THE DIFFERENTIAL EFFECTS OF DIALYSIS AND ULTRAFILTRATE FROM INDIVIDUALS WITH CKD, WITH OR WITHOUT DIABETES, ON PLATELET PHOSPHATIDYLSERINE EXTERNALISATION.

Yingjie Wang1, Werner Beck2, Reinhold Deppisch2, Sally M. Marshall1, Nicholas A. Hoenich1 and Michael G. Thompson1.

1Faculty of Medical Sciences, Framlington Place, Newcastle University, Newcastle-Upon-Tyne, United Kingdom, NE2 4HH.

2Gambro Corporate Research, Hechingen, Germany.

Correspondence and Reprint Requests To: Dr. M.G.Thompson

TEL: +44 191 222 8112
FAX: +44 191 222 0723
E-MAIL: m.g.thompson@ncl.ac.uk

Short title: Dialysis and Platelet PS Externalisation.

Keywords: Cell Signalling, Thrombosis, 5-HT2A/C receptors.
ABSTRACT

Individuals with chronic kidney disease (CKD) and/or diabetes mellitus (DM) are at increased risk of cardiovascular events and have elevated externalisation of phosphatidylserine (PS), (which propagates thrombus formation), in a small, sub-population of platelets. The aim of this study was to examine the effect of (i) removing uraemic toxins by hemodialysis on PS externalisation in patients with either CKD or CKD and DM and (ii) ultrafiltrate (UF) from these individuals on PS externalisation in healthy platelets. PS externalisation was quantified by a fluorescence-activated cell sorter using Annexin V in platelet rich plasma. PS externalisation was elevated 3-fold in CKD patients and returned to basal values during 3 hrs hemodialysis. In contrast, it was elevated 5-fold in individuals with CKD and DM and was still 3-fold above control after 3 hrs treatment. UF significantly increased PS externalisation in a small, sub-population of platelets from healthy controls. The effect of UF from individuals with CKD and DM was significantly greater than that from patients with CKD alone and the responses were partially inhibited by the protein kinase Cδ (PKCδ) inhibitor, rottlerin, and the 5-hydroxytryptamine (5-HT)2A/2C receptor antagonist, ritanserin. The data suggest that uremic toxins present in UF mediate PS externalisation in a small, sub-population of platelets, at least in part, via the 5-HT2A/2C receptor and PKCδ and demonstrate that DM further enhances platelet PS externalisation in CKD patients undergoing hemodialysis. This may explain, at least in part, the additional increase in vascular damage observed in CKD patients when DM is present.
INTRODUCTION

Cardiovascular disease, in particular atherothrombosis, is a common cause of death in patients receiving dialysis and co-morbid conditions such as diabetes mellitus (DM) further influence the incidence of vascular events (13, 19, 20). Progression is associated with vascular endothelial cell injury, atherosclerotic plaque fissuring and rupture (33). An early key event in such progression is increased adherence of monocytes to the damaged vascular endothelium resulting in fatty streak/atheroma development with platelets subsequently attaching firmly to the site of the lesion (29).

In the plasma membrane of resting platelets, the phospholipid, phosphatidylserine (PS), resides in the inner leaflet but, upon activation of Scramblase by Protein Kinase C (PKC), PS translocates to the outer leaflet of the plasma membrane (5, 15). It has become increasingly clear that chronically elevated or prolonged exposure of PS on the cell surface both increases vascular damage and results in the formation of a hypercoagulable environment by stimulating adherence of inflammatory cells to the vascular endothelium (e.g. 22) and providing a catalytic surface for assembly of the prothrombinase complex (29) that accelerates the generation of thrombin (32, 49).

Evidence now suggests that classic platelet agonists such as thrombin and ADP elicit PS externalisation in a small, sub-population of these cells (45, 48) and that the size of this population correlates with prothrombinase activity (48). In addition, increased PS externalisation in a sub-population of platelets has been observed in individuals with chronic kidney disease (CKD) and DM (45), although the factor(s) responsible for this response is/are unknown.

Advanced glycation end products (AGE) arise by covalent modification of cellular and plasma proteins and form a series of heterogeneous compounds (21). AGE are substantially increased in individuals with either CKD or DM when compared to the
general population (25, 44) and may contribute to the development and progression of cardiovascular disease in these patient groups (35, 39).

In a recent study, we showed that Human serum albumin-AGE (HSA-AGE), prepared in vitro, elicits PS externalisation in a small, sub-population of human platelets. Moreover, this response was completely blocked by both the 5-hydroxytryptamine (5-HT)$_{2A/2C}$ receptor antagonist, ritanserin, and the PKCδ inhibitor, rottlerin (45).

In order to translate this work towards the in vivo situation, we have now explored firstly, the effect of hemodialysis on the elevated PS externalisation we observed in patients with CKD/DM and secondly, the ability of ultrafiltrate from these individuals to elicit PS externalisation in platelets from healthy controls and the mechanism(s) involved.
METHODS

Materials and Reagents

Annexin V-fluorescein isothiocyanate (FITC) was purchased from Immunotech (Marseille, France) and the FluoroSpheres for FACScan calibration were obtained from DakoCytomation (Glostrup, Denmark). The fluorescence-activated cell-scanner, CD61 – PerCP and the Caspase 3 inhibitor, Z-DEVD-FMK, were purchased from Becton Dickinson (Cowley, Oxford, UK). Rottlerin and Bisindolylmaleimide 1 were from Merck Biosciences Ltd (Beeston, Nottingham, UK). The 5-HT$_{2A2C}$ receptor antagonist, ritanserin, and all general chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK). The low-flux Fresenius polysulfone® hemodialysis membrane was purchased from Fresenius Medical Care (Bad Homburg, Germany) and the ENDOSAFE® LAL Gel Clot Test was from Charles River (Charleston, USA).

Patients

26 stable individuals (age range 32-85 years) who had been receiving low flux conventional hemodialysis using a Fresenius Polysulfone membrane 3 times per week for at least 3 months gave their informed consent to participate in the study. 8 (6 male and 2 female) of these also had Type 2 DM, whilst the remaining 18 (13 male and 5 female) did not.

The effect of a single, low-flux hemodialysis treatment on platelet PS externalization in individuals with either CKD or CKD and DM

Hemodialysis patients at Newcastle undergo three sessions a week for 4 hours at a time. The individuals selected for this study were treated on Monday, Wednesday and
Friday. There was no preference for any particular day when examining the effect of a single treatment.

A 2.5 ml blood sample was withdrawn pre-dialysis and then 1, 2 and 3 hours after the commencement of hemodialysis from patients with either CKD or CKD and DM using a standard 16G hypodermic needle. This was then immediately added to a tube containing acid citrate dextrose (ACD) and centrifuged at 100g for 15 minutes at 20°C (Beckman J6-MC). The upper phase containing the Platelet Rich Plasma (PRP) was removed and the extent of PS externalisation was determined as described below.

The effect of three low-flux hemodialysis treatments during a seven day period on platelet PS externalisation in individuals with either CKD or CKD and DM

Pre- and 3 hours post-dialysis blood samples (2.5 ml) were withdrawn on Monday, Wednesday and Friday from patients with either CKD or CKD and DM using a standard 16G hypodermic needle. PRP was then prepared as above and the extent of PS externalisation was determined as described below.

Preparation of PRP from healthy control subjects

Blood was obtained from 6 healthy control subjects (2 male and 4 female, age range 28-52 years) who had no history of cardiovascular disease or hypertension and were not using any medication. PRP was then prepared as above and subsequently incubated with ultrafiltrate under various treatment conditions as described below.

Collection of Ultrafiltrate (UF) from individuals with either CKD or CKD and DM

Studies using urea as a candidate molecule to understand solute kinetics in hemodialysis have revealed that it is rapidly removed during the first 30 minutes, but
that its removal rate then gradually declines throughout the rest of dialysis (e.g. 24). We have therefore utilised two time points, 20 minutes and 180 minutes, to investigate effects on PS externalisation in these two different kinetic phases.

To collect pure ultrafiltrate, the dialysis fluid was disconnected from the dialyser for a short period both 20 minutes (UF20) and 180 minutes (UF180) after the start of treatment. The dialysis fluid pathway was evacuated, ultrafiltrate was allowed to flow for 2-3 minutes and 3 mls of ultrafiltrate was collected into an endotoxin-free tube. The system was then reconnected and the treatment continued.

**Effect of ultrafiltrate from individuals with CKD or CKD and DM on PS externalisation**

PRP was prepared from healthy controls as described above and then incubated with increasing volumes (2.5 – 20 µl) of either UF20 or UF180 from individuals with CKD or CKD and DM at 37°C for 10 minutes. Samples were immediately placed on ice and PS externalisation was determined as described below.

**Effect of ADP on PS externalisation in healthy controls and individuals with CKD or CKD and DM during hemodialysis**

PRP was prepared from individuals with either CKD or CKD and DM at 20 minutes and 180 minutes after the commencement of hemodialysis, or from healthy controls. ADP (100 nM) was then added for 1 minute. The extent of PS externalisation was then determined as described below.
Effect of inhibition of Caspase 3 on PS externalisation mediated by ultrafiltrate from individuals with CKD or CKD and DM

PRP prepared from healthy controls was pre-incubated with 20 µM of the Caspase 3 inhibitor, Z-DEVD-FMK, or its negative control, Z-FAD-FMK (17), for 30 minutes and then 10 µl of either UF20 or UF180 from individuals with CKD or CKD and DM was added for 10 minutes at 37°C. The extent of PS externalisation was determined as described below.

Effect of inhibition of Protein Kinase Cδ on PS externalisation mediated by ultrafiltrate from individuals with CKD or CKD and DM

PRP prepared from healthy controls was pre-incubated with 10 µM of the PKCδ inhibitor, rottlerin, for 5 minutes. This concentration has been reported to block PKCδ activity by approx. 80-90% whilst having no effect on representative examples of either the classical e.g. PKCα or atypical e.g. PKCζ isoforms (15). 10 µl of either UF20 or UF180 from individuals with CKD or CKD and DM was then added for 10 minutes at 37°C. The extent of PS externalisation was determined as described below.

Effect of a 5-HT2A/2C receptor antagonist on PS externalisation mediated by ultrafiltrate from individuals with CKD or CKD and DM

PRP prepared from healthy controls was pre-incubated for 30 minutes with 1 µM of the 5-HT2A/2C receptor antagonist, ritanserin (26). 10 µl of either UF20 or UF180 from individuals with CKD or CKD and DM was then added for 10 minutes at 37°C. The extent of PS externalisation was determined as described below.
Measurement of PS externalisation in PRP

PS externalisation was determined using the cell membrane-impermeant, PS-specific, Annexin V-fluorescein isothiocyanate (FITC) conjugate and CD61 – PerCP as a platelet-specific marker with subsequent quantification by a fluorescence-activated cell-scanner (FACScan) supporting Lysis II software as described previously (45).

Statistical analysis

Statistical analysis was undertaken using Minitab 14 (Minitab Inc, State College, PA, USA). Studies with the Caspase 3 / PKCδ inhibitors and the 5-HT_{2A/2C} receptor antagonist were analysed by use of Student’s t-test and are presented as Means ± s.e.m. where *p<0.05, **p<0.01 and ***p<0.001 with respect to the relevant control. Serial measurements with ultrafiltrate from individuals with CKD or CKD and DM were analysed as summary measures (27), i.e. the areas under the concentration or time curves (AUC). All experiments were performed on at least three occasions.
RESULTS

The analysis of PRP prepared from individuals with CKD or CKD and DM pre-dialysis revealed a sub-population of platelets (approx 3% and 10% of the total population respectively) with increased PS externalisation when compared to healthy controls (Figs. 1 and 2). We have therefore characterised this small population in detail and all subsequent data describing the effects of dialysis relate to this fraction.

The degree of pre-dialysis PS externalisation in platelets derived from individuals with CKD and DM was significantly higher than that from patients with CKD alone (161 ± 1 vs 99 ± 5 x 10^2 total fluorescent binding sites, p<0.001; n=3). Analysis of PS externalisation during low flux (Fresenius Polysulfone) dialysis for 3 hours in individuals with CKD showed that, whilst still slightly elevated after 60 minutes, this parameter had returned to basal values after 2 hours (Figs. 1 and 3). In contrast, PS externalization in the platelet sub-population derived from patients with CKD and DM, whilst significantly reduced, was still well above basal values (78 ± 1 vs 30 ± 1 x 10^2 total fluorescent binding sites, p<0.001; n=3) after 3 hours dialysis (Figs. 2 and 3).

When patients undergo dialysis on Monday, Wednesday and Friday, there is a 2 day interval between treatments Monday – Wednesday and Wednesday – Friday, but a 3 day interval between Friday – Monday. We were therefore interested to establish if the 3 day interval, with its likely greater accumulation of metabolites, resulted in increased PS externalization on a Monday when compared with Wednesday/Friday. Analysis showed that the degree of pre-dialysis PS externalization in patients with CKD or CKD and DM was significantly higher on Monday than the two other days of the week when dialysis took place (Fig. 4). Moreover, in spite of the increased PS externalization seen in CKD patients on a Monday, this had returned to basal values following dialysis treatment (Fig. 4a).
When increasing volumes of ultrafiltrate from individuals with either CKD or CKD and DM were added to PRP from healthy controls, statistically significant (p<0.001 in all cases; n=4) increases in PS externalization in a sub-population of platelets (approx 3% and 5% of the total population respectively), were observed (Fig. 5). In contrast, equivalent material prepared from healthy controls using a 30 kDa cut-off filter had no effect (data not shown). A small, but highly significant stimulation (p<0.001, n=4) was also seen when hemodialysis fluid, which had been used to rinse the dialyser, was added to PRP (Fig. 5). We have therefore characterised this small population in detail and all subsequent data relate to this fraction. Optimal responses were observed with 10 µl of ultrafiltrate (data not shown) and analysis demonstrated that the levels of endotoxin present were below the minimum value (0.03 EU/ml) detectable by the test. The effects of UF20 and UF180 taken from individuals with CKD and DM were significantly greater than the corresponding samples generated by patients with CKD alone and, interestingly, UF180 from both patient groups appeared as potent in eliciting PS externalisation as UF20 (Fig. 5).

At this point in our investigation, it was not clear why, with the CKD data for example, PS externalisation had returned to basal values after 180 minutes dialysis (Figs 1 and 2), yet ultrafiltrate removed at the same time point elicited a substantial increase in PS externalisation when added to PRP from healthy individuals (Fig. 5). In order to address this question, we firstly examined the ability of UF20 and UF180 from individuals with CKD to elicit PS externalisation on PRP from both healthy controls and that taken from patients with CKD after 180 minutes hemodialysis. Whilst UF20 and UF180 elicited substantial increases in PS externalisation in PRP from healthy controls, they had no effect on that generated from individuals with CKD following 180 minutes dialysis (Fig. 6a). Similar data were obtained when ultrafiltrate and PRP
Dialysis and Platelet PS Externalisation

from patients with CKD and DM were used (Fig. 6b). This insensitivity could be due to either the uremic status per se of the individuals and/or the hemodialysis process which in some way desensitizes platelets to the active component(s) present in ultrafiltrate.

In order to investigate these observations further, we then examined the ability of ADP, a classic physiological activator of human platelets (16), to elicit PS externalisation in PRP from either healthy controls or individuals with CKD or CKD and DM after 20 or 180 minutes hemodialysis. In platelets obtained from patients with CKD after 20 minutes, the magnitude of the response to ADP was reduced (Fig. 7a) compared to healthy controls (although PS externalisation in the untreated sample was still significantly elevated as reported in Fig. 3a). After 180 minutes hemodialysis, platelets from individuals with CKD were unresponsive to ADP (Fig 7a). Platelets obtained from patients with CKD and DM were unresponsive to ADP at both time points although, as we have shown in Fig 3b, PS externalisation in the untreated samples obtained following both 20 and 180 minutes hemodialysis had not returned to basal values (Fig. 7b). This suggests that hemodialysis results in both (i) the removal of one or more factors able to elicit PS externalisation for at least 180 minutes after the commencement of treatment and (ii) a time-dependent desensitization of the small platelet sub-population to both physiological agonists such as ADP and uremic toxins.

In addition to its role in thrombosis, PS externalisation is also implicated in apoptosis via Caspase 3 – mediated cleavage of PKC δ (11, 30). When PRP from healthy controls was pre-incubated for 30 minutes with the Caspase 3 inhibitor, Z-DVED-FMK (20 µM) and then subsequently challenged for 10 minutes with UF20 or UF180 from individuals with either CKD or CKD and DM, their ability to elicit PS externalisation was unaffected (data not shown) suggesting that the pathway(s)
employed by the active component(s) did not involve Caspase 3. We have previously shown that when used under identical conditions, Z-DVED-FMK completely abolished the effect of ADP (100 nM) added for 1 minute whilst the negative control, Z-FAD-FMK (20 µM), had no effect (45).

The pre-incubation of PRP from healthy controls for 10 minutes with bisindolylmaleimide 1 (10 nM), an inhibitor of both the classical (α, β, γ) and novel (δ, ε) isoforms of PKC (40, 46) resulted in a partial inhibition of the increase in PS externalisation in the small platelet sub-population elicited by the addition of either UF20 or UF180 from individuals with CKD or CKD and DM for 10 minutes (data not shown).

Similarly, pre-incubation of PRP from healthy controls for 5 minutes with the PKCδ inhibitor, rottlerin (10 µM), partially prevented (57-73 % inhibition) the increase in PS externalisation elicited by the addition of either UF20 or UF180 from both patient groups after 10 minutes incubation (Fig. 8) suggesting a role for PKCδ in this response. In all cases, the effects of ultrafiltrate and inhibitor were significantly different from inhibitor alone (p<0.001, n=3).

The pre-incubation of PRP from healthy controls for 30 minutes with ritanserin (1 µM), a 5-HT2A/2C receptor antagonist, also resulted in a partial inhibition (35-73 %) of PS externalisation elicited by 10 minutes incubation with either UF20 or UF180 from both patient groups (Fig. 9). In all cases, the effects of ultrafiltrate and inhibitor were significantly different from inhibitor alone. This suggests that a significant fraction of the effects of ultrafiltrate from individuals with CKD or CKD and DM on PS externalisation in the small sub-population of platelets are mediated via 5-HT2A/2C receptors.
DISCUSSION

Cardiovascular disease, particularly atherosclerosis, is a major cause of morbidity and mortality in patients undergoing current haemodialysis therapies (13, 19, 20). It is clear from our study that, with regard to platelet PS externalisation, there is a distinct difference between the effect of hemodialysis on individuals with CKD and DM when compared to CKD alone. In the former group, PS externalisation in the platelet sub-population had essentially reached a nadir after 3 hours hemodialysis at a value well above that observed in healthy controls whilst, in the latter, it had returned to normal values. It remains to be established if the failure of PS externalisation to return to normal values in individuals with co-morbid conditions such as DM plays a role in the increased vascular damage observed in this patient group (13, 19, 20).

A primary cause of vascular disease in individuals undergoing hemodialysis is the inadequate removal of uremic toxins (3) and AGE, which are amongst the best studied of these toxins (42), may contribute to the development and progression of cardiovascular disease in these patients (35, 39). In support of this hypothesis, we have recently provided evidence suggesting that HSA-AGE elicits PS externalisation, a key step in the generation of thrombin (32, 49), in a small, sub-population of human platelets via the 5-HT2A/2C receptor and PKCδ (45).

Our observation that the effects of UF20 / UF180 from both patient groups on PS externalisation could also be partially blocked by the same inhibitors we employed in our previous study suggests that AGE, acting through a similar mechanism, may also play a role in the responses we report here. With regard to AGE in plasma, most are modified amino acid residues of plasma protein, whilst glycation-free adducts normally comprise less than 5% of the total. The latter increase substantially in uraemia e.g. Nε-carboxymethyl-lysine (CML), Nε-carboxyethyl-lysine (CEL), glyoxal-
Dialysis and Platelet PS Externalisation

HI (G-H1), methylglyoxal (MG-H1) and 3-deoxyglucosone (3DG-H) are elevated many fold and, at the end of a 4 hour dialysis session with a polysulfone membrane, these increases in plasma (and ultrafiltrate) concentrations were reversed to varying degrees, but not normalised (1).

When considering the plasma protein content of AGE, most are not eliminated efficiently by hemodialysis e.g.G-H1 residues, CEL, CML and pentosidine, which are elevated 2-, 3- and 4-fold respectively prior to dialysis, did not change during treatment (1). The potential role / identity of glycation-free adducts and plasma protein AGE in the responses we report here requires further investigation.

In addition to AGE, another potential receptor-mediated mechanism which requires consideration involves release of the contents of both platelet-dense granules and α-granules which occurs when blood comes into contact with artificial material during hemodialysis. The factors released include ADP, Serotonin, Platelet-derived growth factor (PDGF), platelet factor-4 (PF4) and β-thromboglobulin (βTG), some of which do not return to basal values until 20 hours after the end of the hemodialysis session (4, 8). It is possible that autocrine / paracrine loops play a role in our observations.

As well as a possible role for receptor-mediated pathways in the stimulation of PS externalisation we report here, other mechanisms also require consideration. Protein-bound uremic solutes (42, 43) such as p-cresol and indoxyl sulphate, at concentrations commonly found in uremia, have been shown to have biological effects (9). The involvement of such agents is worthy of future study.

Perhaps the most interesting possibility involves a direct effect of one or more uremic toxins on the activity of Scramblase and / or Aminophospholipid Translocase (APLT), which opposes the role of Scramblase and is responsible for the inward
translocation of aminophospholipids such as PS (6). There is increasing evidence that oxidative stress is an important complication in hemodialysis (12) and it is now clear that the activity of both Scramblase and APLT can be modified by alterations to critical –SH groups (7, 10) e.g. the activity of Scramblase is enhanced by oxidative modification of one or more sulphhydryl groups and is suppressed by the reducing agent, dithiothreitol (23). In addition to oxidative stress, there is a growing interest in nitrosative stress (14) and recent evidence suggests that this is present in patients undergoing hemodialysis (1, 28) e.g. 3-nitrotyrosine levels in plasma proteins have been reported to be elevated 3-fold prior to treatment (1). Moreover, a very recent investigation has shown that nitrosative stress both activates Scramblase and inhibits APLT resulting in PS externalisation (41). Intriguingly, in preliminary experiments with dithiothreitol, which both reduces disulphides and de-nitrosylates S-nitrosylated proteins in live cells (38), we observed that this agent was able to partially reverse the increased PS externalisation seen in the sub-population of platelets from individuals with CKD (Wang Y and Thompson MG, unpublished observation). Clearly, a possible link between uremic toxins inducing oxidative / nitrosative stress and the effects on PS externalisation we describe in this manuscript requires further examination.

Regardless of the mechanism(s) involved, our data suggest that, in both patient groups, PS externalisation in the small sub-population of platelets either is, or becomes, desensitized to uremic toxins / ADP during a 3 hour hemodialysis session. A number of other studies have also observed decreased platelet activation following hemodialysis (2, 31, 36, 37). For example, individuals with CKD undergoing hemodialysis (with either a cellulose acetate or polysulfone membrane) had a lower percentage of platelets expressing the adhesion molecule, P-Selectin, in response to
ADP (0-1 µM) at the end of dialysis than at the start (2). In contrast, increased platelet activation following hemodialysis has also been reported (18). The mechanism through which PS externalisation becomes desensitized is not yet clear, but a better understanding of these observations may potentially facilitate the development of novel treatments aimed at manipulating platelet responses in pathophysiological states.

We have previously reported that the effect of HSA-AGE on platelet PS externalisation was independent of Caspase 3 activity (45) and demonstrate similar findings here with UF$_{20}$ and UF$_{180}$ from individuals with CKD or CKD and DM. In nucleated cells, these observations correlate with the efficient propagation and control of coagulation and thrombosis (49), rather than a signal for apoptotic cell removal (11). Although recent studies have identified the presence of Caspase activity (including Caspase 3) in human platelets (34, 47), their precise role in these cells remains unclear.

In conclusion, we have demonstrated distinct differences both between (i) the effects of hemodialysis on PS externalisation in a small, sub-population of platelets in individuals with CKD and those with CKD and DM and (ii) responses of healthy platelets to ultrafiltrate derived from these two patient groups. The metabolites / mechanisms involved and their consequences in relation to the increased vascular damage observed in these individuals requires further investigation.
ACKNOWLEDGEMENTS

This work was supported by scientific grants from the HOSPAL Cardiovascular Research Programme and the Northern Counties Kidney Research Fund.
REFERENCES


FIGURE LEGENDS

Fig. 1. The effect of a single, low-flux hemodialysis treatment on PS externalization in a sub-population of human platelets in individuals with CKD.

PRP was prepared from individuals with CKD either pre-dialysis or 1, 2 and 3 hours after the commencement of dialysis. PS externalisation was then assessed using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. For comparison purposes, a healthy control is also shown and data are presented both as (a) a histogram and (b) a dot-plot.

Fig. 2. The effect of a single, low-flux hemodialysis treatment on PS externalization in a sub-population of human platelets in individuals with CKD and DM.

PRP was prepared from individuals with CKD and DM either pre-dialysis or 1, 2 and 3 hours after the commencement of dialysis. PS externalisation was then assessed using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. For comparison purposes, a healthy control is also shown and data are presented both as (a) a histogram and (b) a dot-plot.

Fig. 3. The effect of a single, low-flux hemodialysis treatment on PS externalization in a sub-population of human platelets in individuals with either CKD or CKD and DM.

PRP was prepared from individuals with (a) CKD or (b) CKD and DM either pre-dialysis or 1, 2 and 3 hours after the commencement of dialysis. PS externalisation was then assessed using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. For comparison purposes, a healthy control is also
shown. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of three independent experiments with statistical analysis by Student’s t-test.

**Fig. 4.** The effect of three low-flux hemodialysis treatments during a seven day period on PS externalisation in a sub-population of human platelets in individuals with either CKD or CKD and DM

PRP was prepared from pre- (■) and post-dialysis (□) blood samples withdrawn on Monday, Wednesday and Friday from patients with either (a) CKD or (b) CKD and DM. PS externalisation was then assessed using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of three independent experiments with statistical analysis by Student’s t-test.

**Fig. 5** The effect of ultrafiltrate from individuals with CKD or CKD and DM undergoing low-flux hemodialysis on PS externalisation in a sub-population of human platelets

PRP from healthy controls was incubated for 10 minutes with increasing concentrations (2.5 – 20 µl) of either unused hemodialysis fluid (HF), hemodialysis fluid used to rinse the dialyser (RS) or ultrafiltrate taken at 20 minutes (UF20) or 180 minutes (UF180) from individuals with either CKD or CKD and DM. PS externalisation was determined using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and statistical analysis was determined as AUC (27). The effects of ultrafiltrate vs HF was significant in all instances (p<0.001) and the response elicited
by both UF$_{20}$ ($p<0.001$) and UF$_{180}$ ($p<0.01$) from individuals with CKD and DM was significantly greater than that seen in individuals with CKD alone.

**Fig. 6.** The effect of ultrafiltrate from individuals with CKD or CKD and DM on PS externalisation in a sub-population of human platelets from both healthy controls and individuals with either CKD or CKD and DM after 180 minutes hemodialysis.

PRP from healthy controls (HP) and individuals with either CKD (a) or CKD and DM (b) following 180 minutes hemodialysis (UP$_{180}$) was incubated for 10 minutes with 10 µl of ultrafiltrate taken at 20 minutes (UF$_{20}$) or 180 minutes (UF$_{180}$) from individuals with either CKD (a) or CKD and DM (b). PS externalisation was determined using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of 3 independent experiments with statistical analysis by Student’s t-test.

**Fig. 7.** The effect of ADP on PS externalisation in a sub-population of human platelets from both healthy controls and individuals with either CKD or CKD and DM after 20 or 180 minutes hemodialysis.

PRP from either healthy controls or that obtained from individuals with either CKD (a) or CKD and DM (b) following 20 or 180 minutes hemodialysis were incubated with (■) or without (□) 100 nM ADP for 1 minute. PS externalisation was determined using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of 3 independent experiments with statistical analysis by Student’s t-test.
Fig. 8. Effect of the PKCδ inhibitor, Rottlerin, on PS externalisation in a subpopulation of human platelets in response to ultrafiltrate taken from individuals with either CKD or CKD and DM after 20 or 180 minutes hemodialysis.

PRP from healthy controls was pre-incubated with or without 10 μM PKCδ inhibitor, rottlerin (Rott), for 5 minutes at 37°C. Ultrafiltrate taken from individuals with (a) CKD or (b) CKD and DM after either 20 (UF20) or 180 (UF180) minutes hemodialysis was then added and the incubation continued for a further 10 minutes. PS externalisation was determined using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of 3 independent experiments with statistical analysis by Student’s t-test.

Fig. 9. Effect of the 5-HT2A/2C receptor antagonist, Ritanserin, on PS externalisation in a sub-population of human platelets in response to ultrafiltrate taken from individuals with either CKD or CKD and DM after 20 or 180 minutes hemodialysis.

PRP from healthy controls was pre-incubated with or without 1 μM 5-HT2A/2C receptor antagonist, Ritanserin (Rit), for 30 minutes at 37°C. Ultrafiltrate taken from individuals with (a) CKD or (b) CKD and DM after either 20 (UF20) or 180 (UF180) minutes hemodialysis was then added and the incubation continued for a further 10 minutes. PS externalisation was determined using Annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total fluorescent binding sites and are shown as Mean ± s.e. of 3 independent experiments with statistical analysis by Student’s t-test.
Fig. 2

Pre-dialysis

Dialysis Time

Total Platelet Events

Fluorescence Intensity (Annexin V)

Fluorescence Intensity (CD-61)
Fig. 3

(a) Fluorescent Binding Sites $\times 10^2$

- Control
- Pre-dialysis
- 1hr-dialysis
- 2hr-dialysis
- 3hr-dialysis

(b) Fluorescent Binding Sites $\times 10^2$

- Control
- Pre-dialysis
- 1hr-dialysis
- 2hr-dialysis
- 3hr-dialysis

Significance levels:
- ****: p ≤ 0.01
- *****: p ≤ 0.001
Fig. 5

AUC of Increasing UF Volume-dependent Fluorescent Binding Sites X 10^4

HF, RS, UF20, UF180, UF20, UF180

CKD, CKD&DM
Fig. 8

(a)

Fluorescent Binding Sites X 10^2

Control  Rottlerin  UF20  Rott+UF20  UF180  Rott+UF180

(b)

Fluorescent Binding Sites X 10^2

Control  Rottlerin  UF20  Rott+UF20  UF180  Rott+UF180
Fig. 9

(a) Fluorescent Binding Sites X 10^2

Control Ritanserin UF20 Rit+UF20 UF180 Rit+UF180

(b) Fluorescent Binding Sites X 10^2

Control Ritanserin UF20 Rit+UF20 UF180 Rit+UF180

** Stars indicate significant differences.