

Platelet morphology and IPF during treatment with haemodialysis

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In subjects treated with haemodialysis (HD) haemostatic balance is disturbed as a consequence of extracorporeal blood circulation. Hypercoagulability is demonstrated to be strongly influenced by surface characteristics of the dialyser membrane and anticoagulation mode. Thrombogenicity may result in reduced HD efficacy, because the membrane area does not function appropriately.

During treatment with HD PLT interaction with the dialysis membrane results in activation and adhesion on the membrane surface and release of platelet-derived factors. In case of PLT activation increased amounts of degranulated PLTs are present in peripheral blood circulation. Degranulated PLTs are less viable and demonstrate a shortened life span. Increased turnover of PLTs might reveal increased amounts of immature PLTs with increased RNA content.

CD62p expression on PLTs, secretion of Platelet Factor 4 (PF4) from alpha granules and release of serotonin from dense granules in plasma are indicative for the degree of PLT activation. Morphological aspects of PLTs in blood smears can be classified by microscopic evaluation.

In a longitudinal study deviations in PLT morphology and RNA content are established in combination with analysis of markers of PLT activation during HD treatment. Results for IPF counts, in conjunction with PLT counts, MPV and PDW are classified in order to elucidate the relationship with specific PLT activation parameters such as CD62p expression on PLTs and release of PF4 and serotonin in plasma.

Results are indicative for continuous PLT activation during HD treatment. The reduction of PLT and IPF counts is indicative for suppressed activity of megakaryopoiesis. Decreases in MPV and PDW indicate a shift of the ratio of reticulated and degranulated PLTs in peripheral blood. The reticulated platelets demonstrate a high quality of viability. Therefore, adherence of reticulated platelets to the dialyser membrane is likely to occur predominantly if compared with adherence to degranulated, less viable PLTs. Uraemia reveals an additional trigger for the occurrence of degranulated PLTs. The increase of PF4 and serotonin concentrations in plasma suggests a mild degree of PLT function damage.

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Introduction

Platelet physiology

Platelets are derived from megakaryocytes, which arise from pluripotent myeloid stem cells. Platelet production is controlled by thrombopoietin which is involved in the final maturation of the megakaryocyte. Thrombopoietin has multiple actions in megakaryocyte development. It shares some structural homology with erythropoietin and is produced principally by the liver. It increases the size and number of megakaryocytes, stimulates the expression of platelet-specific markers, and is a potent megakaryocyte colony-stimulating factor. Megakaryocytes undergo endomitosis, the megakaryocyte cytoplasm then fragments into small pieces, called proplatelets. The edges of the megakaryocyte break off to produce approximately 3,000 platelets which are released into the peripheral blood circulation. Platelets are needed for normal blood clotting. The first part of the coagulation pathway, known as primary haemostasis involves platelets. Damage to blood vessel walls exposes

collagen which is normally present under the endothelium. Platelets bind to the collagen and this adhesion is strengthened further by the large multimeric circulating protein, von Willebrand factor (vWF), which forms links between the platelet glycoprotein and collagen. The platelets are then activated and release the contents of their granules into the plasma, thus activating other platelets. The tissue factor pathway which involves the generation of thrombin is induced by the tissue factor and factor VIIa complex which activates factor X which then leads to the production of small amounts of thrombin. Thrombin generation is amplified by the activation of the intrinsic (or contact pathway) coagulation factors. The thrombin burst leads to the formation of a clot in which fibrin and activated platelets are stabilised by factor XIIIa. Common routine laboratory tests which are used to assess the coagulation system are the prothrombin time and the activated partial prothrombin time. However, these tests do not provide any information about primary haemostasis. The process of platelet activation and aggregation is illustrated in figure 1.

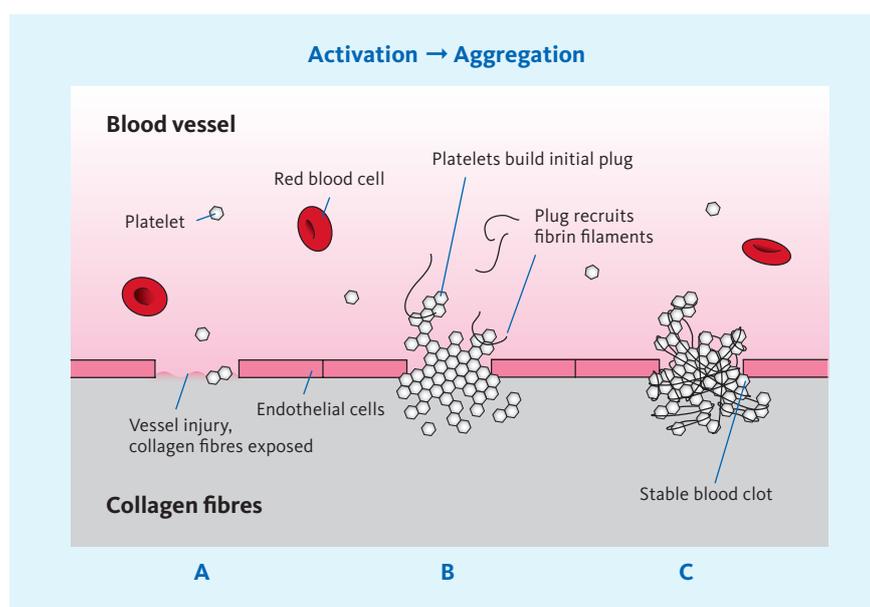


Figure 1 Damage to blood vessel walls exposes collagen. Circulating platelets bind to the collagen (A). The platelets become activated and release the contents of their granules into plasma, activating other platelets and coagulation factors (B) which ends in the production of thrombin and a final fibrin plug (C).

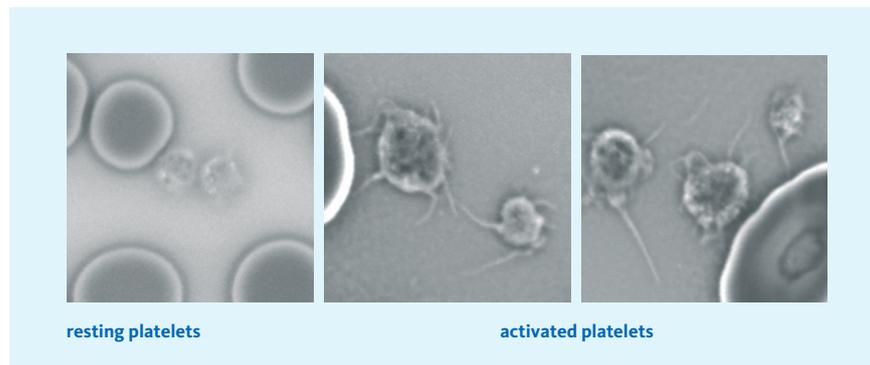


Figure 2 The changes in the shape of the platelet membrane when activation occurs.

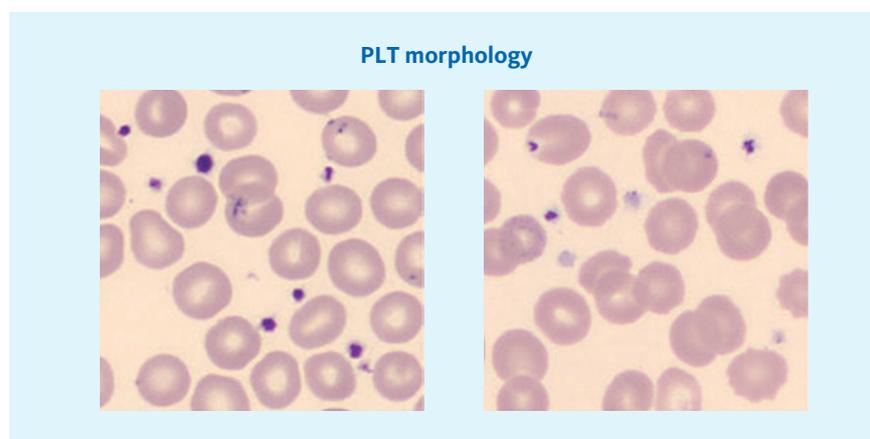


Figure 3 Normally stained platelets in the peripheral blood on the left and degranulated platelets on the right which show a lower uptake of stain and appear paler.

By electron microscopy it is possible to see the changes in the shape of the platelets that occur on activation (figure 2). Resting platelets have smooth membranes, but when activated there is a change in the shape of the membranes.

Following activation, platelets release the contents of their granules, and this can be seen by both electron microscopy and on the conventional microscopic examination of a stained blood film. Platelets normally appear as deep purple/blue dots, but degranulated platelets take up much less stain and appear much paler (figure 3).

Renal failure and haemodialysis

In end stage renal failure (ESRF) only 5–10% of normal kidney function remains. As a consequence, the urea concentration rises in blood and the patient retains fluid and oedema occurs. Urea is toxic and has to be removed from the blood by haemodialysis (HD); this cleans the blood and ultrafiltrates the hydrated body. There are different types of dialysing membranes available and different anticoagulants can be used during HD. Blood flow rate through the membrane will vary from patient to patient. This and other factors such as the rate of blood flow through the artificial membrane and the surface area of the membrane affect the degree of activation of platelets, coagulation factors, complement and granulocytes during HD.

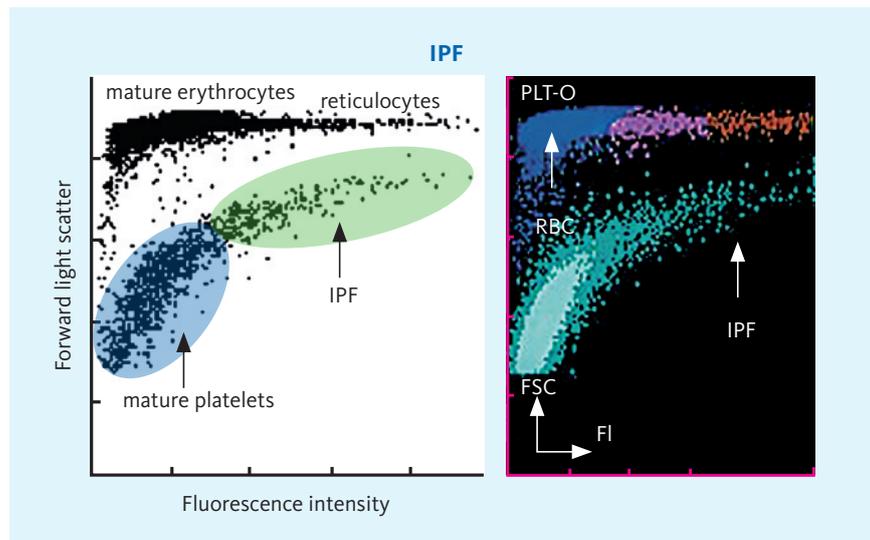


Figure 4 Location of the IPF in the optical platelet count cluster, measured in the reticulocyte channel. The IPF show higher fluorescent intensity and are larger in size than mature platelets.

A high degree of activation indicates biocompatibility of the artificial membrane, the efficiency of treatment is poor. Activation therefore needs to be minimised to improve efficiency and cause fewer complications for the patient. Different types of membranes can cause activation of different blood components; however, which membrane is used is dependant upon the patient's particular clinical situation.

Hypothesis for the study

Platelets become activated during HD due to contact with the artificial membranes. Platelet activation can be measured using flow cytometry and monoclonal antibodies such as anti CD62p. This antibody binds to P-selectin from platelet alpha granules, which are exposed on the surface of endothelial cells and platelets after activation. PF4 levels are also a measure of activation, this is released from alpha granules. Serotonin can also be measured which is released in platelet activation from the dense bodies. The morphological appearance of platelets in a stained peripheral blood film can be used to judge platelet

activation, as degranulated platelets appear paler compared to inactivated platelets with their intact granules.

As a consequence of activation platelets have a less flexible membrane and a shortened lifespan in the peripheral blood. A heterogeneous population of platelets, young (reticulated or immature) and older platelets exist in the circulation under these circumstances. The immature platelets (IPF) can be measured on the Sysmex XE-2100 using flow cytometry and a polymethine dye. The IPF have higher fluorescence due to the staining of residual RNA and are larger in size. Figure 4 demonstrates the location of the IPF in the optical platelet scattergram. The IPF is expressed as a percentage of the platelet count or as an absolute count $\times 10^9/L$. Previously published studies have reported that in HD treatment activation of platelets does not produce statistically significant changes in the mean platelet volume (MPV) or platelet distribution width (PDW) [1, 2, 3]. However, we hypothesise that there is a fall in MPV and PDW during HD as a result of a change in the ratio of immature platelets and activated, degranulated platelets, in peripheral blood.

Study Aims

To establish differences in platelet morphology, IPF, platelet size and platelet activation between normal healthy subjects and patients undergoing HD three times a week.

Patients

- A group of 20 subjects (age 28–82 years) in ESRF being treated by HD three times a week. Patients excluded from the study were those with active inflammation, thrombocytopenia, autoimmune disease or malignancy, as well as the use of specific categories of drugs (immunosuppressive, calcium antagonists, serotonin receptor antagonists, coumarin derivatives and aspirin).
- A reference group of 20 apparently healthy subjects (age 20–50 years) to establish reference ranges for the various parameters and morphological appearance of platelets.
- A group of uraemic patients (age 36–91 years) with serum urea concentrations of 20–30 mmol/L in order to establish the pathophysiological effect of urea on platelet characteristics.

HD protocol

A low flux polysulphone® F8 membrane (Fresenius, Bad Homburg, Germany) was used with the anticoagulant, Fragmin® (2000–5000 U intravenous bolus injection). Blood samples were collected at time point zero, before starting HD, one minute after starting HD and then after 5, 30, 60 and 150 minutes.

Platelet parameters measured

1. Sysmex XE-2100

platelet count, MPV, PDW and IPF.

2. Platelet morphology

two peripheral blood films stained with May-Grünwald-Giemsa on the Sysmex SP-100. Slides were evaluated for morphological aspects of platelets using the CellaVision® DM96 digital imaging analyser (CellaVision AB, Lund, Sweden). Density of staining of the platelet granules was scored (<25% of staining considered to be activated or 'empty' platelets) as well as the number of platelets with each score.

3. Platelet activation

Activation marker CD62p was measured by flow cytometry. PF4 and serotonin concentrations were measured with an ELISA.

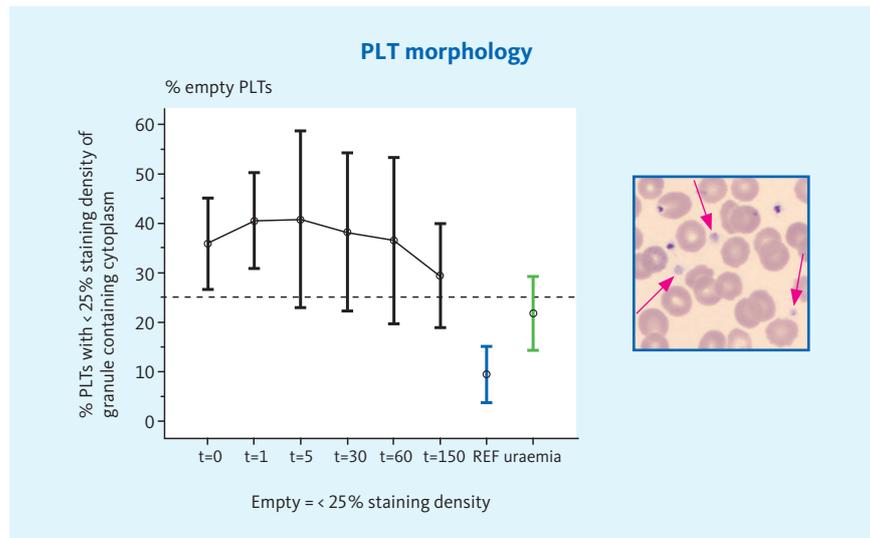


Figure 5 Activated (degranulated platelets) demonstrated by pale staining in the peripheral blood film, in patients undergoing HD, healthy subjects and a group of uraemic patients.

Results

1. Platelet count, IPF, PDW and MPV

The platelet count and IPF was initially lower in the patients receiving HD treatment than in the healthy individuals. During treatment, by time 150 minutes, there is a statistically significant decrease (student t-test for paired data, $p < 0.001$) in the platelet count amounting to a mean drop of 8% of the initial PLT count at $t = 0$. Immediately after starting extra-corporal blood circulation ($t = 1$ minute), a statistically significant decrease of IPF counts is observed amounting to a mean drop of 22% of the initial IPF value at $t = 0$ ($p = 0.019$). PLT counts reveal a smaller decrease amounting to a mean drop of 13% within the first minute. At $t = 150$ minutes a statistically significant decrease in IPF count with a meandrop of 9% of the initial level is demonstrated ($p = 0.031$). PDW and MPV fell by one unit (fL) over the HD treatment period.

2. Platelet morphology

Degranulated platelets with pale staining were present in much higher numbers in patients undergoing HD treatment than in healthy individuals. The uraemic group also showed more degranulated platelets than healthy individuals but not as many as those undergoing HD. Degranulation increases during HD treatment after five minutes but then falls over time until the end of treatment, but always remains high compared to the two other groups (figure 5).

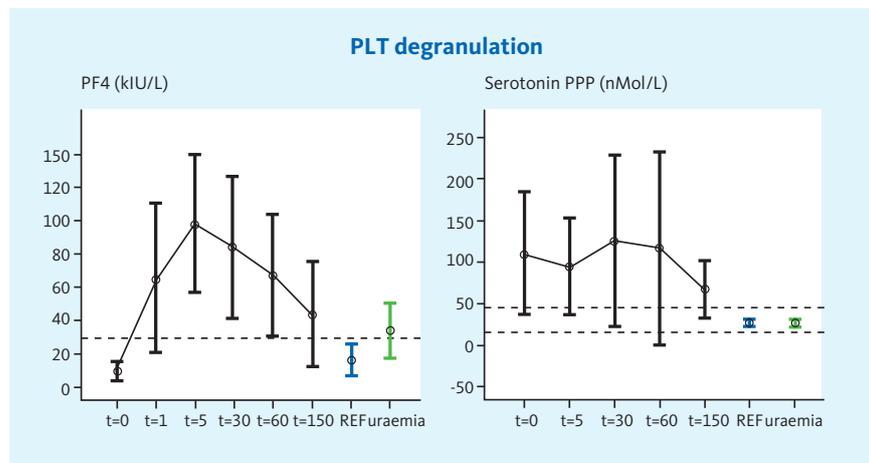


Figure 6 PF4 and serotonin levels before and during the course of HD treatment in patients with ESRF compared to the healthy reference group and patients with uraemia.

3. Platelet activation

In the ESRF patients CD62p is normal before starting treatment but then increases by up to almost two-fold at 30 minutes of treatment before falling back to normal by the end of treatment at 150 minutes. PF4 appears to be normal before HD begins but immediately after starting treatment increases by up to ten fold at five minutes. It then decreases but does not return to normal during the course of treatment, results are shown graphically in figure 6. The reference and uraemic groups of patients have normal PF4 levels. In the ESRF patients serotonin levels are high, even before treatment, since it is not being excreted by the diseased kidneys. There are slight variations in the plasma serotonin levels during treatment (figure 6), but with no significant rise in the levels. This could be because serotonin is a small molecule which may pass through the pores of the dialysis membrane.

Conclusions

Reduced total platelet counts and IPF levels are indicative of suppressed megakaryopoiesis in the bone marrow as a result of long term HD treatment. Results of this study demonstrate a decrease in platelet volume (MPV and PDW) during the course of a HD cycle. This is probably due to the adherence of the immature, more reactive, platelets to the dialyser membrane. Additionally, due to the repeated activation of platelets in the HD subjects, increased numbers of degranulated platelets, with a smaller volume than normal platelets, are seen in the circulation. Patients with a high urea concentration (without ESRF) also show platelets with reduced granule content.

In subjects having HD treatment, suppressed activity of the megakaryocytes is accompanied by reduced platelet volumes due to activation of platelets by contact with the dialysis membrane. The results show there is continuous activation of platelets during haemodialysis. This needs to be monitored as thrombogenicity may result in reduced haemodialysis because the membrane surface cannot function efficiently.

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