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Citation for published version (APA):
Schoorl, M. (2014). Activation of platelets and coagulation during haemodialysis

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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$_2$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 F1+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™ 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 $t = 30 = \frac{Ht_{t = 0}}{Ht_{t = 30}} \times \text{value}_{t = 30}$. 
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 β-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 β-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 β-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 adhesion 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.
REFERENCES


