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

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Summary

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 interact with markers of endothelial integrity during HD treatment. In the current study uraemia and HD induced changes, with particular emphasis on PLT depletion, activation of coagulation and reduced endothelial integrity were investigated.

Methods
To identify PLT depletion, peripheral blood slide smears were screened by light microscopy for qualitative evaluation of PLT granule containing cytoplasm, as indicated by its staining density. Activation of coagulation was investigated by means of thrombin-antithrombin (TAT) and fibrinogen concentrations. To identify endothelial integrity proendothelin (proET-1) 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 PLTs was obviously increased in the subjects’ group with end-stage CKD if compared with the group with renal failure. 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 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 morphology assist in the early detection of changes in the anticoagulant properties of the endothelium to one with procoagulant activity.
Keywords

platelet activation, coagulation, proendothelin, end-stage kidney disease, renal failure

Abbreviations

CKD  Chronic Kidney Disease
ET-1  Endothelin-1
HD  Haemodialysis
PLT  Platelet
TAT  trombin-antitrombin
**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 uremic toxins. Uremic toxins are classified in three groups: water-soluble compounds with low molecular weight, such as urea, middle molecular weight substances and protein-bound uremic toxins. (5) Protein-bound uremic toxins are poorly eliminated by haemodialysis (HD) treatment. Systemic exposure of the endothelium to uremic toxins may lead to activation of the endothelial cells and to features associated with systemic inflammation like hypertension and atherosclerosis. (6) However the mechanisms by which increased uraemia might influence activation of endothelial cells have not been elucidated, uremic toxins are able to act as specific pathogenic agents for inducing endothelial dysfunction. (6,7)

Intact endothelium tissue 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 the endothelial cells resulting in a procoagulant surface. (8) The activated endothelium tissue promotes coagulation. Tissue factor originating from the endothelium plays an important role in the transformation of an anticoagulant surface to a procoagulant surface. (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 haemodialysis (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.\textsuperscript{(11,12,13,14)} Thrombin is involved in the activation of PLTs, neutrophils and monocytes, and acts on endothelium cells in order to release a variety of vasoactive and inflammatory mediators.\textsuperscript{(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 depletion, activation of coagulation and reduced endothelial integrity.
Methods

Patients

A group of 20 subjects with end-stage CKD (age 28-82 years) from the dialysis 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). Low flux polysulfone® F8 dialyser membranes (Fresenius Medical Care, Bad Homburg, Germany) were applied. 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 3500 IU). 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 ethics committee. Written informed consent was obtained from participants.

For the purpose of comparison and clinical interpretation with regard to the pathophysiological effect of uremia, additional investigations are 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
Blood samples from HD subjects were drawn from the arterial line before starting a HD session. For establishment of proET-1 levels in plasma, blood samples were collected into K₂EDTA-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 2500g in order to separate plasma from the cellular fraction. Plasma aliquots were stored at –70°C until analysis.

**Analytical methods**

**PLT morphology**

Peripheral blood slide smears were prepared in duplicate for evaluation of PLT morphology aberrations. 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 the presence of PLT aggregates and qualitative evaluation of morphological PLT aberrations with application of a CellaVision™ DM96 analyzer (CellaVision AB, Lund, Sweden). As previously established, a staining density >75% of the granule containing cytoplasm in >50% of PLTs is considered to be the lower limit of the reference range.(15) Granule depleted PLTs are 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.(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 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**

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 calculated by application of the 2-tailed Student t-test for unpaired data. A p-value < 0.05 was considered to be statistically significant.
Results

Mean results of 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 1-3. Plasma concentrations of proET-1 are demonstrated in Figure 1. Results for parameters reflecting aberrations in PLT morphology 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. ProET-1 concentrations in the subjects with end-stage CKD were statistically significant increased in comparison with subjects with renal insufficiency (207 ± 23 pMol/L, *p*=0.017).

ProET-1 results for both groups were obviously increased in comparison with the reference group of apparently healthy subjects (37 ± 22 pMol/L, *p*=0.000) (Figure 1). In 45% of the subjects with end-stage CKD proET-1 concentrations were above 250 pMol/L.

*PLT morphology*

Microscopic evaluation of stained blood slides did not reveal PLT aggregates. 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 PLTs yielded >75% staining density. Subjects with renal failure showed also a marked decrease of the PLTs with appropriate staining density (43±12%, *p*=0.000 compared with end-stage CKD)

At the contrary, in the group of reference subjects 70 ± 12% of the PLTs are established to reveal appropriate staining density (*p*=0.000).

PLTs with a staining density amounting to <25% of the granule containing cytoplasm are classified as depleted. In the subjects' group with end-stage CKD 46±11% of the PLTs reveal
depleted granule staining (Figure 2). In the subjects’ group with renal failure 25±6% of the PLTs reveal depleted granule staining (p=0.000, compared with end-stage CKD). In the reference subjects’ group only 9±6% of the PLTs reveal depleted granule staining (p= 0.000).

TAT, fibrinogen

TAT plasma concentrations and fibrinogen concentrations in the group with end-stage CKD and renal failure are demonstrated in Figure 3. 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. 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 from the results of the group with end-stage CKD (p=0.014).

Association between proET-1 and modifications in PLT morphology or markers indicating activation of coagulation

Establishment of the interdependence of proET1 results with additional parameters reflecting aberrations in PLT morphology reveal a negative relationship with PLTs with appropriate staining density r=0.840, p=0.000 (Figure 4).

For results concerning activation of coagulation a similar trend is detected. A statistically significant positive trend 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=0.338 (p=0.016) and r=0.311 (p=0.028) respectively.

Nine patients (45%) of the subjects’ group with end-stage CKD yielded pro-ET1 concentrations exceeding 250 pMol/L. Results for aberrations in PLT morphology in the subjects’ group with pro-ET1 concentrations exceeding 250 pMol/L demonstrated statistically significant deviations if compared with the subjects’ group with proET-1 concentrations below 250 pMol/L (p=0.008), whereas markers for activation of coagulation did not differ.
Discussion

ProET-1 plasma concentrations in subjects with end-stage CKD and renal failure are investigated for a possible association between reduced endothelial integrity and aberrations in PLT morphology or markers indicating activation of coagulation.

Results of the study demonstrate that ProET-1 plasma concentrations were 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.(16,17) 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%. (18) However, on the basis of age related shifts, the increase in proET-1 concentrations in the groups with end-stage CKD and renal failure was obviously higher than expected. ProET-1 plasma concentrations in the subjects’ group with end-stage CKD demonstrated 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.(16) ET-1 concentrations are demonstrated to correlate with blood pressure, suggesting that ET-1 may yield hypertension.(19) Endothelial dysfunction is considered to be associated with increased incidence of cardiovascular disease.(16) Together with inflammation, hyperhomocysteinaemia and anaemia, cardiovascular disease yields an additional risk factor in subjects with end-stage CKD.(20)

Moreover, results of our study demonstrated that the amount of depleted PLTs is obviously increased in the subjects’ group with end-stage CKD if compared with the group with renal failure. Concentrations of TAT and fibrinogen revealed results within the reference range.
Subjects with end-stage 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.(21,22,23) During HD treatment, blood constituent are exposed to foreign surfaces within the extracorporeal circuit, including the wall of blood lines, the artificial dialyzer membrane and mechanical forces of the roller pump. Within the extracorporeal circuit endothelium is lacking and activation of PLTs and biomarkers concerning activation of coagulation are released by mechanical triggers.(24) In order to prevent clotting in the extracorporeal circuit during haemodialysis, a bolus of low molecular weight heparin or unfractionated heparin is supplied just before the start of HD treatment. Despite appropriate anticoagulation treatment, the rather unphysiological conditions within the extracorporeal circuit, amplified by pre-dialysis increased uraemia related factors, induce PLT activation. Increase of PLT activation and procoagulant activity is demonstrated in HD patients.(25,26) PLTs are activated within the ECC, as detected by increase in the expression of CD62p and release of β-TG within the ECC.(24) The inner negatively charged wall of the ECC induces activation of FXII and hence the intrinsic coagulation pathway. Increased concentrations of thrombin-antithrombin complexes as well as prothrombin fragment 1+2 during HD treatment indicate that thrombin has been generated.(12,13,14) Additionally, it has been demonstrated that thrombin is involved in the activation process of platelets, neutrophils, and monocytes, and acts on endothelium in order to release a variety of vasoactive and inflammatory mediators.(9)
Conclusions

Results of our study demonstrate that uraemia is associated with endothelial damage and aberrations in PLT morphology in subjects with CKD. Uraemic toxins, especially protein-bound toxins, are likely pathogenic agents inducing endothelial damage in CKD. (27) In 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 endothelial cells amongst others due to uraemia. Biomarkers like proET-1 and aberrations in PLT morphology assist in the early detection of changes in the anticoagulant properties of the endothelium to one with procoagulant activity. Therefore, the level of proET-1 concentration and the amount of depleted PLTs might be an important link between the degree of reduced endothelial integrity and the severity of CKD. In subjects with end-stage CKD deterioration of integrity of the endothelium and aberrations in PLT morphology are aggravated, because of the repetitive PLT activation in the extracorporeal circuit during HD treatment.
Conflict of interest

The authors have no financial or other competing interests.

Authors' contributions

Marianne Schoorl (MiS) participated in the design of the study, analysis of laboratory parameters for platelet depletion, activation of coagulation and endothelial integrity and data interpretation and has written the current manuscript.

Margreet Schoorl (MgS) participated in the analysis of laboratory parameters for platelet depletion and activation of coagulation and has read and approved the manuscript.

Menso J Nubé (MN) participated in the design of the study, provided intellectual content of hemodialysis and importance of the work, helped with a draft version of the manuscript and has read and approved the manuscript.

Piet CM Bartels (PB) participated in the design of the study and design and data interpretation, provided intellectual content of platelet depletion, activation of coagulation and endothelial integrity and importance of the work, has critically revised the draft versions of the manuscript and gave final approval of the current manuscript.

Acknowledgements

The authors have no acknowledgements to make.
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Legend to Figure 1
Box plots representing pro-ET-1 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 indicate the upper level of the reference range for apparently healthy subjects.

Legend to Figure 2
Box plots representing the percentage of PLTs with <25% (left)) and >75% (right) staining intensity of the granule 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 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 (0) and (*). The horizontal dashed line indicate the upper (left) and lower (left) level of the reference range for apparently healthy subjects.

Legend to Figure 3
Box plots representing TAT (left) and fibrinogen (right) 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 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 values corresponding with
values between 1.5 and 3 times the box length are designated as (0). The horizontal dashed line indicate the upper level of the reference range for apparently healthy subjects.

**Legend to Figure 4**

Relationship between results concerning aberrations in PLT morphology and pro-ET1 concentrations in the groups with end-stage kidney disease (●), renal failure (o) and apparently healthy subjects (■) respectively.
Figure 1:
Figure 2
Figure 4