital image masks of EM evaluation, markedly exceed the small alterations in PLT volume as assessed by haemocytometry. It may result from decreased activity of megakaryopoiesis accompanied with PLT volume reduction as a result of frequent PLT activation. Also, increased urea concentrations may reveal an additional cause for occurrence of PLTs with reduced density of granules in the cytoplasm. In this respect it is interesting to note that the masking procedure in this study was relatively difficult to perform due to the clustering of PLTs in several electron microscopic TIFFs. Possibly, PLTs in an uraemic environment may be extremely sensitive for the fixation procedure applied for EM, resulting in abnormal dehydration and shrinkage. In conclusion, EM evaluation showed that PLTs from an HD subject are considerably smaller and substantial less granular than PLTs from a healthy reference subject. These observations support the results of increased amounts of PLTs with decreased staining density of granule-containing cytoplasm by light microscopic evaluation and the hypothesis of PLT depletion in subjects with chronic intermittent HD.

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Coagulation activation, depletion of platelet granules and endothelial integrity in case of uraemia and haemodialysis treatment

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ABSTRACT

Background: During haemodialysis (HD) treatment, increase of platelet (PLT) activation and induction of procoagulant activity is demonstrated. Although the role of the endothelium and its direct interaction with coagulation and homeostasis is known, it is not elucidated how PLT activation markers and activation of coagulation coincide with markers of endothelial integrity during HD treatment. In the present study uraemia and HD induced changes, with particular emphasis on PLT granules depletion, activation of coagulation and endothelial integrity were investigated.

Methods: To detect depletion of PLT granules, peripheral blood slide smears were screened by light microscopy for qualitative evaluation of PLT granule containing cytoplasm, as indicated by its granules staining density. Activation of coagulation was investigated by establishment of thrombin-antithrombin (TAT) and fibrinogen concentrations. To evaluate endothelial integrity proendothelin (proET-1) plasma concentrations were established.

Results: Results of our study demonstrate that proET-1 plasma concentrations were obviously increased in the subjects’ group with end-stage chronic kidney disease (CKD) and renal failure if compared with a group of apparently healthy subjects. The amount of depleted PLT granules was obviously increased in the subjects’ group with end-stage CKD if compared with the group with renal failure. Mean plasma concentrations of TAT and fibrinogen revealed results within the reference range.

Conclusions: It is demonstrated that uraemia is associated with endothelial damage and aberrations in PLT granules morphology in subjects with HD treatment. We hypothesize that increased proET-1 concentrations reflect ongoing stress on endothelial cells amongst others due to uraemia. Biomarkers like proET-1 and aberrations in PLT granules morphology assist in the early detection of procoagulant activity of the endothelium.
BACKGROUND

Subjects with chronic kidney disease (CKD) are at risk of cardiovascular diseases and suffer from accelerated atherosclerosis [1,2]. Maintaining the functional integrity of the endothelium is important in prevention or delay of vascular diseases [3]. The vascular endothelium plays a pivotal role in the modulation of vascular tone, initiation of coagulation, fibrinolysis activity and release of inflammatory mediators [4]. The endothelium of subjects with CKD is continuously exposed to uraemic toxins. These toxins are classified in three groups: water-soluble compounds with low molecular weight, such as urea, middle molecular weight substances and protein-bound uraemic toxins [5]. Proteinbound uraemic toxins are poorly eliminated by haemodialysis (HD) treatment. Systemic exposure of the endothelium to uraemic toxins may lead to activation of the endothelial cells and to features associated with systemic inflammation like hypertension and atherosclerosis [6,7]. However, the mechanisms by which increased uraemia might influence activation of endothelial cells have not been elucidated. Intact endothelium demonstrates anticoagulant activity [8]. An essential function of endothelium is to provide an anti-thrombotic surface which inhibits activation of the coagulation cascade [8]. Bacterial endotoxins or inflammatory cytokines, such as IL-1, and glycosylated proteins are able to activate endothelial cells [8]. Activated endothelium has procoagulant properties and promotes coagulation. Tissue factor originating from the endothelium plays an important role in the transformation of anticoagulant endothelium to procoagulant endothelium [8,9]. Agonists capable of inducing release of tissue factor include thrombin, endotoxins, cytokines, hypoxia, shear stress and oxidized lipoproteins. Shear stress and metabolic stimuli, in particular complement, granulocytes, platelets and free radicals, induce secretion of endothelin-1 (ET-1) and endothelial cell deterioration [10]. Subjects with end-stage CKD are on regular HD treatment for two or three times a week. Despite appropriate anticoagulation treatment, increase of platelet (PLT) activation and induction of procoagulant activity is demonstrated during HD treatment [11-14]. Thrombin is involved in the activation of PLTs, neutrophils and monocytes, and acts on endothelial cells in order to release vasoactive and inflammatory mediators [9]. Although the role of the endothelium and its direct interaction with coagulation and homeostasis is known, it is not elucidated how PLT activation markers and activation of coagulation interact with markers of endothelial integrity during HD treatment. In the current study we report on uraemia and HD induced changes, with particular emphasis on PLT granules depletion, activation of coagulation and endothelial integrity.

METHODS

Patients
A group of 20 subjects with end-stage CKD (age 28–82 years) from the Haemodialysis unit of the Medical Center Alkmaar participated in the study. Patients were on regular HD treatment for at least 1 year (median 30 months, range 12–80 months). The etiology of chronic renal insufficiency was hypertensive nephrosclerosis (n = 8), diabetic nephropathy (n = 5), adult dominant polycystic kidney disease (n = 3), IgA nephropathy (n =
1), tubulo-interstitial nephritis (n = 1), chronic pyelonephritis (n = 1) and membranous nephropathy (n = 1). Criteria for exclusion were subjects with an age of < 18 years, a life expectancy < 3 months, active inflammation, thrombocytopenia, autoimmune disease or malignancy as well as supplementation of drugs interfering with PLT function or anticoagulation (immunosuppressive drugs, calcium antagonists, serotonin receptor antagonists, coumarin derivatives and salicylates). The study protocol was approved by the local Medical Ethical Committee (METC Noord-Holland, The Netherlands). Written informed consent was obtained from participants.

For the purpose of comparison and clinical interpretation with regard to the pathophysiological effects of uraemia, additional investigations were performed in a group of 20 subjects with renal insufficiency (aged 36–85 years, GFR < 80 ml/min).

A reference group of 20 subjects (laboratory technicians, aged 20–50 years), was selected in order to establish reference range intervals for parameters reflecting activation of coagulation and endothelial integrity.

**Blood sampling**

During this study blood samples from the subjects’ group on regular HD treatment were taken from the fistula (t0) before the administration of LMWH. For establishment of proendothelin-1 (proET-1) levels in plasma, blood samples were collected into K_EDTA tubes (Vacutainer®, Becton Dickinson, Plymouth, UK). Sodium citrate tubes (0.109 Mol, Vacutainer®, Becton Dickinson, Plymouth, UK) were applied for establishment of trombin-antitrombin (TAT) and fibrinogen plasma concentrations. Blood samples for determination of concentrations of proET-1, TAT and fibrinogen were centrifuged at 2-8°C for 20 minutes at 2500 g in order to separate plasma from the cellular fraction. Plasma aliquots were stored at −70°C until analysis.

**Analytical methods**

**Morphology of PLT granules**

Peripheral blood slide smears were prepared in duplicate for evaluation of aberrations in the morphology of PLT granules. Slide smears were stained according to May-Grünwald-Giemsa methodology on a Sysmex SP-100 analyzer (Sysmex Corporation, Kobe, Japan). Slide smears were microscopically screened for qualitative evaluation of morphological PLT granules aberrations with application of a CellaVision™ DM96 analyzer (CellaVision AB, Lund, Sweden). As previously established, a staining density >75% of the PLT granules containing cytoplasm in >50% of PLTs was considered to be the lower limit of the reference range [15]. Depleted PLTs granules were defined as PLTs with a staining density amounting to <25% of the PLT granules containing cytoplasm. The upper limit of the reference interval for depleted PLT granules was determined at <20% of PLTs [15].

**TAT, fibrinogen and proET-1 plasma concentration**

TAT plasma concentrations were assayed with ELISA (Enzygnost® TAT micro and Enzygnost® F1 + 2 monoclonal, Siemens Healthcare Diagnostics Inc., Marburg, Germany). ProET-1 concentrations were established by means of a commercial LIA-kit (B.R.A.H.M.S. CT-proET-1, B.R.A.H.M.S. AG, Hennigsdorf, Germany). Fibrinogen concentrations were established on a ACL-TOP analyzer (Instrumentation Laboratory, Milan, Italy) in accor-
dance with the Clauss method by adding excess of thrombin to diluted plasma in order to convert fibrinogen to fibrin (Instrumentation Laboratory, Milan, Italy).

**Statistical evaluation**
Statistical evaluation of data was performed with application of SPSS software 14.0 for Windows. Statistical significance of deviations between mean values of the group of HD subjects and the group with renal failure and the reference group of laboratory technicians was established by applying the one-way analysis of variance (one-way ANOVA). A p-value < 0.05 was considered to be statistically significant. Correlation coefficients (r) were calculated and expressed as Pearson’s coefficients.

**RESULTS**
Mean results for the groups of subjects with end-stage CKD and renal insufficiency together with the results of the reference group of laboratory technicians are depicted in the Figures 1, 2 and 3. Plasma concentrations of proET-1 are demonstrated in Figure 1. Results for parameters reflecting aberrations in the morphology of PLT granules are demonstrated in Figure 2. Results for parameters concerning activation of coagulation (TAT and Fibrinogen) are demonstrated in Figure 3.

**proET-1**
ProET-1 concentrations in subjects with end-stage CKD (mean ± SD) amounted to 244 ± 47 pMol/L. One-way Anova statistics revealed a positive correlation regarding the reference group and the groups with renal insufficiency and end-stage CKD (p = 0.000). ProET-1 concentrations in the subjects with end-stage CKD (207 ± 23 pMol/L, p = 0.017) were significantly increased in comparison with subjects with renal insufficiency. ProET-1 results for both groups were obviously increased in comparison with the reference group of apparently healthy subjects (37 ± 22 pMol/L) (Figure 1). In 45% of the subjects with end-stage CKD proET-1 concentrations were above 250 pMol/L (Table 1).

**Morphology of PLT granules**
In the group of reference subjects 70 ± 12% of the PLTs are established to reveal appropriate staining density of the PLT granules. In subjects with chronic HD treatment staining density of the granule containing PLT cytoplasm decreased to a minimum score (Figure 2). Only 19 ± 10% of the PLTs yielded > 75% PLT granules staining density (p = 0.000). Subjects with renal failure showed also a marked decrease of the PLTs with appropriate staining density of the PLT granules (43 ± 12%, p = 0.000).
PLTs with a staining density amounting to <25% of the PLT granules containing cytoplasm are classified as depleted. In the reference subjects’ group only 9 ± 6% of the PLTs reveal depleted PLT granules (p = 0.000) (Figure 2). In the subjects’ group with end-stage CKD 46 ± 11% of the PLTs reveal depleted granules staining. In the subjects’ group with renal failure 25 ± 6% of the PLTs reveal depleted granules staining.
tat, fibrinogen
TAT plasma concentrations and fibrinogen concentrations in the group with end-stage CKD and renal failure are demonstrated in Figure 3. One-way Anova statistics revealed a positive correlation regarding the reference group and the groups with renal insufficiency and endstage CKD (p = 0.010). Mean results for TAT and fibrinogen concentrations in the group with end-stage CKD were within the reference range and did not statistically differ from the results of the reference group. However, in the group with end-stage CKD increased TAT or fibrinogen concentrations were established in respectively 15% and 40% of the subjects. Statistically significant increases for fibrinogen and TAT concentrations were established in the subjects with renal failure in comparison with the reference group (p = 0.000), whereas only TAT results differ significantly from the results of the group with end-stage CKD (p = 0.014).
Figure 2. **PLT granules staining density.** Box plots representing the percentage of PLTs with <25% (A) and >75% (B) staining intensity of the granules containing cytoplasm established in subjects with end-stage CKD (n = 20) and renal failure (n = 20). For comparison, results for a group of 20 apparently healthy subjects (REF) are depicted. The box extends form the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to the largest and smallest observed values within 1.5 box lengths. Outlying and extreme values corresponding with values between 1.5 and 3 times the box length or > 3 times the box length, respectively, are designated as (0) and (*). The horizontal dashed lines indicate the upper (A) and lower (B) level of the reference range for apparently healthy subjects. Statistically significant deviations between groups (p < 0.05) are indicated by .
Figure 3. Coagulation biomarkers TAT and fibrinogen. Box plots representing TAT (A) and fibrinogen (B) plasma concentrations established in subjects with end-stage CKD (n = 20) and renal failure (n = 20). For comparison, results for a group of 20 apparently healthy subjects (REF) are depicted. The box extends from the 25th to the 75th percentile. The line inside the box indicates the median value. Whiskers extend to the largest and smallest observed values within 1.5 box lengths. Outlying values corresponding with values between 1.5 and 3 times the box length are designated as (0). The horizontal dashed line indicates the upper level of the reference range for apparently healthy subjects. Statistically significant deviations between groups (p < 0.05) are indicated by – – –.
Correlation between proET-1 and modifications in PLT morphology or markers indicating activation of coagulation

 Establishment of the interdependence of proET-1 results with additional parameters reflecting aberrations in the morphology of PLT granules reveal a negative correlation with PLTs with appropriate PLT granules staining density $r = -0.84$, $p = 0.000$ (Figure 4). For results concerning activation of coagulation a similar correlation is detected (Table 2). A statistically significant positive correlation to increased values for proET-1 with TAT and fibrinogen has been established in the group of reference subjects and the subjects groups with renal failure and end-stage CKD of $r = 0.34$ ($p = 0.016$) and $r = 0.31$ ($p = 0.028$) respectively (Table 2).

Nine patients (45%) of the subjects' group with endstage CKD yielded proET-1 concentrations exceeding 250 pMol/L (Table 1). Results for aberrations in the morphology of PLT granules in the subjects’ group with proET-1 concentrations exceeding 250 pMol/L demonstrated statistically significant deviations if compared with the subjects’ group with proET-1 concentrations below 250 pMol/L, whereas markers for activation of coagulation did not differ (Table 1).

![Figure 4. Correlation between PLT granules staining density and biomarker proET-1. Relationship between results concerning PLT granules staining density and proET-1 concentrations in the groups with end-stage kidney disease (●), renal failure (○) and apparently healthy subjects (■) respectively.](image-url)
TABLE 1. *PLT* granules staining density and coagulation activation parameters in end-stage CKD subjects with proET-1 concentrations of <250 and >250 pMol/L

| End-stage CKD | proET-1 <250 proET-1 >250 | statistical significance |
|---------------|--------------------------|-------------------------|
|               | pMol/L (n = 11) pMol/L (n = 9) | mean ± SD | mean (SD) |
| proET-1 (pMol/L) | 210 ± 18 | 285 ± 40 | p = 0.000 |
| PLTs with <25% granules staining density (%) | 33 ± 13 | 46 ± 8 | p = 0.008 |
| PLTs with >75% granules staining density (%) | 34 ± 15 | 18 ± 10 | p = 0.001 |
| TAT (μg/L) | 3.8 ± 1.0 | 3.8 ± 1.4 | NS |
| Fibrinogen (g/L) | 4.1 ± 1.3 | 3.9 ± 1.2 | NS |

Evaluation of proET-1, PLT granule staining density, TAT and fibrinogen (mean value with standard deviation) established in the group with end-stage CKD. Results of statistically significant evaluation are indicated as p-values. NS = deviation not statistically significant.

TABLE 2. *Pearson* correlation coefficients (r) and statistical significance (p) between biomarker proET-1, PLT granules staining density and biomarkers indicating activation of coagulation

| Pearson correlation (r) | Statistical significance |
|-------------------------|-------------------------|
| proET-1 and PLTs with <25% granules staining density | 0.79 | p = 0.000 |
| proET-1 and PLTs with >75% granules staining density | −0.84 | p = 0.000 |
| proET-1 and TAT | 0.34 | p = 0.016 |
| proET-1 and Fibrinogen | 0.31 | p = 0.028 |
ProET-1 plasma concentrations in subjects with endstage CKD and renal failure were investigated for a possible association between reduced endothelial integrity and aberrations in the morphology of PLT granules or markers indicating activation of coagulation. The in vivo inactive biomarker proET-1 reflects the level of the bioactive peptide ET-1. ProET-1 is the precursor of ET-1, which reveals stability ex vivo [16]. Results of the study demonstrate that ProET-1 plasma concentrations are obviously increased in the subjects’ group with end-stage CKD and renal failure if compared with a group of apparently healthy subjects. Results are in accordance with the findings of other authors, who demonstrated increasing endothelin plasma concentrations with progression of renal failure [17,18]. Altered expression of microcirculation parameters with age is a commonly occurring phenomenon. In the age of 25 till 65 years concentrations of proET-1 increase by 20% [16]. However, on the basis of age related shifts, the increase in proET-1 concentrations in the groups with end-stage CKD and renal failure are obviously higher than expected. ProET-1 plasma concentrations in the subjects’ group with end-stage CKD demonstrate a statistically significant increase if compared with the subjects’ group with renal insufficiency. Endothelial injury is considered to initiate increased secretion of ET-1 and to effectuate vasoconstriction, increased intraglomerular pressure, and decreased glomerular filtration [17]. ET-1 concentrations are demonstrated to correlate with blood pressure, suggesting that ET-1 may contribute to hypertension [18]. Reduced endothelial integrity is considered to be associated with increased incidence of cardiovascular disease [17]. Together with inflammation, hyperhomocysteinaemia and anaemia, cardiovascular disease yields an additional risk factor in subjects with end-stage CKD [19]. Moreover, results of our study demonstrate that the amount of depleted PLT granules is obviously increased in the subjects’ group with end-stage CKD if compared with the group with renal failure. Subjects with endstage CKD are on regular HD-treatment. Pathophysiological mechanisms inducing activation of coagulation are based on Virchow’s triad including modifications in vessel wall, blood flow and composition of blood components [20-22]. During HD treatment, blood constituents interact with the foreign surfaces within the extracorporeal circuit (ECC), including the wall of blood lines, the artificial dialyzer membrane and mechanical forces of the roller pump. Within the ECC endothelium is lacking and activation of PLTs and biomarkers inducing activation of coagulation are released by mechanical triggers [23]. In order to prevent clotting in the ECC during HD, a bolus of low molecular weight heparin or unfractionated heparin is supplied at the start of each HD session. Despite appropriate anticoagulation treatment, the rather unphysiological conditions within the ECC, amplified by pre-dialysis increased uraemia related factors, induce PLT activation. Increase of PLT activation and procoagulant activity is demonstrated in HD patients [24,25]. PLTs are activated within the ECC, as detected by increase in the expression of CD62p and release of β-tromboglobulin (β-TG) within the ECC [23]. PLT activation is associated with exposure of phosphatidylserine on the PLT exterior. Platelet factor-4 and β-TG are released from PLTs as a result of a defect in their granules membrane mainly as a consequence of the bloodmembrane contact during HD and return only slowly postdialytic to control values [26]. In this study mean concentrations of TAT and fibrinogen reveal results within the referen-
ce range. However, in the group with end-stage CKD increased TAT or fibrinogen concentrations are established in respectively 15% and 40% of the subjects, indicating activation of the coagulation pathway or an acute phase response already before the beginning of a new HD session. The inner negatively charged wall of the ECC induces activation of FXII and subsequently the intrinsic coagulation pathway. Increased concentrations of TAT as well as prothrombin fragment 1 + 2 treatment during HD indicate that thrombin is generated [12-14]. Additionally, it has been demonstrated that thrombin is involved in the activation process of PLTs, neutrophils, and monocytes, and acts on endothelium in order to release a variety of vasoactive and inflammatory mediators [9].

With respect to different biomarkers of activation of PLTs and the coagulation pathway dissimilar results could be obtained. Although activation of PLTs and the coagulation pathway is present, concentrations of activation and release products staying within the reference values could also be detected as a result of different release times, presence of neutralizing agents and removal by the dialyzer membrane [11,14].

Results of our study demonstrate that uraemia is associated with endothelial damage and aberrations in the staining density of PLT granules in subjects with CKD. Uraemic toxins, especially protein-bound toxins, are likely pathogenic agents inducing endothelial damage in CKD [27]. In case of renal failure, endothelial damage and cardiovascular complications like hypertension are closely linked [28,29]. Concerning interpretation of our experimental data, we hypothesize that increased proET-1 concentrations reflect ongoing stress on the endothelium amongst others due to uraemia. Biomarkers like proET-1 and aberrations in the staining density of PLT granules contribute in the early detection of procoagulant activity of the endothelium. Therefore, the level of proET-1 concentration and the amount of depleted PLT granules will add an important link to the degree of endothelial integrity and the severity of CKD.

**CONCLUSIONS**

In this study it has been demonstrated that uraemia is associated with endothelial damage and aberrations in appropriate staining density of PLT granules in subjects with HD treatment. In subjects with end-stage CKD deterioration of integrity of the endothelium and depletion of PLT granules are aggravated, because of the frequent PLT activation in the ECC during HD treatment.
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ASPECTS OF PLATELET DISTURBANCES IN HAEMODIALYSIS PATIENTS

MINIREVIEW

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Patients with mild-to-chronic kidney disease (CKD) exhibit a variety of haemostatic disorders, ranging from an increased clotting tendency and reductions in the levels of natural inhibitors of coagulation to defective fibrinolysis. In addition, platelet (PLT) abnormalities are common. In this minireview, we report on aspects of haemodialysis (HD)-induced PLT activation. It is demonstrated that PLTs from HD patients are exhausted due to repeated stimulation of HD treatment and recurrent release of PLT degranulation products. During HD, additional aberrations of the haemostatic process occur. Besides deviations of coagulation and fibrinolysis, PLT activation and a reduction in their granule content have been observed during HD treatment. As HD treatment is carried out three times per week, month after month, chronic HD patients may suffer persistently from coagulation defects and PLT disorders on top of the alterations induced by the uraemic state itself. PLT activation occurs together with thrombin and fibrin generation. However, macro fibrin depositions in clot devices are not demonstrated, microaggregates occur not only in the extracorporeal circuit (ECC) but are also present in the blood circulation. As vascular access thrombosis is a frequent complication in patients with HD treatment, it is believed that hypercoagulability could result from vascular changes combined with PLTs and activation of coagulation factors.
INTRODUCTION

In this review, aspects of platelet (PLT) disturbances and haemodialysis (HD) treatment are considered.

The haemostasis system contributes to a wide range of body defense systems which are essential for normal life. It impedes both the loss of blood and the disturbance of blood flow, but also provides for the repair of injured vasculature and tissue. Attacks by microorganisms are prevented by the formation of a temporary PLT and fibrin plug, which is dissolved at a later stage. In the case of haemostasis, processes are initiated to induce the formation of connective tissue and vessel wall revascularization. The main reactants implicate PLTs, vessel wall, coagulation factors, fibrinolysis factors, inhibitors of coagulation and fibrinolysis, calcium ions and phospholipids.

Patients with chronic kidney disease (CKD) exhibit a variety of haemostatic disorders, ranging from increased clotting tendency and reductions in the levels of natural inhibitors of coagulation to defective fibrinolysis [1–3]. Not only increased PLT activation has been described, but also defective PLT function occurs. Clinically, these disturbances are associated with the paradoxical observation of a thrombotic diathesis as well as an increased bleeding tendency. The pathogenesis of bleeding in uraemia is considered multifactorial. However, the major defects involve primary haemostasis because abnormalities in PLT–PLT and PLT–vessel wall interactions appear to be of crucial importance. Changes in PLT function are partially due to uraemic toxins present in circulating blood. Despite decreased PLT function, abnormalities of blood coagulation and fibrinolysis are less consistent and are more indicative of a hypercoagulable state carrying the risk of cardiovascular and thrombotic complications [4–6].

HD treatment improves PLT abnormalities and reduces, but does not eliminate, the risk of haemorrhage. The interaction between blood and artificial dialyser membrane surfaces and the use of anticoagulants may induce chronic activation of PLTs, leading to PLT exhaustion and aberrations in PLT function. The risk of bleeding may be minimized by using a low dose of high-molecular weight heparin (HMWH), the use of low-molecular weight heparin (LMWH) or regional anticoagulation with citrate to prevent clotting in the extracorporeal circuit (ECC) [7].

Despite their haemorrhagic tendency, uraemic patients have an activated coagulation system that is more prominent in those who are treated by HD. Uraemic subjects on HD are exposed to thrombotic complications related to their vascular access. Percutaneous cannulation, central vein catheters and native vein or prosthetic arteriovenous fistula are all associated with thrombotic occlusion. The risk factors for a hypercoagulable state include enhanced PLT aggregability, increased concentrations of (TAT), prothrombin fragment F1 + 2, FVIIIc and von Willebrand Factor (vWF). It is suggested that coagulation aberrations and frequent PLT activation in the case of HD treatment contribute to increased risk of cardiovascular disease [2, 8]. Cardiovascular events related to thrombosis are a predominant cause of death and account for an important morbidity in patients with CKD.
HAEMOSTASIS

The process of blood coagulation activation can be divided into several stages. Steps mediated by the interaction of PLTs, vessel wall and plasma coagulation proteins are indicated as primary haemostasis (Figure 1), whereas the stage of fibrin formation, mediated by activated coagulation factors, is indicated as secondary haemostasis (Figure 2). The haemostatic system is balanced in order to maintain blood in a fluid state under physiological conditions, and to stop blood loss in the case of vascular injury. At the site of injury, PLTs adhere to the vessel wall within a period of seconds. Within a period of minutes, PLTs aggregate and blood coagulation is initiated. Haemostatic plug formation occurs within a period of minutes due to the synergistic action of multiple plasma factors and cellular elements.

PLT MORPHOLOGY, PLT ACTIVATION AND ACTIVATION OF THE COAGULATION PATHWAY

Laboratory parameters reflecting PLT characteristics, PLT activation and degranulation which are of relevance in detecting aberrations in haemostasis in subjects with HD treatment are demonstrated in Table 1. Evaluation of these markers is performed in order to assess poor dialyser membrane biocompatibility in the case of extracorporeal blood circulation [9–11]. PLT counts feature the balance between the activity of thrombopoiesis and the rate of PLT removal. The results of haemocytometric parameters demonstrate small changes in PLT count, MPV, platelet distribution width (PDW) and p-LCR during HD (Figure 3) [12]. In blood smears, the degree of PLT aggregation and morphological aspects of PLTs, more specific PLT granular density, can be classified by light microscopic evaluation [12]. Whereas granules containing PLT cytoplasm stain light purple or pink, PLT cytoplasm turns faintly grey after discharge of the granule content. In healthy individuals, >70% of PLTs reveals a staining density of >75% [13]. In HD patients, obviously decreased values are found [12].

The degree of PLT activation is detected by longitudinally monitoring CD62p (Figure 3). PLT activation is indicated by an increase in the expression of CD62p on the PLT surface. CD62p (140 kD) is a granule membrane protein, exposed on the PLT surface membrane in the case of activation (Figures 1 and 3). The process of activation results in a partial release of PLT granules content in plasma, such as PLT Factor 4 (PF4) and β-thromboglobulin (β-TG) (Figures 1 and 3). Thus, PF4 and β-TG concentrations are markers concerning PLT degranulation which may be used in order to assess biocompatibility of extracorporeal blood treatment.

Activation of coagulation can be detected by monitoring the activity of coagulation factors in plasma and by means of coagulation markers such as TAT or prothrombin fragment F1 + 2 (Figure 3). During HD both the coagulation system and PLTs are activated (Figure 3). Activation of coagulation is a rather complicated multifactorial process initiated with interaction of PLTs, vWF and the vessel wall. PLTs are additionally activated due to contact with the artificial membrane during treatment with HD and heparin recruitment at the beginning of a dialysis session. PLT activation increases when thrombin generation is
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Initiated by a release of the tissue factor from endothelium, Factor VIIa and Factor XIIa (Figure 2) [13, 14].

The coherence between PLT aggregation, PLT degranulation as detected by morphological evaluation and release of the PLT content as detected by establishment of concentrations of PF4 and β-TG is not yet elucidated [15]. Morphological PLT aberrations and PLT counts were considered simultaneously with HD-induced stimulation of markers for activation of PLTs and plasma coagulation factors. Surprisingly, interdependency indicating interaction could not be established [15]. In fact, neither deviations in PLT counts, CD62p nor any parameter of PLT degranulation correlated at any time interval with plasma concentrations of coagulation markers, such as TAT and prothrombin fragment F1 + 2 [15]. Within the ECC, endothelium is lacking and activation of PLTs and biomarkers concerning activation of coagulation are induced by mechanical triggers. It is conceivable
that the highly unphysiological conditions within the ECC, adherence and release of activated PLTs from the dialyser membrane, explain the lack of a clear association between deviations in PLT activation and the coagulation system.

**ASPECTS OF EXTRACORPOREAL BLOOD CIRCULATION**

Extracorporeal blood circulation is a complicated process because intravascular coagulation is induced [16]. Factors concerning thrombogenicity include reduction of blood flow, modifications in the blood vessel wall, changes in blood composition and biocompatibility of artificial membranes, respectively.

**Dialysis procedure**

The dialysis procedure itself will interfere with haemostasis. Compared with HD, both haemofiltration and haemodiafiltration (HDF) procedures demonstrate increased plasma concentrations of thrombin–antithrombin and D-dimers [17]. Recently, it was shown that PLT activation, as measured by upregulation of CD62p, is increased during HDF when compared with HD. Surprisingly, decreased β-TG levels are possibly due to loss of this substance through the high-flux dialyser membrane and excretion into the dialysate [18].

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**Figure 2.** Three phases of coagulation from an analytical point of view. (i) Primary haemostasis: based on an interaction of platelets, vWF and endothelium. (ii) Thrombin generation: induced by tissue factor (TF) and Factor VIIa. The TF–VIIa complex activates the conversion of Factor X to Factor Xa and leads to the production of a small amount of thrombin. Thrombin is amplified by activation of intrinsic coagulation factors (VIIIa, IXa, Xla) and Factor V. The thrombin burst results in clot formation. (iii) Clot formation: the clot is formed by fibrin and activated PLTs and stabilized by Factor XIIIa. All three components are activated by thrombin, which is the key enzyme for clot formation.
Artificial dialyser membranes

During HD treatment, blood of the subjects is exposed to foreign surfaces within the ECC, including the wall of blood lines and the artificial dialyser membrane, and also highly unphysiological mechanical forces of the roller pump. Furthermore, the effectiveness of the anticoagulation treatment results in an efficient HD process [11]. Acute-phase response parameters and a concomitantly increased degree of hypercoagulability are demonstrated to be exaggerated by definite characteristics of the dialyser membrane, for instance membrane permeability and chemical composition. Cuprophan and polyacrylonitrile membranes induce a higher degree of intradialytic PLT activation when compared with polysulphone and cellulose-triacetate membranes [9, 10, 19, 20].

ASPECTS OF ANTI-CLOTTING AGENTS

PLT activation resulting in thrombus generation in the ECC is a multifactorial process, in which the dialysis membrane and other parts of the ECC, such as needle, pump and shunt, play a role. Probably the most important factor in PLT activation is the used anti-clotting agent like the kind of heparin (HMWH, LMWH) or trisodiumcitrate (TSC) [11, 16, 21]. To prevent clotting, in clinical practice a bolus of LMWH or unfractionated heparin is administered just before the start of HD. Despite adequate anticoagulation treatment, an increase of PLT activation and pro-coagulatory activity is shown in HD patients. During HD, PLTs are activated within the ECC, as release of β-TG within the ECC [18]. Previously, it was reported that PLT counts decline during HD. By taking blood samples at various time intervals from various sites, it is demonstrated that PLTs will aggregate and disaggregate across the entire length of the ECC, but only in small numbers and not

| TABLE 1. Mini-overview of laboratory applications and physiological findings before, during and at the end of a HD treatment |
|---------------------------------------------------------------|
| **PLT characteristics** | **HD treatment** | **Physiological findings** | **Reference** |
| PLT | PS, LMWH | n − ↓, n − ↓, n − ↓ | [12, 15] |
| PDW, MPV | PS, LMWH | n, n − ↓, n − ↓ | [12, 15] |
| % PLTs with <25% staining density | PS, LMWH | ↑, ↑ − ↑↑, ↑ | [15] |
| % PLTs with >75% staining density | PS, LMWH | ↓, ↓ − ↓↓, ↓ | [12, 15] |
| PLT activation and degranulation CD62p% | PS, LMWH | n − ↑, ↑ − ↑↑, n − ↑ | [11, 12, 15, 18, 21] |
| PLT degranulation PF4 (IU/mL) | PS, LMWH | n, ↑↑↑, ↑ − ↑↑ | [11, 12, 15, 18, 21] |
| β-BTG (IU/mL) | PS, LMWH | ↑, ↑↑, ↑ − ↑↑ | [15, 18] |
| Coagulation TAT, F1 + 2, fibrinogen | PS, LMWH | n − ↑, n − ↑, ↑ − ↑↑ | [9, 10, 11, 15] |
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sufficiently to explain a drop in PLT counts [21]. Apparently, activated PLTs stick to the artificial dialyser membrane. As a result of PLT activation during HD treatment micro PLT aggregates and thrombin generation occur. PLT aggregates are detected by microscopic evaluation on stained blood slides. Impaired PLT function is detected by means of a PLT function analyser (PFA-100™). The PFA-100™ system simulates in vitro haemodynamic conditions of PLT adhesion and aggregation in a vascular lesion. Within a group of HD patients, prolonged closure times with a PFA-100™ system are demonstrated as a result of PLT aggregates which do not fit tightly to the membrane (own observation, not published). Functionally, PLTs in the case of uraemia are less responsive to ADP-induced stimulation, as demonstrated with PLT aggregation tests in PRP plasma [22, 23]. Moreover, fibrinogen, which inhibits PLT function by competitive binding to the fibrinogen receptor GP IIb–IIIa on PLTs, is increased in uraemic plasma [24]. PLT aggregates block pores of the dialyser membrane, reducing exchange of harmful substances like urea. In practice, visible clotting in the air trap device is not observed.

It has been recently shown that the anti-clotting agent, which is administered during HD, affects the degree of PLT activation and degranulation [21]. In a comparative study between unfractionated heparin (UH), LMWH and TSC, it is demonstrated that UH and Fragmin® induce substantial increases in PLT activation, whereas PLT characteristics remain unaltered during HD with TSC [11, 21].

Figure 3. Overview of results for parameters reflecting PLT count (10⁹/L), PLT activation, PLT degranulation and coagulation before and at 5, 30, 60 and 150 min after starting HD treatment. Anti-clotting agent: low-molecular weight heparin; membrane characteristics: polysulphone. For comparison, the laboratory data of a reference group of 20 healthy controls (age 20–50 years) (REF) are also demonstrated [12, 15]. HD, haemodialysis; LMWH, lowmolecular weight heparin; PF4, platelet factor 4 (kIU/L); PLT, platelet; TAT, thrombin antithrombin (μg/L)
CONSEQUENCES OF REPETITIVE PLT ACTIVATION

PLT disturbancies are common in CKD patients and aggravate during HD treatment. Immediately after starting HD treatment, PLTs are being activated and release their content. PLT activation is illustrated by up-regulation of surface receptors and release of degranulation products. PLT activation results in increased turnover of PLTs. Usually the PLT count decreases slightly during the first minutes [25]. During HD treatment, statistically significant changes in PLT counts could not be detected. It is hypothesized that exhausted PLTs are continuously being removed from the circulation and new PLTs are released simultaneously [12]. The results of morphological analysis support the hypothesis that PLTs from HD patients are chronically exhausted [12, 15].

Alterations vary according to the stage of CKD, anticoagulant strategy and dialysis procedure. In spite of application of unfractionated heparin and LMWH during dialysis, haemostatic imbalance increases during the course of treatment [11, 16, 21]. Although significant activation of PLTs occurs during HD treatment, short-term and long-term effects of PLT activation on the microcirculation are mainly unknown [25]. It appears that during HD interactions between the anti-clotting agents, the haemostatic system and the endothelium exert a protective effect, at least against activation of the tissue factor coagulation pathway [26].

As PLT disturbances are already detected in CKD patients not yet on dialysis, although to a lesser extent, it is not elucidated whether HD-induced alterations of the haemostatic system will contribute to an increased risk of cardiovascular disease [12].

In clinical practice, alterations in PLT function are associated with a paradoxical observation of both a procoagulatory state, as demonstrated by recurrent vascular access failure, and increased bleeding tendency, as illustrated by a higher risk of gastrointestinal blood loss [27, 28]. Hypercoagulability is associated with cardiac disease, cerebral spill, pulmonary embolism as well as thrombus formation in vascular access for dialysis, particularly in the case of fistula with polytetrafluorethylene.

Vascular complications represent 20–25% of hospitalizations of patients with HD treatment [29]. Stenosis is due to gradual hyperplasia of the intima and muscular layers of the vessels. Stenosis, in turn, results in blood flow reduction, which favours hypercoagulability. The majority of HD patients with recurrent problems of vascular access revealed higher amounts of circulating activated PLTs. Activated PLTs predispose to hypercoagulability yielding frequent occurrences of thrombosis in the vascular access [27]. Although PLTs play an important role in the explanation of HD-related symptoms and diseases, most data provide limited support to the above mentioned clinical effects, because of a lack of a clinically outcome study.
CONCLUSIONS

Three main conclusions can be drawn with respect to HD-induced PLT activation. First, already before dialysis both activation markers on the PLT surface area and the PLT granule content are markedly decreased below the reference range. PLTs from chronic HD patients are chronically exhausted due to repeated stimulation and activation in the course of HD treatment and recurrent release of PLT degranulation products.

Second, during HD treatment, several alterations of the haemostatic process additionally occur. Besides deviations of coagulation parameters during HD, PLTs are activated (CD62p) and a concomitant reduction in the granule content is observed (% PLTs with >75% staining density, PF4, β-TG). Because changes occur three times a week, chronic HD patients reveal persistent coagulation defects and PLT activation in addition to alterations induced by a severe uraemic state itself.

Third, during HD treatment, PLT activation combined with thrombin and fibrin generation occurs. However, fibrinogen or fibrin depositions on clot devices could not be demonstrated. Nevertheless, microaggregates occur not only in the ECC but also in the blood circulation.

As vascular access thrombosis is a frequent complication in patients with HD treatment, it is believed that hypercoagulability could result from vascular changes combined with PLTs and coagulation factors activation. Unfortunately most data provide only limited support for linking repetitive PLT activation during HD treatment with HD-related symptoms and clinical outcomes. Treatment-related changes in the haemostatic balance might contribute to an in-vivo procoagulatory state. Hypercoagulability might be a risk factor for vascular access thrombosis and may contribute to an increased risk of cardiovascular disease. Much needs to be clarified in this interesting field before the contribution of dialysis-induced PLT alterations could be discussed in the light of clinically relevant end points, e.g. myocardial infarction, mortality, gastrointestinal bleeding.
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GENERAL DISCUSSION AND FUTURE PERSPECTIVES
1. INTRODUCTION

In end-stage kidney disease, haemodialysis treatment is applied to remove excess fluid and waste products, such as urea and creatinine, from the blood. During haemodialysis, blood of a patient flows through an extracorporeal circuit, which consists of needles, blood lines, dialyzer, bubble trap and roller pump. Although all of these factors may play a role in the bio(in)compatibility of the system, it has been well documented that the dialyzer - with a surface area of 1-2 m² - is crucial in this respect. In addition, both the administered anticoagulant, usually low molecular weight heparin, and microbial contamination of the dialysate may contribute to the level of bio(in)compatibility.

Despite the efficiency of modern dialyzers and major improvements in the bio-compatibility of the materials used, various side effects occur during haemodialysis, including activation of several types of blood cells (platelets, leucocytes), protein systems (coagulation and complement) and the endothelium.

Haemodialysis affects the haemostatic system in two ways:
1. By contact of blood constituents with the entire inner surface of the extracorporeal circuit.
2. By the anticoagulation procedure to prevent clotting in the extracorporeal circuit.

Effects of the roller pump and the bubble trap as well as the role of the anticoagulation procedure preventing platelet activation have been studied by others in our research group. It was demonstrated that the increase in platelet factor 4 occurs only to a limited extent within the dialyzer, but primarily as a result of detachment from the endothelium due to the administered heparin. From these studies it also appeared that anticoagulation with trisodiumcitrate completely inhibits haemodialysis-induced platelet activation and platelet degranulation.

In this thesis, attention is focussed on activation of platelets and the coagulation system using several types of dialyzer membranes (Table I). A variety of parameters regarding platelet morphology, platelet activation, platelet degranulation and markers for activation of coagulation were analyzed both before and during haemodialysis treatment with low-flux polysulphon (F-8), high-flux polysulphon (F-60), high flux acrylonitrile (AN-69) and low-flux poly-methyl-metacrylaat (PMMA) dialyzer membranes. At the start of all sessions a bolus injection of low molecular weight heparin was applied. An overview of the results reflecting activation of the coagulation system, platelet morphology, platelet activation and degranulation of platelets before and during a haemodialysis session is represented in Table II.

Markedly elevated plasma levels of soluble cell adhesion molecules (sICAM-1, sVCAM-1) and von Willebrand factor have been observed in individuals with chronic kidney disease. Moreover, during haemodialysis treatment an increase in the levels of vascular cell surface molecules was observed. Therefore, it is conceivable that haemodialysis treatment promotes vascular injury, thus contributing to the increased cardiovascular risk in end-stage kidney disease. With regard to endothelial cell activation, the biomarker
| Chapter | Type of anticoagulation | Dialyzer membrane | Blood sampling: before/during HD treatment | Platelet haemocytometry | Platelet morphology | Platelet activation or degranulation | Activation of coagulation | Endothelial integrity | Reference groups |
|---------|-------------------------|--------------------|------------------------------------------|------------------------|---------------------|-------------------------------------|----------------------------|---------------------|------------------|
| 2       | Bolus LMWH, at start HD treatment | AN-69 high flux | Before / t= 1, 5, 30, 150 minutes | -                      | -                   | -                                   | FXII, TAT, F1+2, TpP, Fibrinogen | -                   | -                |
| 3       | Bolus LMWH, at start HD treatment | PMMA / F-60 high flux | Before / t= 5, 30, 150 minutes | -                      | -                   | -                                   | FXII, TAT, F1+2, TpP, Fibrinogen | healthy / uraemia   | -                |
| 4       | Bolus LMWH, at start HD treatment | F8 low flux | Before / t=1, 5, 30, 150 minutes | PLT count, PDW, MPV, p-LCR, IPF | Light-microscopy | -                                   | -                          | -                   | healthy          |
| 5       | Bolus LMWH, at start HD treatment | F8 low flux | Before / t=1, 5, 30, 60, 150 minutes (afferent line) + t= 5, 30 minutes (afferent line) | PLT count, PDW, MPV, p-LCR, IPF | -                   | Platelet CD62p expression, plasma PF4, β-TG | -                          | -                   | -                |
| 6       | Bolus LMWH, at start HD treatment | F8 low flux | Before / t= 5, 30, 150 minutes | PLT count, IPF | Light-microscopy | Platelet CD62p expression, serotonin content; plasma PF4, β-TG | TAT, F1+2 | -                   | healthy          |
| 7       | Bolus LMWH, at start HD treatment | F8 low flux | Before / t= 5, 30, 150 minutes | PLT count, PDW, MPV, p-LCR | Light-microscopy | electron microscopy | -                          | -                   | -                |
| 8       | Bolus LMWH, at start HD treatment | F8 low flux | Before | - | Light-microscopy | -                                   | TAT, Fibrinogen | ProET-1 | healthy / uraemia |

PLT, platelet; p-LCR, platelet large cell ratio; PF4, platelet factor 4; TAT, thrombin-antithrombin complexes; PDW, platelet distribution width; IPF, immature platelet fraction; β-TG, β-thromboglobulin; F1+2 = prothrombin fragment 1+2; TpP, thrombus precursor protein; MPV, mean platelet volume; proET-1, proendothelin-1; HD, haemodialysis; LMWH, low molecular weight heparin.
proendothelin-1 was investigated together with activation of the coagulation system and platelet granule depletion both in patients not yet on haemodialysis and in patients with haemodialysis treatment (Table II).

2. HAEMODIALYSIS-INDUCED CHANGES REGARDING THE COAGULATION SYSTEM

The effects of several types of dialysis membranes on activation of the coagulation system were evaluated before and during a session of haemodialysis treatment (Chapters 2, 3, 6).

**High flux AN-69 dialyzer membrane**
Already before the start of haemodialysis treatment increased concentrations of fibrinogen and prothrombin fragment F1+2 were established. During a haemodialysis session a FXII decrease amounting to 25% of the initial FXII level occurred within the first minute. Subsequently, only a slight increase towards the initial FXII level was detected. At the end of haemodialysis treatment moderately increased concentrations of thrombin-antithrombin complexes and prothrombin fragment F1+2 were established, whereas changes in fibrinogen concentrations were not observed.

**Low flux PMMA dialyzer membrane**
Concentrations of prothrombin fragment F1+2 were already increased before the haemodialysis treatment. During haemodialysis treatment FXII levels decreased. Slightly increased concentrations of prothrombin fragment F1+2 and fibrinogen were established, whereas increased generation of thrombin-antithrombin complexes was not detected.

**High-flux F-60 dialyzer membrane**
Obvious decreases of FXII exceeding the lower level of the reference range were observed within 5 minutes after starting haemodialysis treatment. Subsequently, only a slight increase amounting to 20% of the initial FXII level was detected. Changes in plasma concentrations of prothrombin fragment F1+2 and fibrinogen were not observed. In 40% of the patients slightly increased concentrations of thrombin-antithrombin complexes were established during a haemodialysis session.

**Low-flux F-8 dialyzer membrane**
Concentrations of prothrombin fragment F1+2 were already increased before the start of a haemodialysis session. During haemodialysis steadily ongoing increases of prothrombin fragment F1+2 and thrombin-antithrombin complexes were established.

From our data the following conclusions can be drawn. Patients with end-stage kidney disease already have signs of activation of the coagulation system prior to a haemodialysis session. During haemodialysis with various types of dialysis membranes even further stimulation of the coagulation system occurs, which, however, appears highly dependent on the individuals under study. As the above mentioned investigations were performed over a time span exceeding a pe-
TABLE II. Overview of findings for laboratory markers of platelet (PLT) haemocytometry, morphology, activation or degranulation as well as activation of coagulation before, during and at the end of haemodialysis session presented in Chapters 2 to 7.

| Blood sampling | Before HD (T=0) | During HD (T=1, 5, 30, 60 minutes) | End HD (T=150 minutes) | Uraemia reference group |
|----------------|----------------|-----------------------------------|------------------------|-------------------------|
|                | AN-69 high flux | PMMA high flux                    | F8 low flux            | AN-69 high flux         | PMMA high flux         | F8 low flux            | AN-69 high flux         | PMMA high flux         | F8 low flux |
| PLT haemocytometry |                |                                   |                        |                         |                         |                        |                         |                         |             |
| PLT count      |               | N -                              | -                      | N -                     | -                      | N -                   | -                      | N -                     | -            |
| PDW            |               | N                                |                        | N                       |                        | N                     |                        | N                       |              |
| MPV            |               | N                                |                        | N                       |                        | N                     |                        | N                       |              |
| P-LCR          |               | N -                              | -                      | N                       |                        | N -                   | -                      | N -                     | -            |
| IPF            |               |                                   |                        |                         |                         |                       |                        |                         |              |
| PLT morphology |                |                                   |                        |                         |                         |                       |                        |                         |              |
| % PLT's with a staining density of granule-containing cytoplasm of: |               | F8 -                              | -                      | F8 -                   | -                      | F8 -                  | -                      | F8 -                     | -            |
| - >75%         |               | -                                |                        | -                      |                        | -                     |                        | -                      |              |
| - <25%         |               | -                                |                        | -                      |                        | -                     |                        | -                      |              |
| PLT activation or degranulation |                |                                   |                        |                         |                         |                       |                        |                         |              |
| CD62p          |               | N -                              | -                      | N -                    |                        | N -                  | -                      | N -                     | -            |
| PF4            |               | N                                |                        | N -                    | -                      | N -                  | -                      | N -                     | -            |
| β-TG           |               | N -                              | -                      | N -                    |                        | N -                  | -                      | N -                     | -            |
| Serotonin in PRP |             |                                   |                        |                         |                         |                       |                        |                         |              |
| Activation of coagulation |            |                                   |                        |                         |                         |                       |                        |                         |              |
| FXII           | N              | N -                              | N -                   | N -                    | -                      | N -                  | -                      | N -                     | -            |
| Fl+2           | ↑              | N                                | N -                   | -                      | (T1)                  | N -                   | -                      | N -                     | -            |
| TAT            | N              | N                                | N -                   | -                      | (T30)                 | N -                   | N -                    | N -                     | -            |
| Fibrinogen     | N -           | N -                              | N -                   | N -                   | (T30)                | N -                   | N -                    | N -                     | -            |
| TpP            | N -           | N                                | N -                   | -                      | (T30)                | N -                   | N -                    | N -                     | -            |

PLT, platelet; p-LCR, platelet large cell ratio; PF4, platelet factor 4; TAT, thrombin-antithrombin complexes; PDW, platelet distribution width; IPF, immature platelet fraction; β-TG, β-thromboglobulin; F1+2, prothrombin fragment 1+2; MPV, mean platelet volume; PRP, platelet-rich plasma; HD, haemodialysis
period of 10 years and comparative studies are lacking, it is hardly possible to pass judgment on the differences between the various types of dialysis membranes used. If anything, it appears that high-flux polysulphon (F-60) and poly-methyl-metacrylaat (PMMA) dialysis membranes are less bio-incompatible than low-flux polysulphon (F-8) and high flux AN-69, a copolymer of acrylonitrile and sodium methallylsulphonate, dialysis membranes.

3. HAEMODIALYSIS-INDUCED CHANGES REGARDING PLATELETS

Studies concerning platelet number and activation status were performed with low-flux polysulphon F-8 dialyzer membranes (Chapters 4-8). Altered characteristics of platelets, indicated by deviations in RNA content, volume and morphology, were established before and during haemodialysis sessions (Chapters 4, 5, 6, 7) to investigate whether the number of circulating platelets in the low normal range in the patients on haemodialysis treatment is due to platelet disappearance during the haemodialysis session, whether this disappearance is a balance between an increase in the number of new platelets versus a higher overall removal, and whether the disappearance is associated with platelet activation during the haemodialysis session. In Chapter 8 effects of uraemia and haemodialysis-induced changes, with particular emphasis on platelet granule depletion were investigated.

3.1 Platelet haemocytometry and platelet morphology

The data on platelet count, morphology and granular staining in the various studies are presented in Table III.

| Table III. Overview of changes in platelet numbers and characteristics during a haemodialysis session. |
|---------------------------------------------------------------|
|                                                                  |
| Before HD (mean value) | End HD | % increase / decrease | PLT number increase / decrease |
|------------------------|--------|------------------------|-------------------------------|
| PLT (150 - 400 x10⁹/L) | 198    | 182                    | -8                            | -16                            |
| IPF (4 - 17 x10⁹/L)    | 6.7    | 6.1                    | -9                            | -0.6                           |
| MPV (9 - 11.5 fl)      | 10.5   | 9.5                    | -10                           |                                |
| PDW (10.5 - 14 fl)     | 12.3   | 11.0                   | -11                           |                                |
| p-LCR (<36 %)          | 29     | 27.2                   | -6                            |                                |
| % PLTS with 75% staining density (> 50%)                        | 19     | 29                     | +50                           | +15                            |
| % PLTS with 25% staining density (< 20%)                        | 36     | 29                     | -20                           | -18                            |

PLT, platelet; IPF, immature platelet fraction; MPV, mean platelet volume; PDW, platelet distribution width, p-LCR, platelet large cell ratio; HD, haemodialysis; Reference ranges are presented between the parentheses.
On average, patients on haemodialysis treatment already had a low normal platelet count. The platelet count decreased slightly during a haemodialysis session. The number of immature platelets in the circulation was also low normal prior to haemodialysis and further decreased during the haemodialysis session. This implies a reduced megakaryopoiesis, increased removal of young platelets or a combination thereof in the period between the haemodialysis sessions.

Compared to apparently healthy subjects, minor deviations in mean platelet volume, platelet distribution width and platelet large cell ratio were established during but not prior to the haemodialysis sessions. Electron microscopy studies revealed that the platelet surface area in a haemodialysis patient was considerably smaller if compared with an apparently healthy subject. It is not elucidated why the reduced mean platelet volume, as derived from digital image analyses of the electron microscopy findings, markedly exceeded the small alterations in platelet volume as assessed by haemocytometry. Possibly, platelets in an uraemic environment may be extremely sensitive to the fixation procedure applied for electron microscopy imaging, resulting in abnormal dehydration and shrinkage.

Platelets with a granule staining density of less than 25% have been arbitrarily defined as depleted. The reference range amounts to a maximum number of 20% of the platelets in the circulation.

In patients with chronic kidney disease (without haemodialysis treatment) depleted granule staining density was present in 25% of the platelets, in patients with end stage kidney disease 36% of the platelets.

Also, the granularity of platelets determined as the area of dense bodies per platelet was substantially less (31%) in comparison with platelets of a healthy subject. The observations obtained by electron microscopy support the findings of increased numbers of platelets with decreased staining density of granule-containing cytoplasm by light microscopic evaluation and the hypothesis of granular depletion of platelets in patients with end stage kidney disease.

### 3.2 Platelet activation

Before the start of a haemodialysis session, results for platelet surface expression of p-selectin (CD62p), concentrations of serotonin in platelets and concentrations of platelet factor 4 in plasma were within the reference range. β-Thromboglobulin concentrations were already markedly increased. This finding is interpreted as a result from ongoing platelet activation. In chronic kidney disease the normal half life of β-thromboglobulin of approximately 100 minutes is considerably increased which may cause the increased β-thromboglobulin concentration. Therefore, the increased β-thromboglobulin concentrations prior to haemodialysis should not be interpreted as ongoing platelet activation between the haemodialysis sessions. Platelet factor 4 concentrations in the plasma prior to the haemodialysis session were normal, but this protein binds to the endothelium and is therefore not a suitable marker for ongoing platelet activation. Overall, based upon the absent platelet surface expression of p-selectin we conclude that persistent activation of platelets between the haemodialysis sessions is not substantial.

To investigate the effect of the dialysis membranes on platelet activation, platelet characteristics, p-selectin, β-thromboglobulin and platelet factor 4 were estimated in samples from
the afferent and efferent lines of the dialyzer (Chapter 5). Platelet numbers decreased immediately after the first passage of blood through the dialyzer, most probably due to adherence to the foreign material of the dialyzer membrane. Platelet activation, as detected by increased p-selectin, also occurred at the first passage. The p-selectin expression and plasma concentrations of β-thromboglobulin further increased in the extracorporeal circuit within 30 minutes. Platelet factor 4 concentrations demonstrated an immediate steep increase after the start of the haemodialysis sessions. This is ascribed to its detachment from the endothelium by the low molecular weight heparin bolus administration.

Before the start of a haemodialysis session, concentrations of serotonin in platelet-rich plasma, i.e. plasma plus platelets, were situated in the lower quartile of the reference range. Plasma concentrations of serotonin before the start of a haemodialysis session were increased, possibly due to its reduced clearance. The platelet serotonin content was decreased. During a haemodialysis session platelet serotonin concentrations steadily decreased further to 35% of the initial level, indicating ongoing platelet secretion of their granular content into the plasma. Plasma serotonin concentrations decreased to 45% of the initial level. Considering the low molecular weight (176 D), serotonin is most likely efficiently removed from the plasma during a haemodialysis session.

3.3 Conclusions

The data on the platelet morphology, immaturity, granular content and activation parameters may indicate the following. Prior to haemodialysis the patients had, on average, a low normal number of platelets in their circulation. The number of immature platelets was also low normal, which reflect an impaired megakaryopoiesis, an increased removal or a combination thereof. The platelet serotonin content was reduced prior to a haemodialysis session, but the serotonin content decreased dramatically during the session, indicating granular release. The period between the haemodialysis sessions may be too short to refill the platelets with serotonin. We do not consider the low serotonin content of the platelets to indicate ongoing platelet activation between the haemodialysis sessions, because the platelets do not express p-selectin on their surface prior to the haemodialysis session.

During haemodialysis, the number of platelets decreased already at the first passage of the blood through the dialyzer (from 198 to 173 x10⁹/L) and subsequently increased somewhat to 182 at t=150 min. The initial decrease is likely to be caused by the adherence of the platelets to the dialyzer. The subsequent increase in platelet count may be due to platelets detaching from the dialyzer, but also due to a release from platelet stores in the body. The platelet granular density staining indicates removal of less density stained platelets and enrichment of density stained platelets after the haemodialysis session. These data indicate that at least some of the platelet increase during is haemodialysis is due to platelet release from some storage compartments, e.g. the bone marrow. The reduction in the number of immature platelets after the haemodialysis would counterdict this conclusion, but the decrease may be due to increased removal of such platelets in the dialyzer. This is supported by our finding of the reduction in MPV after the haemodialysis session, as immature platelets are larger than older platelets. Thus, the slight reduction in platelet count after a haemodialysis session is a result of platelet removal, especially within the dialyzer, and simultaneously some release of new platelets from platelet storage sites.
4. HAEMODIALYSIS-INDUCED CHANGES REGARDING ENDOTHELIAL INTEGRITY

An essential function of the endothelium is to provide a surface which prohibits activation of the coagulation cascade in the resting state. Another function is to promote coagulation activation by the activated endothelial cells. Tissue factor expression on the surface of the endothelial cells plays an important role in the transformation of their anticoagulant into the procoagulant state. The endothelial cells of patients with chronic kidney disease are continuously exposed to uraemic toxins, which may cause their activation and manifestation of features associated with systemic inflammation. However, the mechanisms by which uraemia might activate endothelial cells have not yet been elucidated.

During haemodialysis various blood cell types and protein systems are stimulated, depending on the type of dialyzer and the degree of dialysate contamination. As a result, pro-inflammatory cytokines, such as IL-6 and TNF-α are released. Degranulation of granulocytes and platelets leads to the discharge of various granule products, such as myeloperoxidase and platelet factor 4, respectively. Stimulated white blood cells and activated platelets leave the dialyzer, enter the systemic circulation of the patients, and may activate and/or damage endothelial cells. As a consequence, haemodialysis treatment induces oxidative stress, prothrombotic changes, and signs of inflammation.

Proendothelin-1 concentrations in patients with chronic kidney disease and end stage kidney disease were investigated to establish whether patients on haemodialysis have a reduced endothelial integrity (Chapter 8). The concentration of the in vivo inactive biomarker proendothelin-1 reflects the level of the bioactive peptide endothelin-1. Proendothelin-1 is the precursor of endothelin-1, which in contrast to endothelin-1 reveals stability ex vivo. Plasma concentrations of proendothelin-1 in chronic kidney disease and end stage kidney disease were some five-fold increased compared to a reference group from apparently healthy individuals. In subjects 65 years of age the concentrations of proendothelin-1 are some 20% increased as compared to individuals 25 years of age, and this increase is gradual over the years. Even taking the age-related increase into account, the increase in proendothelin-1 plasma concentrations in the subjects with chronic kidney disease and end stage kidney disease was obviously higher, indicating reduced endothelial integrity.

Regarding the markedly increased concentrations of proendothelin-1 and reduction of endothelial integrity, a study of Grooteman et al demonstrated that endothelial cell adhesion molecules (sICAM-1 and sVCAM-1) and von Willebrand factor are also markedly increased in patients with end-stage kidney disease and do not change after 4 weeks on haemodialysis treatment. It was demonstrated that each haemodialysis session results in a marked increase in von Willebrand factor within 24 hours, whereas the concentration of endothelial cell adhesion molecules (sICAM-1) did not change. In addition, concentrations of cell adhesion molecules and von Willebrand factor varied notably between individual patients. It was concluded that endothelial dysfunction appears to be far more dependent on patient-related factors, such as co-morbidity, chronic kidney dysfunction and prescribed medication, than on the haemodialysis treatment itself.
5. CONCLUSION

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. Clinically relevant are the prolonged bleeding from the dialysis fistula and the prothrombotic state. To summarize the findings of the studies we performed, new insights have been obtained with respect to haemodialysis-induced activation of platelets and coagulation:

- Already before the start of a haemodialysis session the patients have, on average, a low normal platelet count. This may reflect impaired megakaryopoiesis, but also a lack of ability of the body to replenish the removed platelets during the frequent haemodialysis sessions.
- Because platelet surface expression of p-selectin prior to the haemodialysis is absent, we hypothesize that ongoing platelet activation between the haemodialysis sessions does not occur.
- The staining density of the platelet granule content prior to haemodialysis reveals a marked decrease below the reference range. Platelets in patients with end stage kidney disease thus demonstrate chronic depletion of their granular content, not due to ongoing activation between the haemodialysis sessions. Most likely the reduced granular content is due to their repeated activation during the haemodialysis treatment.
- The overall slight decrease in the platelet count after a haemodialysis session is most likely due to platelet adherence to the dialyzer membrane, which is not fully compensated by an influx of platelets from storage pools.
- Prior to haemodialysis, the patients already reveal an activated coagulation system, as indicated by slightly increased plasma concentrations of thrombin-antithrombin complexes and prothrombin fragment F1+2.
- During a haemodialysis session, extensive activation of the coagulation system is observed in spite of the anticoagulant regime and the relative bio-compatibility of current dialyzer membranes. This seems to be particularly initiated via the factor XII dependent pathway.
- Some evidence of an impaired integrity of the endothelium was obtained as indicated by the increased plasma concentrations of pro-endothelin 1.
- Activation of platelets during haemodialysis sessions and activation of the coagulation system during and between haemodialysis sessions evidently yields a continuous prothrombotic state of the patients.

6. FUTURE PERSPECTIVES

Introduction

As described in the preceding paragraphs, both the uremic state and the haemodialysis procedure itself are associated with a variety of platelet abnormalities and disorders of the coagulation system. Clinically these alterations may contribute to the seemingly paradoxical findings of both a pro-thrombotic state and a bleeding tendency in patients with chronic kidney disease. Moreover, as the haemostatic system has been implicated in the development of atherosclerosis in non-renal patients, it is conceivable that the patho-
physiological derangements of haemostasis in uraemic patients contribute to the extremely high burden of cardiovascular disease in this patient group. Therefore, alleviation of these abnormalities may have a favorable influence on both short and long-term complications in patients with chronic kidney disease not yet on haemodialysis and patients with haemodialysis treatment.

**Short term complications**

As mentioned before, a bolus of un-fractionated heparin or low-molecular weight heparin is administered to the patients shortly before the start of a haemodialysis session in order to prevent clotting in the extracorporeal circuit. In everyday clinical practice, the starting dose of the anticoagulant is mainly based on the weight of the individuals, and modified during consecutive sessions in case of visible clotting in the bubble trap or an increasing pressure in the afferent line. However, whereas a higher dose (or a second bolus halfway the session) of the anticoagulant may temper clotting and lower the intra-dialyzer pressure, the chance on fistula bleeding at the end of the haemodialysis session may increase. Therefore, anti-factor Xa monitoring in high risk patients may diminish or even prevent this unwanted side-effect of chronic haemodialysis treatment. Other strategies include the more liberal use of trisodium citrate, which prevents platelet factor 4 release from the endothelium, a combination of citrate containing dialysate with low dose low molecular weight heparin, administration of nafostat mesylate (NFM), and pre-dilution haemodiafiltration (HDF). As these alternative approaches may alleviate or circumvent the above mentioned problems, further research in this field is mandatory.

**Long term complications**

**Fistula stenosis**

A relatively frequent complication of haemodialysis treatment is occlusion of the arteriovenous access, either a graft when synthetic materials are used or a fistula when an artery is directly connected with a vein. Whereas a graft is most frequently used in the United States of America, in the Netherlands in 90% of the patients a fistula is created as arteriovenous access. Thrombosis that occurs within one month after fistula construction is most often due to technical errors or premature use. The major predisposing factor for fistula thrombosis is a stenosis in the venous outflow tract, which is the result of progressive, fibromuscular intimal hyperplasia and perivenous fibrosis. Clinically, fistula thrombosis is characterized by a diminished blood flow, recirculation and hence a decreased efficiency of dialysis. Whether the compromised haemostasis, as described in this thesis, contributes to its development is not yet elucidated. Although treatment with aspirin and dipyridamole has been shown to reduce the time to first thrombosis in PTFE grafts, neither treatment with coumarins nor prescription of anti-platelet agents, showed a reduction in the occurrence rate of fistula failure. In this respect it is interesting to note that vascular remodeling and neointimal formation was reduced after targeted delivery of nonspecific factor Xa inhibitors, such as heparin and low molecular weight heparin, coupled to an antifibrin antibody. Whether non-heparin containing modulators of haemostasis can prevent or delay the development of fistula stenosis is currently unknown and demands further research.
Cardiovascular disease
The risk of mortality is extremely high in haemodialysis patients, cardiovascular causes accounting for 40%-50%. Apart from classical risk factors, such as increased cholesterol and hypertension, several non-classical risk factors have been identified which aggravate or contribute to the vascular abnormalities in chronic kidney disease. Whereas in the previous decade most attention was paid to an abnormal homocystein metabolism, oxidative stress and chronic micro-inflammation, currently, disorders of mineral metabolism are the main research topics in this area.

However, both experimental and clinical data indicate that platelets and the coagulation system are involved in classical atherogenesis and atherothrombosis. In numerous clinical trials, administration of antiplatelet or anticoagulant therapy has been associated with attenuation or even regression of plaque growth. A systemic micro-inflammatory environment, as described in patients with chronic kidney disease, has been shown to induce a phenotypic switch to a pro-atherogenic endothelium. As discussed in chapter 8, in patients with chronic kidney disease not only elevated levels of pro-endotheline were found, but also high levels of soluble adhesion molecules (sICAM-1, sVCAM-1), suggesting endothelial activation. The resulting enhanced expression of cell-adhesion molecules induces adherence of platelets and the secretion of various atherogenic mediators, including cytokines. Binding between adherent platelets and leucocytes support activation and transmigration of monocytes, which is considered critical for plaque formation.

In patients with chronic kidney disease various uraemia specific pro-atherogenic conditions have been identified, such as hyperhomocysteinemia, oxidative stress, micro-inflammation and disorders of mineral metabolism. Whether uraemic derangements in platelets characteristics and disorders of coagulation contribute to the vascular abnormalities, which are commonly observed in patients with chronic kidney disease, is conceivable but incompletely understood. On the other hand, depletion and exhaustion of platelets may protect against platelet-induced damage to vascular wall. Future research in this field may help to unravel the complex interactions between disorders of haemostasis and other inducers of uraemic vasculopathy. Whether interventions in the haemostatic system can alleviate the high burden of cardiovascular disease in this population is also a subject for future research.
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Activatie van bloedplaatjes en het stollingssysteem gedurende hemodialyse.
1. INLEIDING

Wereldwijd krijgt 7% van de bevolking boven de leeftijd van 30 jaar en 25-35% van de mensen ouder dan 65 jaar te maken met een chronische nierziekte. Bovendien is de morbiditeit en mortaliteit door cardiovasculaire oorzaken in patiënten met een chronische nierziekte tot 30 keer hoger dan in de algemene bevolking. Een chronische nieraandoening wordt o.a. gekenmerkt door een sterk verminderde nierfunctie en hoge bloeddruk. Patiënten met een chronische nierziekte hebben een verhoogd risico op het ontwikkelen van eindstadium nierfalen, waarin de nierfunctie is teruggebracht tot slechts 10-15%. Eindstadium nierfalen is een ernstige aandoening waarbij nierfunctievervangende therapie, zoals hemodialyse, noodzakelijk is.

Hemodialyse wordt toegepast om overtollig vocht en afvalstoffen zoals uremische toxines en kreatinine uit het bloed te verwijderen. Tijdens hemodialyse stroomt bloed van een patiënt door een zogenaamd extracorporeel circuit, dat uit naalden, slangen, kunstnier, luchtvangers en een rollerpomp bestaat. Hoewel al deze componenten een rol kunnen spelen in de bio(in)compatibiliteit van het dialysesysteem is de kunstnier ofwel het dialysemembraan, met een oppervlakte van 1-2 m², daarvan de belangrijkste factor. Daarnaast kunnen het toegeediende anticoagulans, meestal laagmoleculair gewicht heparine, en microbiële verontreiniging van het dialysaat bijdragen tot de bio(in)compatibiliteit. Bio(in)compatibiliteit is het geheel van ongewenste reacties dat ontstaat door contact van het extracorporele circuit met het bloed van de patiënt. Hoewel hemodialyse technisch steeds verder wordt verbeterd en meer biocompatible kunstniermaterialen worden gebruikt, blijft de functie van de kunstnier inferieur aan die van de normale nier. Interacties tussen de kunstnier en het bloed van de patiënt leiden tot ongewenste bijwerkingen waaronder het activeren van verschillende soorten bloedcellen (bloedplaatjes, leukocyten), eiwitsystemen (stolling en complement) en het endotheel.

Hemodialyse kan het hemostase systeem op twee manieren beïnvloeden:
1. Door contact van bloed met het extracorporale circuit.
2. Door de antistollingsprocedure om stolling in het extracorporele circuit te voorkomen.

Activatie van bloedplaatjes gedurende een hemodialyse behandeling en de rol van rollerpomp, luchtvangers en antistollingsprocedure hierbij is bestudeerd door anderen in onze onderzoeksgroep. Aangetoond werd dat de toename van plaatjesfactor 4 (PF4) voornamelijk een gevolg is van ‘losweken’ vanaf het endotheel als reactie op het toegeediende antistollingsmiddel heparine. Tevens bleek uit deze onderzoeken dat activatie en degranulatie van bloedplaatjes gedurende hemodialyse vrijwel niet optreedt bij gebruik van trinatriumcitraat als antistollingsmiddel.

In dit proefschrift is de aandacht gericht op de activatie van bloedplaatjes en het stollingssysteem bij gebruik van verschillende soorten kunstniermembranen (Tabel I). Diverse parameters met betrekking tot de morfologie, activatie en degranulatie van bloedplaatjes en activatie van het stollingssysteem werden bestudeerd, zowel vóór als tijdens een he-
TABEL I. Samenvatting van de studies in Hoofdstuk 2 tot 8.

| Hoofdstuk | Type antistolling | Dialysemateriaal | Bloedafname: voor/tijdens HD behandeling | Trombocytenteller: hemocyto metrie | Trombocytenteller: morfologie | Trombocytenteller: activatie of degranulatie | Activatie van het stollingsysteem | Endotheliale integriteit | Referentie groepen |
|-----------|-------------------|------------------|------------------------------------------|----------------------------------|-------------------------------|--------------------------------------------|-------------------------------|----------------|-------------------|
| 2         | Bolus LMWH, voor begin HD sessie | AN-69 high flux | Voor / t= 1, 5, 30, 150 minuten | -                             | -                             | -                           | FXII, TAT, F1+2, TpP, Fibrinogeen | -                           | -                |
| 3         | Bolus LMWH, voor begin HD sessie | PMMA / F-60 high flux | Voor / t= 5, 30, 150 minuten | -                             | -                             | -                           | FXII, TAT, F1+2, TpP, Fibrinogeen | -                           | gezonden / uremie |
| 4         | Bolus LMWH, voor begin HD sessie | F8 low flux | Voor / t=1, 5, 30, 150 minuten | PLT aantal, PDW, MPV, p-LCR, IPF | Lichtmicroscopie | -                           | -                             | -                           | gezonden |
| 5         | Bolus LMWH, voor begin HD sessie | F8 low flux | Voor / t=1, 5, 30, 150 minuten (afferente lijn) + t=5, 30 minuten (afferente lijn) | PLT aantal, PDW, MPV, p-LCR, IPF | -                             | CD62p expressie op trombocyten, plasma PF4, β-TG | -                             | -                           | gezonden |
| 6         | Bolus LMWH, voor begin HD sessie | F8 low flux | Voor / t= 5, 30, 150 minuten | PLT aantal, IPF | Lichtmicroscopie | CD62p expressie op trombocyten serotonine hoeveelheid; plasma PF4, β-TG | TAT, F1+2 | -                           | gezonden |
| 7         | Bolus LMWH, voor begin HD sessie | F8 low flux | Voor / t= 5, 30, 150 minuten | PLT aantal, PDW, MPV, p-LCR | Lichtmicroscopie, elektronen microscopie | -                           | -                             | -                           | gezonden |
| 8         | Bolus LMWH, voor begin HD sessie | F8 low flux | Voor | -                             | Lichtmicroscopie | -                           | TAT, Fibrinogeen | ProET-1 | gezonden / uremie |

PLT, platelet / bloedplaatje; IPF, immature platelet fraction / fractie onrijpe bloedplaatjes; MPV, mean platelet volume / gemiddelde bloedplaatjes volume; PDW, platelet distribution width / bloedplaatjes verdelingscurve; p-LCR, platelet large cell ratio / verhouding grote bloedplaatjes; PF4, plaatsesfactor 4; TAT, thrombine-antithrombine complex; β-TG, β-thromboglobuline; F1+2 = prothrombine fragment 1+2; TpP, thrombus precursor protein; proET-1, proendotheline-1; HD, hemodialyse; LMWH, low molecular weight heparin / laagmoleculair gewicht heparine.
modialyse sessie met low-flux polysulphon (F-8), high-flux polysulphon (F-60), high-flux acrylonitril (AN-69) en low-flux poly-methyl-metacrylaat (PMMA) kunstnierenmembra- 

en. Aan het begin van elke hemodialyse sessie werd een bolus injectie van laagmoleculair 

gewicht heparine gegeven. Een overzicht van resultaten van de activatie van het stollings- 

systeem, morfologie van bloedplaatjes en activatie en degranulatie van bloedplaatjes voor 

eindtijd hemodialyse, is weergegeven in Tabel II.

Bij patiënten met een chronische nierziekte zijn aanzienlijk verhoogde plasmaconcen-

traties van oplosbare adhesiemoleculen (sICAM-1, sVCAM-1) en von Willebrand factor 

aangetoond.4,5,6 Bovendien is tijdens een hemodialyse sessie een toename aan vCAM-1 in 

plasma aangetoond.7 Het is daarom denkbaar dat een hemodialyse behandeling schade 

daan de vaatwand bevordert, en daarmee bijdraagt aan het verhoogde cardiovasculaire ri-

sico bij patiënten met eindstadium nierfalen. Met betrekking tot endotheelcel activatie, 

is de biomarker proendothelin-1 onderzocht tezamen met activatie van het stollingssysteem 

e depletie van bloedplaatjes granulae bij patiënten die nog geen hemodialyse ondergaan 

e bij patiënten met hemodialyse behandeling (Tabel II).

2. HEMODIALYSE-GEÏNDECUERDE VERANDERINGEN TEN 

AANZIEN VAN HET STOLLINGSSYSTEEM

De effecten van verschillende soorten dialysemembranen op de activatie van het stollings-

systeem werden voor en tijdens een hemodialyse sessie bestudeerd (hoofdstukken 2,3,6).

High-flux AN-69 kunstnierenmembraan

Reeds vóór het begin van een hemodialyse behandeling werden verhoogde plasmaconcentra-

traties fibrinogeen en protrombine fragment F1+2 vastgesteld. Ten opzichte van het ini-

tiële FXII niveau werd tijdens hemodialyse binnen de eerste minuut een 25% FXII daling 

waargenomen. Vervolgens werd een lichte stijging naar het oorspronkelijke FXII niveau 

gedetecteerd. Aan het einde van de hemodialyse behandeling werden matig verhoogde 

concentraties van trombine-antitrombine complex en protrombine fragment F1+2 vast-

gesteld, terwijl veranderingen in fibrinogeen concentraties niet werden waargenomen.

Low-flux PMMA kunstnierenmembraan

Concentraties van protrombine fragment F1+2 waren reeds voor de start van een hemo-

dialyse behandeling verhoogd. Gedurende een hemodialyse behandeling daalde de FXII 

concentratie. De concentraties van protrombine fragment F1+2 en fibrinogeen namen iets 

toe, terwijl een verhoogde concentratie van trombine-antitrombine complex niet werd 

waargenomen.

High-flux F-60 kunstnierenmembraan

In de plasma FXII concentratie werd binnen 5 minuten na het starten van een hemodi-

alyse behandeling een forse daling tot onder de referentiewaarde vastgesteld. Vervolgens 

werd een lichte stijging gedetecteerd tot 20% van het initiële FXII niveau. Veranderin-

gen in plasmaconcentraties van protrombine fragment F1+2 en fibrinogeen werden niet
TABEL II. Samenvatting van de resultaten van trombocyten parameters voor hemocytometrie, morfologie en activatie of degranulatie en parameters voor activatie van het stollingssysteem voor, tijdens en aan het einde van een hemodialyse sessie in Hoofdstuk 2 tot 7.

| Bloedafname               | Voor HD (T=0) | Tijdens HD (T=1, 5, 30, 60 minuten) | Eind HD (T=150 minuten) | Uremie referentie groep |
|---------------------------|---------------|-----------------------------------|--------------------------|--------------------------|
|                           | AN-69         | PMMA                              | F-60                     | F8                       | AN-69                  | PMMA                  | F-60 | F8            | AN-69 | PMMA | F-60 | F8 | Referentie  |
|                           | high flux     | high flux                          | high flux                | low flux                 | high flux              | high flux             | high flux | low flux | high flux  | high flux | high flux | high flux | low flux | group       |
| Trombocyten: hemocytometrie |               |                                    |                          |                          |                        |                        |                   |            |                |        |        |        |    |            |
| PLT aantal                | N - ↓         | N - ↓                              | N - ↓                    | N - ↓                    | N - ↓                  | N - ↓                  | N - ↓ | N - ↓ |
| PDW                       | N             | N                                  | N                        | N                        | N                      | N                      | N     | N    |
| MPV                       | N             | N                                  | N                        | N                        | N                      | N                      | N     | N    |
| P-LCR                     | N - ↓         | N                                  | N - ↓                    | N - ↓                    | N - ↓                  | N - ↓                  | N - ↓ | (T1) |
| IPF                       |               |                                    |                          |                          |                        |                        |       |      |
| Thrombocyten: PLT morfologie |               |                                    |                          |                          |                        |                        |       |      |
| % PLTs met een kleurdcichtheid van de granula in het cytoplasma |               |                                    |                          |                          |                        |                        |       |      |
| - >75%                    |               |                                    |                          |                          |                        |                        |       |      |
| - <25%                    |               |                                    |                          |                          |                        |                        |       |      |
| Thrombocyten: activatie of degranulatie |               |                                    |                          |                          |                        |                        |       |      |
| CD62p                     |               |                                    |                          |                          |                        |                        |       |      |
| PF4                       | N             | N                                  | N - ↑                    | N - ↑                    | N - ↑                  | N - ↑                  | N - ↑ | (T30) |
| β-TG                      | N - ↑         | N                                  | N - ↑                    | N - ↑                    | N - ↑                  | N - ↑                  | N - ↑ | (T5) |
| Serotonin in PRP          | N - ↓         | N                                  | N - ↓                    | N - ↓                    | N - ↓                  | N - ↓                  | N - ↓ | (T30) |
| Activatie stollingssysteem |               |                                    |                          |                          |                        |                        |       |      |
| FXII                      | N             | N - ↓                              | N - ↓                    | N - ↓                    | N - ↓                  | N - ↓                  | N - ↓ | N - ↓ |
| F1+2                      | ↑             | ↑                                  | ↑                       | ↑                       | ↑                      | ↑                      | ↑     | ↑    |
| TAT                       | N             | N                                  | N - ↑                    | N - ↑                    | N - ↑                  | N - ↑                  | N - ↑ | (T60) |
| Fibrinogeen               | N - ↑         | N - ↑                              | N - ↑                    | N - ↑                    | N - ↑                  | N - ↑                  | N - ↑ | N - ↑ |
| TpP                       | N - ↑         | N                                  | N - ↑                    | N - ↑                    | N - ↑                  | N - ↑                  | N - ↑ | N - ↑ |

PLT, platelet / bloedplaatje; IPF, immature platelet fraction / fractie onrijpe bloedplaatjes; MPV, mean platelet volume / gemiddelde bloedplaatjes volume; PDW, platelet distribution width / bloedplaatjes verdelingscurve; p-LCR, platelet large cell ratio / verhouding grote bloedplaatjes; TAT, thrombine-antithrombine complex; β-TG, β-thromboglobuline; F1+2 = prothrombine fragment 1+2; TpP, thrombus precursor protein; PRP, platelet-rich plasma / plaatjesrijk plasma; HD, hemodialyse.
waargenomen. In 40% van de patiënten werden tijdens hemodialyse enigszins verhoogde concentraties van trombine-antitrombine complex vastgesteld.

**Low-flux F-8 kunstnier membraan**
Concentraties van protrombine fragment F1+2 waren reeds voor de start van een hemodialyse behandeling verhoogd. Gedurende de gehele hemodialyse behandeling werd een toename van protrombine fragment F1+2 en trombine-antitrombine complex vastgesteld.

Uit onze resultaten kunnen de volgende conclusies worden getrokken. Hemodialyse patiënten hebben al tekenen van activatie van het stollingssysteem voorafgaand aan een hemodialyse sessie. Tijdens hemodialyse met verschillende soorten dialysemembranen treedt een nog verdere stimulering van het stollingssysteem op, die overigens sterk patiëntafhankelijk blijkt te zijn.

Aangezien de hierboven vermelde onderzoeken in een periode van meer dan 10 jaar zijn uitgevoerd en vergelijkende studies ontbreken, is het vrijwel niet mogelijk om een oordeel te geven over de verschillen tussen de diverse soorten dialysemembranen. Het lijkt erop dat high-flux polysulphon (F-60) en poly-methyl-metacrylaat (PMMA) dialysemembranen meer biocompatibel zijn dan low-flux polysulphon (F-8) en high-flux AN-69, een copolymer van acrylonitril en natriummethallylsulfonaat, dialysemembranen.

## 3. HEMODIALYSE-GEÏNDOUCEERDE VERANDERINGEN TEN AANZIEN VAN BLOEDPLAATJES

Studies betreffende bloedplaatjes aantallen en activatie van bloedplaatjes werden uitgevoerd met een low-flux polysulphon F-8 dialysemembraan (**hoofdstukken 4-8**). Veranderingen in eigenschappen van bloedplaatjes, aangeduid met afwijkingen in RNA inhoud, grootte en morfologie werden vóór en tijdens hemodialyse sessies (**hoofdstukken 4,5,6,7**) bestudeerd om de vraag te beantwoorden of het aantal bloedplaatjes in de lage referentie-range in patiënten die hemodialyse therapie ondergaan een gevolg is van het verdwijnen van bloedplaatjes gedurende een hemodialyse sessie, of dit verdwijnen een balans weergeeft tussen een toename van het aantal nieuwe bloedplaatjes versus een hogere verwijdering van de totale populatie bloedplaatjes, en of het verdwijnen samenvaalt met de activatie van bloedplaatjes gedurende de hemodialyse sessie. In **hoofdstuk 8** werden effecten van uremie- en hemodialyse-geïnduceerde veranderingen op het verlies van granulaire inhoud van bloedplaatjes onderzocht.

### 3.1 Hemocytometrie en morfologie van bloedplaatjes

De resultaten van het aantal bloedplaatjes en kenmerken van bloedplaatjes (morfologie en granulaire kleuring), die met behulp van de verschillende onderzoeken zijn verkregen, zijn weergegeven in Tabel III.
Gemiddeld hadden hemodialyse patiënten al voor de start van een hemodialyse sessie een laag normaal aantal bloedplaatjes. Tijdens hemodialyse werd een lichte daling van het aantal bloedplaatjes vastgesteld. Ook het aantal onrijpe bloedplaatjes (IPF) in de circulatie was voor de start van een hemodialyse behandeling laag normaal en daalde verder tijdens de sessie. Deze resultaten impliceren een verminderde megakaryopoïese, een verhoogde eliminatie van jonge bloedplaatjes uit de circulatie, of een combinatie daarvan in de periode tussen twee hemodialyse behandelingen.

Tijdens maar niet vóór de start van een hemodialyse sessie werden, in vergelijking met ogenschijnlijk gezonde personen, kleine afwijkingen in het gemiddelde bloedplaatjesvolume (MPV), de bloedplaatjesverdelingscurve (PDW) en de verhouding grote bloedplaatjes (p-LCR) vastgesteld. Elektronenmicroscopie studies gaven aan dat het oppervlak van de bloedplaatjes van hemodialyse patiënten aanzienlijk kleiner is in vergelijking met ogenschijnlijk gezonde personen. Het is niet duidelijk waarom het verlaagde gemiddelde bloedplaatjesvolume zoals afgeleid uit digitale beeldanalyse van de elektronenmicroscopische bevindingen, de kleine veranderingen in bloedplaatjesvolume zoals beoordeeld met behulp van hemocytometrie (MPV), aanzienlijk overtreffen. Mogelijk zijn de bloedplaatjes in het uremische milieu zeer gevoelig voor de fixatie procedure die noodzakelijk is voor de elektronenmicroscopische beeldvorming, en resulteert dit in abnormale uitdroging en krimp. Bloedplaatjes met een kleurdichtheid lager dan 25% zijn arbitrair gedefinieerd als ‘leeg’. De referentiewaarde bedraagt maximaal 20% van het aantal bloedplaatjes. Bij patiënten met een chronische nierziekte (zonder hemodialyse behandeling) was 25% bloedplaatjes met een kleurdichtheid lager dan 20% aanwezig. Bij hemodialyse patiënten was het aantal ‘lege’ bloedplaatjes 36%.

**Tabel III.** Overzicht van veranderingen in het aantal bloedplaatjes en kenmerken van bloedplaatjes tijdens hemodialyse.

|               | voor HD | eind HD | % toename / afname | bloedplaatjes aantal toename / afname |
|---------------|---------|---------|--------------------|-------------------------------------|
| PLT (150 - 400 x10⁹/L) | 198     | 182     | -8                 | -16                                 |
| IPF (4 - 17 x10⁹/L)    | 6,7     | 6,1     | -9                 | -0,6                                |
| MPV (9 - 11,5 fl)     | 10,5    | 9,5     | -10                |                                     |
| PDW (10,5 - 14 fl)    | 12,3    | 11,0    | -11                |                                     |
| p-LCR (<36 %)         | 29      | 27,2    | -6                 |                                     |
| % bloedplaatjes met 75% kleurdichtheid (> 50%) | 19      | 29      | +50                | +15                                 |
| % bloedplaatjes met 25% kleurdichtheid (< 20%) | 36      | 29      | -20                | -18                                 |

PLT, platelet / bloedplaatje; IPF, immature platelet fraction / fractie onrijpe bloedplaatjes; MPV, mean platelet volume / gemiddelde bloedplaatjes volume; PDW, platelet distribution width / bloedplaatjesverdelingscurve; p-LCR, platelet large cell ratio / verhouding grote bloedplaatjes; HD, hemodialyse; Referentiewaarden zijn tussen haakjes weergegeven.
Ook de mate van aanwezigheid van de granula in de bloedplaatjes, gedefinieerd als het oppervlak van de dense bodies per bloedplaatje, was aanzienlijk lager (31%) in vergelijking met bloedplaatjes van ogenschijnlijk gezonde personen. De elektronenmicroscopische bevindingen ondersteunen de lichtmicroscopische bevindingen van een verhoogd aantal bloedplaatjes met verminderde kleurdichtheid en de hypothese van verlies van granulaire inhoud bij hemodialyse patiënten.

3.2 Activatie van bloedplaatjes

Voor de start van een hemodialyse behandeling bleken de p-selectine expressie op bloedplaatjes (CD62p), concentratie serotonin in bloedplaatjes en concentratie plaatjesfactor 4 in plasma binnen de referentiewaarden. β-Tromboglobuline concentraties waren voor de start van een hemodialyse sessie reeds sterk verhoogd, hetgeen normaal gesproken wordt geïnterpreteerd als voortdurende activatie van bloedplaatjes. Echter, bij een chronische nierziekte is de normale halfwaardetijd van β-tromboglobuline (100 minuten) aanzienlijk toegenomen. Daardoor is het niet mogelijk de verhoogde β-tromboglobuline concentraties voorafgaand aan een hemodialyse sessie te interpreteren als voortdurende activatie van bloedplaatjes tussen twee hemodialyse behandelingen. Concentraties plaatjesfactor 4 in plasma vóór hemodialyse waren ook binnen de referentiewaarden, maar dit eiwit bindt aan het endotheel en is daarom geen geschikte merker voor continue activatie van bloedplaatjes. Op basis van de afwezige expressie van p-selectine op de bloedplaatjes concluderen we dat activatie van bloedplaatjes tussen hemodialyse behandelingen niet substantieel is.

Het effect van dialysemembranen op de morfologie van bloedplaatjes, activatie van bloedplaatjes, p-selectine expressie op bloedplaatjes, β-tromboglobuline plasmaconcentratie en plaatjesfactor 4 plasmaconcentratie werd onderzocht in monsters van de afferente en effereente lijnen van de kunstnieren (hoofdstuk 5). Waarschijnlijk als gevolg van hechting aan de lichaamsvreemde stoffen van de dialysemembraan nam het aantal bloedplaatjes onmiddellijk na de eerste passage van het bloed door de kunstnien af. Activatie van bloedplaatjes, zoals gedetecteerd door verhoogde p-selectine expressie, gebeurde ook meteen na de eerste passage van het bloed door de kunstnier. In het extracorporele circuit namen de p-selectine expressie op bloedplaatjes en plasmaconcentraties β-tromboglobuline binnen 30 minuten verder toe. De plasmaconcentratie plaatjesfactor 4 toonde na de start van een hemodialyse sessie onmiddellijk een sterke stijging. Dit wordt echter toegeschreven aan het loslaten van plaatjesfactor 4 vanaf het endotheel als reactie op de bolus injectie van het antistollingsmiddel – (laagmoleculair gewicht) heparine – bij de start van de hemodialyse sessie.

Voor de start van een hemodialyse sessie, lagen de concentraties serotonin in plaatjesrijk plasma, dat wil zeggen plasma plus bloedplaatjes, in het onderste kwartiel van de referentierange. Plasmaconcentraties serotonin voor de start van een hemodialyse sessie waren verhoogd, mogelijk vanwege een verminderde klaring. De concentratie serotonin in de bloedplaatjes was verlaagd. Tijdens hemodialyse daalde de serotonin concentraatie geleidelijk verder tot 35% van het oorspronkelijke niveau, hetgeen aangeeft dat voortdurende secretie van de inhoud van de bloedplaatjes granules in het plasma plaatsvindt. Plasma serotonin concentraties daalden tot 45% van het oorspronkelijke niveau. Hoogst-
waarschijnlijk wordt serotonine, gelet op het lage molecuulgewicht (176D), gedurende de hemodialyse behandeling efficiënt uit het plasma verwijderd.

3.3 Conclusies

De resultaten van de morfologie, onrijpheid, granulaire inhoud van bloedplaatjes en parameters voor activatie van bloedplaatjes leiden tot de volgende conclusies. Vóór hemodialyse hadden patiënten gemiddeld een laag normaal aantal bloedplaatjes in de circulatie. Het aantal onrijpe bloedplaatjes was laag normaal, hetgeen wijst op een verminderde megakaryopoïese, een verhoogde eliminatie uit de circulatie, of een combinatie daarvan. De serotonine concentratie in bloedplaatjes was voor aanvang van een hemodialyse sessie verlaagd. Tijdens de sessie daalde de serotonine concentratie in de bloedplaatjes drastisch, hetgeen wijst op release van de granulaire inhoud door de bloedplaatjes als onderdeel van hun activering. De periode tussen de hemodialyse sessies is waarschijnlijk te kort om de bloedplaatjes weer met serotonine te vullen, waardoor ze voor de aanvang van een hemodialyse sessie al een verlaagde serotonine inhoud hebben. Voortdurende activatie van bloedplaatjes tussen twee hemodialyse behandelingen als oorzaak van de verlaagde serotonine concentratie in de bloedplaatjes is niet waarschijnlijk, doordat er voorafgaand aan een hemodialyse sessie geen verhoogde p-selectine expressie op de bloedplaatjes werd aangetoond. Het aantal bloedplaatjes nam onmiddellijk af na de eerste passage van het bloed door de kunstnier (van 198 tot 173 x10⁹/L). Daarna steeg het aantal enigszins tot 182 x10⁹/L op T=150 minuten. De initiële afname wordt waarschijnlijk veroorzaakt door hechting van de bloedplaatjes aan de kunstnier. De daaropvolgende toename van het aantal bloedplaatjes kan een gevolg zijn van het weer loslaten van bloedplaatjes van de kunstniersmembraan, maar kan ook een gevolg zijn van het vrijkomen van bloedplaatjes uit opslagplaatsen in het lichaam. De granulaire kleurdichtheid van bloedplaatjes indiceert het verwijderen van bloedplaatjes met een verminderde kleurdichtheid en verhoging van de kleurdichtheid van bloedplaatjes na een hemodialyse sessie. Deze resultaten geven aan dat tenminste een deel van de bloedplaatjes stijging tijdens een hemodialyse sessie een gevolg is van het vrijkomen van bloedplaatjes uit een aantal lichaamscompartimenten, bijvoorbeeld het beenmerg. De vermindering van het aantal onrijpe bloedplaatjes na hemodialyse is in tegenspraak met deze conclusie. De afname van het aantal onrijpe bloedplaatjes kan echter te wijten zijn aan een verhoogde verwijdering van deze bloedplaatjes in de kunstnier. Dit wordt ondersteund door onze bevinding van verlaging van MPV na hemodialyse, immers onrijpe bloedplaatjes zijn groter dan oudere bloedplaatjes. Aldus is de lichte daling van het aantal bloedplaatjes na een hemodialyse behandeling een resultante van het verwijderen van bloedplaatjes in de kunstnier en het tegelijkertijd vrijkomen van nieuwe bloedplaatjes uit opslagplaatsen in het lichaam.
4. HEMODIALYSE-GEÏNDEUCEERDE VERANDERINGEN MET BETREKKING TOT INTEGRITEIT VAN HET ENDOTHEEL

Een essentiële functie van het endotheel is het bieden van een celopervlak dat in normale omstandigheden activatie van het stollingssysteem voorkomt. Bij beschadiging van het oppervlak dienen geactiveerde endotheelcellen juist activatie van het stollingssysteem te bevorderen. Expressie van weefselfactor op het oppervlak van de endotheelcellen speelt een belangrijke rol bij de transformatie van een anticoagulante status naar een procoagulante status van het endotheel. De endotheelcellen van patiënten met een chronische nierziekte worden continu blootgesteld aan uremische toxines die activatie van endotheelcellen teweeg kunnen brengen en verschijnselen van systemische ontsteking kunnen veroorzaken. De mechanismen waarmee uremie endotheelcellen kunnen activeren zijn echter tot nu toe nog niet opgehelderd.

Afhankelijk van het type kunstnijver en de mate van contaminatie van het dialysaat worden tijdens hemodialyse verschillende typen bloedcellen en eiwitsystemen gestimuleerd, met als gevolg dat pro-inflammatoire cytokinen, zoals IL-6 en TNF-α, vrijkomen. Degranulatie van granulocyt en bloedplaatjes leidt tot de uittuut van verschillende stoffen uit de granula van deze cellen, zoals myeloperoxidase en plaatjesfactor 4.1 Gestimuleerde witte bloedcellen en geactiveerde bloedplaatjes verlaten de kunstnijver en komen vervolgens in de bloedcirculatie van de patiënt, waar zij endotheelcellen kunnen activeren en/of beschadigen. Als gevolg hiervan induceert hemodialyse oxidatieve stress, protrombotische veranderingen en ontstekingsverschijnselen.

Concentraties proendotheline-1 werden bij patiënten met een chronische nierziekte en bij hemodialyse patiënten onderzocht om vast te stellen of er bij patiënten die hemodialyse ondergaan sprake is van een verminderd intact zijn van het endotheel (hoofdstuk 8). De concentratie van de in-vivo inactieve biomarker proendotheline-1 weerspiegelt het niveau van het bioactieve peptide endotheline-1. Proendotheline-1 is de voorloper van endotheline-1, die in tegenstelling tot endotheline-1, ex-vivo stabiel blijkt te zijn.12 Plasmaconcentraties van proendotheline-1 bij patiënten met een chronische nierziekte en hemodialyse patiënten waren ongeveer vijfmaal verhoogd in vergelijking met een referentiegroep van ogenschijnlijk gezonde personen. Met de leeftijd neemt de concentratie proendotheline geleidelijk toe. Bij personen ouder dan 65 jaar zijn de concentraties van proendotheline-1 ongeveer 20% toegenomen ten opzichte van personen van 25 jaar oud. Gelet op de leeftijd gerelateerde toename van proendotheline-1, zijn de concentraties proendotheline-1 bij patiënten met een chronische nierziekte en hemodialyse patiënten duidelijk hoger, hetgeen bij een verminderd intact zijn van het endotheel kan passen.

Ten aanzien van de sterk verhoogde concentraties proendotheline-1 en het verminderd intact zijn van het endotheel heeft een studie van Grooteman et al. aangetoond dat bij hemodialyse patiënten de plasmaconcentraties celadhesiometers (sICAM-1 en sVCAM-1) van het endotheel en van Willebrand factor ook duidelijk verhoogd zijn en niet veranderen gedurende 1 maand van hemodialyse behandelingen. Tevens werd aangetoond dat elke hemodialyse sessie binnen 24 uur resulteert in een duidelijke toename van von Willebrand factor in plasma, terwijl de concentratie van de celadhesiometers
(sICAM-1) in plasma stabiel is. De concentraties celadhesiemoleculen en van Willebrand factor varieerden sterk binnen de patiëntengroep. Uit de studie van Grooteman et al. werd geconcludeerd dat endotheel dysfunctie veel meer afhankelijk lijkt te zijn van patiënt-gebonden factoren, zoals co-morbiditeit, chronische nier-dysfunctie en voorgeschreven medicatie, dan van de hemodialyse behandeling.6

5. Conclusie

Hemodialyse patiënten kunnen in twee tegengestelde richtingen complicaties krijgen in hun hemostase: bloeding en stolling. Klinisch relevant zijn het langdurige bloeden uit de shunt en de prothrombotische toestand van deze patiënten. Om onze bevindingen van de uitgevoerde studies samen te vatten, zijn er nieuwe inzichten verkregen met betrekking tot hemodialyse-geïnduceerde activatie van bloedplaatjes en stolling:

• Reeds voor de start van een hemodialyse sessie hebben patiënten gemiddeld een laag normaal aantal bloedplaatjes, hetgeen een vermindere megakaryopoïse kan weerspiegelen, maar ook een onvoldoende vermogen van het lichaam om de tijdens de frequente hemodialyse sessies verwijderde bloedplaatjes aan te vullen.

• Omdat voorafgaand aan een hemodialyse sessie expressie van p-selectine op het oppervlak van bloedplaatjes afwezig is, treedt voortdurende activatie van bloedplaatjes tussen twee hemodialyse sessies niet op.

• De kleurdichtheid van de granula in bloedplaatjes is, voorafgaand aan een hemodialyse sessie, duidelijk onder de referentiewaarden. Bloedplaatjes van hemodialyse patiënten zijn voor wat betreft de inhoud van de granula dus chronisch ‘leeg’, maar dit is niet vanwege hun voortdurende activatie tussen twee hemodialyse sessies. Waarschijnlijk is de beperkte kleurdichtheid van de granula een gevolg van herhaalde activatie tijdens de hemodialyse sessies.

• De gemiddelde lichte afname van het aantal bloedplaatjes na een hemodialyse sessie is waarschijnlijk een gevolg van adhesie van bloedplaatjes aan de kunstniermembranen, die niet volledig wordt gecompenseerd door instroom van nieuwe bloedplaatjes uit lichaamscompartimenten.

• Voor de start van een hemodialyse sessie hebben hemodialyse patiënten reeds een geactiveerd stollingssysteem, zoals blijkt uit de licht verhoogde plasmaconcentraties van trombine-antitrombine complex en protrombine fragment F1+2.

• Tijdens een hemodialyse behandeling vindt uitgebreide activatie van het stollingsysteem plaats, ondanks het toedienen van het antistollingsmiddel laagmoleculair gewicht heparine en het gebruik van kunstniermembranen met een hoge biocompatibiliteit. Activatie van het stollingssysteem lijkt voornamelijk te worden geïnitieerd via de factor XII-afhankelijke route.

• Een aanwijzing voor een verminderd intact zijn van het endotheel werd verkregen door de verhoogde plasmaconcentraties proendotheline-1.

• Activatie van bloedplaatjes tijdens en bloedstolling zowel tijdens als tussen hemodialyse sessies leidt tot een continue prothrombotische toestand van de patiënt.
6. TOEKOMSTPERSPECTIEVEN

Introductie
Zoals uitvoerig in de voorgaande paragrafen beschreven zijn zowel de uremie als de hemodialyse behandeling geassocieerd met een verscheidenheid aan effecten op de bloedplaatjes en het stollingssysteem. Klinisch kunnen dergelijke veranderingen bijdragen aan de schijnbaar paradoxale bevinding van zowel een pretrombotische toestand als een bloedingsneiging bij patiënten met een chronische nierziekte. Aangezien het hemostase systeem betrokken is bij de ontwikkeling van atherosclerose bij patiënten zonder nierziekte, is het denkbaar dat de pathofysiologische verstoringen van de hemostase bij patiënten met uremie bijdragen tot de hoge mate van cardiovasculaire ziekten die in deze patiëntengroep voorkomen. Vermindering van deze afwijkingen kan daarom een gunstige effect hebben op zowel de korte als lange termijn complicaties bij patiënten met een chronische nierziekte (nog niet op hemodialyse) en bij patiënten met hemodialyse behandeling.

Korte termijn complicaties
Zoals eerder vermeld, wordt aan patiënten kort voor de start van een hemodialyse sessie een bolus injectie van ongefractioneerd heparine of laagmoleculair gewicht heparine toegediend om stolling in het extracorporele circuit te voorkomen. In de klinische praktijk wordt de dosis anticoagulans voornamelijk gebaseerd op het gewicht van de patiënt. De dosis anticoagulans wordt aangepast bij zichtbare stolling in de luchtvanger of een toenemende druk in de afferente lijn tijdens opeenvolgende hemodialyse sessies. Echter, terwijl een hogere dosering anticoagulans (of een tweede bolus halverwege de sessie) de stolling en de druk in het extracorporele circuit kunnen verminderen, neemt de kans op bloedingen bij de shunt aan het einde van de hemodialyse sessie toe. Derhalve zou monitoring van anti-factor Xa in hoog-risico patiënten dit ongewenste neveneffect van chronische hemodialyse behandeling kunnen verminderen of zelfs voorkomen. Andere strategieën betreffen een groter gebruik maken van trinatriumcitraat, dat plaatjesfactor 4 afgifte uit het endotheel voorkomt, een combinatie van citraat-houdende dialysevloeistof en een lage dosis laagmoleculair gewicht heparine, toediening van nafostat mesylaat (NFM) en pre-dilution hemodiafiltratie (HDF). Aangezien deze alternatieve benaderingen bovengenoemde complicaties zouden kunnen verminderen of vermijden, is verder onderzoek op dit gebied noodzakelijk.

Lange termijn complicaties

Fistel stenose
Een relatief frequente complicatie van hemodialyse behandeling is occlusie van de arterioveneuze toegang. Occlusie kan zowel voorkomen bij een shunt van synthetisch materiaal (graft) als bij een shunt waarbij een slagader direct is verbonden met een ader (fistel). In de VS hebben hemodialyse patiënten voornamelijk een graft, terwijl in Nederland circa 90% van de hemodialyse patiënten een fistel als arterioveneuze toegang heeft. Trombose die binnen een maand na de fistel constructie optreedt, is meestal te wijten aan technische fouten of voortijdig gebruik. De belangrijkste predisponerende factor voor een fistel trombose is een stenose in het veneuze deel van de shunt, die het resultaat is van gelei-
delijke fibromusculaire intimale hyperplasie en periveneuze fibrose. Klinisch wordt een fistel trombose gekenmerkt door een verminderde bloedstroom, recirculatie en daardoor een minder efficiënte hemodialyse. Of de verstoorde hemostase, zoals beschreven in dit proefschrift, bijdraagt bij aan de ontwikkeling van een stenose is niet goed bekend. Hoe- wel behandeling met aspirine en dipyridamol heeft aangetoond dat de tijdsduur tot het optreden van een eerste trombose bij PTFE (Poly-Tetra-Fluor-Ethyleen) grafts verkort is, heeft behandeling met coumarinederivaten of het voorschrijven van trombocytenaggregatieremmers geen invloed op de snelheid waarmee fistel-falen optreedt.

In dit verband is het interessant om op te merken dat vaatherstel en vorming van nieuwe intima verminderd was door de doelgerichte toediening van aspecifieke factor Xa -remmers zoals heparine en laagmoleculair gewicht heparine, middels hun koppeling aan een antifibrine antilichaam. Of geen-heparine bevattende modulatoren van hemostase de ontwikkeling van fistel stenose kunnen voorkomen of vertragen is momenteel niet bekend, maar nodigt uit tot verder onderzoek.

**Hart-en vaatziekten**

Het risico op sterfte is bij hemodialyse patiënten extreem hoog; 40-50% overlijdt ten gevolge van een cardiovasculaire aandoening. Naast de klassieke risicofactoren als een hoog cholesterol en hoge bloeddruk, zijn ook verschillende niet-klassieke risicofactoren geïdentificeerd die bijdragen aan vasculaire afwijkingen bij chronische nierziekten of die verergeren. In het voorgaande decennium waren belangrijke onderzoeksthema’s op dit gebied een afwijkende homocysteine stofwisseling, oxidatieve stress en chronische microontsteking. Momenteel gaat de meeste aandacht echter uit naar staornissen in het mineraalmetabolisme.

Zowel uit experimentele als klinische gegevens blijkt dat bloedplaatjes en het stollingsysteem betrokken zijn bij klassieke atherogenese en atherotrombose. In tal van klinische studies, is toediening van anti-plaatjes of antistollingstherapie geassocieerd met vermindering of zelfs regressie van plaquegroei. Er is aangetoond dat een systemische micro-inflammatoire omgeving, zoals beschreven in patiënten met een chronische nierziekte, een fenotypische overgang van het endotheel naar een pro-atherogene oppervlak kan induce ren. Zoals besproken in hoofdstuk 8 komen bij patiënten met een chronische nierziekte niet alleen verhoogde concentraties proendotheline-1 voor, maar ook hoge concentraties oplosbare adhesiemoleculen (sICAM-1, sVCAM-1), hetgeen activatie van het endotheel suggerereert. De hieruit voortvloeiende verhoogde expressie van celadhesiemoleculen induceert adhesie van bloedplaatjes en secretie van verschillende atherogene mediatoren, waaronder cytokinen. Binding tussen de aan het endotheel geadhereerde bloedplaatjes en leukocyten ondersteunen de activatie en transmigratie van monocyt en, die als cruciaal voor de vorming van plaques wordt beschouwd.

Zoals eerder beschreven, zijn bij patiënten met een chronische nierziekte verschillende uremie-specifieke pro-atherogene aandoeningen beschreven, waaronder hyperhomocysteineemie, oxidatieve stress, micro-ontsteking en stoornissen van het mineraalmetabolisme. Dat uremie naast vasculaire abnormaliteiten, die gewoonlijk worden waargenomen bij patiënten met een chronische nierziekte, ook een bijdrage levert aan de granulaire afwijkingen van bloedplaatjes en activatie het stollingssysteem, is denkbaar maar nog niet volledig begrepen. Anderzijds kan de verlaagde concentratie en uitputting van de gruu-
lare inhoud van de bloedplaatjes mogelijk bescherming bieden tegen bloedplaatjes-geïn-
duceerde beschadiging van de vaatwand. Toekomstig onderzoek op dit gebied kan helpen
om de complexe interacties tussen aandoeningen van hemostase en andere stimuli van
uremische vasculopathie te ontrafelen. Of interventie in het hemostase systeem de hoge
incidentie van hart-en vaatziekten in deze patiëntenpopulatie kan verminderen, is ook een
onderwerp voor toekomstig onderzoek.

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Marianne Schoorl is geboren op 6 januari 1959 te Zuid-Scharwoude (gemeente Lange- dijk). Na de middelbare school aan de Rijksscholengemeenschap Noord-Kennemerland te Alkmaar, waar zij haar atheneum diploma behaalde in 1978, volgde zij de Hoger Beroepsopleiding aan het Bakhuis Roozeboom Instituut te Beverwijk. Zij behaalde haar diploma in de richting Klinische Chemie A in juni 1980. Daarna ging zij werken als analist in het klinische chemisch laboratorium van het Medisch Centrum Alkmaar. Vanaf september 1982 heeft zij in de avonduren de analist B opleiding aan het Ir. W. van den Broek Instituut te Amsterdam gevolgd. Zij behaalde haar diploma medisch-chemische differentiatie in juni 1984, waarna zij ging werken als research-analist.

Het klinisch chemisch laboratorium ontwikkelde zich vanaf 1989 tot het Laboratorium voor Klinische Chemie, Hematologie en Immunologie. Toen de mogelijkheid bestond om met het HBO-B diploma ook de zogenaamde Ing.-titel te verkrijgen, volgde zij vanaf 1992 aan de Hogeschool te Amsterdam de Hoger Laboratoriumonderwijs opleiding Clinical and Medical Biochemistry. In 1993 werd het diploma behaald met de afstudeeropdracht Ontstekings- en stollingsparameters bij patiënten met Morbus Crohn en Colitis Ulcerosa. In 1999 werd de cursus Industriële statistiek aan de Universiteit te Eindhoven afgerond. Daarna heeft zij zich verder gespecialiseerd in het vakgebied en toegepast wetenschappelijk onderzoek verricht naar o.a. activatie en aggregatie van trombocyten en stolling, ontstekingsparameters en nutriënten in combinatie met de erytropoïese activiteit. Successen werden behaald met o.a. de IAMLt Scientific Iatoron Award 2002, Sysmex Outstanding Science Award 2007 en Pieter van Foreest Penning 2007.

Een eerste link met kwaliteit werd in 1992 gelegd door het volgen van de opleiding Opzetten van een kwaliteitssysteem volgens de CCKL-Praktijkrichtlijn. Na het behalen van laboratoriumaccreditatie in 1995 heeft Marianne zich verder gespecialiseerd op het gebied van Total Quality Management en Auditing. Opleidingen hiervoor werden gevolgd bij het Instituut Nederlandse Kwaliteit (INK, 2001, 2011) en bij de Raad van Accreditatie (RvA, 2010). Externe activiteiten worden tot heden verricht als extern auditor bij INK en RvA en als valued professional bij INK. Focus van Marianne ligt op een adequate kwaliteitsaanpak voor verbeter- en veranderingstrajecten. Vanuit het Laboratorium voor Klinische Chemie, Hematologie & Immunologie verschenen door de jaren heen publicaties op het gebied van kwaliteitsbewustzijn, competentie-management, bedrijfscultuur, integrale kwaliteitszorg, balanced score card, auditing, logistiek en communicatie. Successen werden in het laboratorium behaald met de INK Onderscheiding (2002), NVKC Posterprijs (2004) en nominatie voor de Pieter van Foreest Innovatieprijs (2012).

In 2001 werd zij naast haar werkzaamheden op het gebied van kwaliteit en research bestuurslid van de Nederlandse Vereniging van bioMedisch Laboratoriummedewerkers (NVML) met de aandachtsgebieden nascholing en kwaliteit. In 2003 werd zij benoemd tot voorzitter, een taak die zij tot medio 2010 heeft vervuld.

Vanaf 2010 werden plannen uitgewerkt voor de bewerking van een proefschrift. De voltooiing van dit proefschrift biedt mogelijkheden om de voor haar belangrijke succesbepalende factoren creativiteit, inspiratie en innovatief vermogen nog verder te ontplooiën in haar werk als hoofd Expertisecentrum in het Laboratorium voor Klinische Chemie, Hematologie & Immunologie van het Medisch Centrum Alkmaar.
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Activation of Platelets and Coagulation during Haemodialysis
In end-stage kidney disease, haemodialysis treatment is applied to remove excess fluid and waste products, such as urea and creatinine, from the blood. In this thesis, attention is focussed on activation of platelets and the coagulation system using several types of dialyzer membranes. A variety of parameters regarding platelet morphology, platelet activation, platelet degranulation and markers for activation of coagulation were investigated both before and during haemodialysis treatment with low-flux polysulphon (F-8), high-flux polysulphon (F-60), high flux acrylonitrile (AN-69) and low-flux polymethyl-metacrylaat (PMMA) dialyzer membranes.

With regard to endothelial cell activation, the biomarker proendothelin-1 was investigated together with activation of the coagulation system and platelet granule depletion both in patients not yet on haemodialysis and in patients with haemodialysis treatment.

Marianne Schoorl is currently working as manager of Quality & Research in the Department of Clinical Chemistry, Haematology & Immunology at the Medical Center Alkmaar. The completion of this thesis offers her opportunities to further develop important success factors such as creativity, inspiration and innovative capacity.