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A comparative study on the haemostatic changes in kidney failure patients: Pre- and post- haemodialysis and haemodiafiltration

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ABSTRACT

Background: Individuals with kidney failure have a compromised haemostatic system making them susceptible to both thrombosis and bleeding.
Objectives: Assessment of primary haemostasis in patients treated with either haemodialysis (HD) or haemodiafiltration (HDF) was performed through the measurement of several coagulation-based tests, both pre- and post-dialysis.
Patients/methods: 41 renal failure patients and 40 controls were recruited. Platelet aggregometry, Factor XIII (FXIII), Fibrinogen, Von Willebrand Factor (VWF) and Soluble P-Selectin (sP-Sel) levels were measured.
Results: Maximum platelet aggregation was diminished in renal patients irrespective of aspirin intake. Post-dialysis, platelet function was exacerbated. Pre-dialysis FXIII levels were similar to the healthy cohort and became elevated post-dialysis. This elevation could not be explained by the relative decrease of water by dialysis. Fibrinogen levels were already elevated pre-dialysis and further increased post-dialysis. This elevation was associated with the relative decrease of water by dialysis. VWF levels in males were similar to the healthy cohort and became elevated post-dialysis. This elevation was associated with dialysis-related water loss. VWF antigen and activity in female patients were already elevated pre-dialysis and further increased post-dialysis with the exception of VWF activity in HDF treated female patients. sP-Sel levels were lower than those of the healthy cohort and became similar to the healthy cohort post-dialysis. This elevation could not be explained by the relative decrease of water by dialysis.
Conclusions: Whilst platelet aggregometry was diminished, we noted elevated clotting factors such as fibrinogen, FXIII and VWF with no significant differences between HD and HDF-treated patients.

Lay summary

This article describes how kidney failure interferes with the blood clotting system in an opposing manner, leading to both blood clotting development and bleeding risks. This is supported by a case-control approach where several tests which help identify such risks were performed on 41 kidney failure patients and on 40 individuals without kidney dysfunction. The tests were performed on blood taken before and after dialysis and the intake of blood thinning medication (Aspirin). One particular strong points of this study is that the removal of water by dialysis was also taken into account when comparing the patient and control results. This study helps to confirm risks of blood clotting as a result of diminished function of platelets, that are cell fragments responsible to help with blood clotting. Conversely risks of spontaneous development of blood clots were identified by significantly increased levels of clotting factors that are involved in the formation of blood clots. This research is therefore important as it provides insight on the subject especially to clinicians managing patients that are experiencing the

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aforementioned symptoms.

1. Introduction

Under normal physiological conditions, a haemostatic equilibrium is continually maintained through a delicate balance between the opposing procoagulant and anticoagulant systems [1]. Disruption of this equipoise can lead to either bleeding or thrombosis, depending on which mechanism is affected. In end stage renal disease (ESRD), also known as kidney failure, this haemostatic equilibrium is disrupted [2,3].

Kidney failure is defined as the permanent and final stage of chronic kidney disease where the glomerular filtration rate (GFR) is <15 mL/min/1.73 m^2 [4]. Since some of the roles of the kidneys include removing uraemic toxins, osmoregulation and electrolyte balance, kidney failure is associated with multiple comorbidities and sequelae [5]. These include, changes in pharmacodynamics and pharmacokinetics, anaemia, biochemical alterations, as well as coagulopathy [6]. The association between renal dysfunction and coagulopathy was first mentioned in Giovanni Battista Morgagni's "Opera Omnia" in 1764, yet to date, such haemostatic complications are still not fully understood [7]. This is because the pathogenesis of coagulopathy in kidney failure is multifactorial, with alterations affecting primary haemostasis, the coagulation cascade as well as the fibrinolytic system. This results in kidney failure individuals being at a paradoxical risk of both bleeding and thrombosis [3,8].

Increased incidence of thrombotic events in kidney failure is associated with uraemic toxins such as phenolic acid and guanidinosuccinic acid, which accumulate due to the inability of renal excretion [9,10]. This uraemic *milieu* causes damage to the endothelial cells which results in disruption of their anti-thrombotic properties. Chronic inflammation leads to the release of the procoagulant tissue factor and microparticles from the endothelium, hence making kidney failure individuals prone to thrombotic events [10,11,12]. Additionally, clotting factors including fibrinogen and Von Willebrand Factor (VWF) were found to be elevated in several studies concerning renal failure, and this is also associated with a procoagulant state [13]. Comorbidities in kidney failure are quite prevalent and include diabetes mellitus (DM) and cardiovascular disease (CVD), and these further increase the risk of thrombosis. The latter comorbidity is in fact the most common contributor to both morbidity and mortality in kidney failure. To lessen the risk of thromboembolic events, these patients are often prescribed antiplatelets or anticoagulants [14,15].

Uraemic toxins however have a counter-effect on haemostasis, causing numerous kidney failure patients to experience bleeding events. Uraemic thrombocytopathy is associated with the disturbance and impairment of the three primary haemostatic processes; platelet adhesion, activation, and aggregation [16,17]. Such interferences are related to quantitative and/or qualitative defects of platelet receptors, such as the glycoprotein (GP) Ib receptor which is required for platelets to bind to the endothelium through VWF [18]. Another affected receptor is the GPIIb/IIIa complex, which is responsible for platelet aggregation, as it adjoins adjacent platelets through VWF and fibrinogen [19].

Kidney dialysis is a form of renal replacement therapy and is the current preferred therapeutic method in kidney failure. Dialysis is a selective blood filtration system where uraemic toxins, metabolic waste products and excess water are removed from the body while maintaining electrolytes and blood pressure at safe levels [20,21]. Despite regular dialysis sessions, coagulopathy remains a common complication in kidney failure. This is because dialysis is not only incapable of completely correcting these coagulation disruptions but may also exacerbate the paradoxical risk of bleeding and thrombosis [9]. Thromboembolic and cardiovascular complications are attributed to chronic inflammation, endothelial cell damage, and increased blood viscosity after treatment as a result of dialysis-related water loss [10,22,23]. Conversely, bleeding episodes may be attributed to platelet exhaustion, after being activated by the contact with the extracorporeal surfaces of the dialyser [24].

The aim of this study was tripartite; (i) To determine if haemodialysis (HD) and haemodiafiltration (HDF) have a different effect on primary haemostasis by comparing results between the two dialysis modalities. Coagulation-based tests performed included platelet function testing (platelet aggregometry) and the measurement of several clotting factors, including, fibrinogen, VWF antigen and VWF activity, factor XIII (FXIII), as well as by measuring soluble P-Selectin (sP-Sel). To evaluate both the (ii) long-term and (iii) short-term haemostatic effects of kidney failure treated with HD and HDF. Long-term effects were assessed by comparing the obtained results to controls with preserved kidney function. Short-term effects were assessed by comparing pre-dialysis versus post-dialysis results. The relative decrease in plasma volume due to water loss by dialysis was accounted for. This was done through the formulation of a novel formula which uses changes in albumin levels to compensate for the overestimation of increased factor levels as a result of dialysis related-water loss. This was to determine whether any increase in factor level was due to a true pathophysiological effect or due to water loss by dialysis itself.

2. Materials and methods

2.1. Study subjects

With informed consent and permission from the University Research Ethics Committee (UREC) [FORM V:15062020 7675], a total of 81 participants were recruited between July 2021 and January 2022. This was a pilot study to inform future studies. Forty one participants were kidney failure patients: 21 treated with HD and 20 treated with HDF. All patients were treated with a disposable gamma sterilised α-Polysulfone membrane filter (Diacap® Pro, Braun, Germany) at Mater Dei Hospital (Msida, Malta). A total of 40 individuals with preserved kidney function (eGFR >60 mL/min/1.73 m^2) were recruited as controls: 20 of whom were regularly taking aspirin which was prescribed either as a prophylactic or as part of their cardiac rehab medication. The aspirin control group was included since most kidney failure patients are prescribed this drug. Exclusion criteria for all participants included individuals <18 years, pregnant females, individuals on anticoagulants (other than aspirin), individuals suffering from malignant disease, and severely anaemic patients (Hb: <8 g/dL). Recruited patients and controls were sex matched to reduce bias. For controls, blood samples were collected once by venipuncture whereas for patients, samples were collected from a pre-implanted vascular access before and immediately after the dialysis session. Demographic data such as age and gender as well as medical data such as aspirin intake and past history of thrombosis and/or bleeding were obtained on the study population through a questionnaire. Blood parameters such as haemoglobin levels, platelet count and eGFR were obtained from laboratory results (Table 1).

2.2. Platelet aggregometry

Peripheral blood samples were collected in two 15 mL conical tubes containing buffered sodium citrate concentration 0.9 % (Bio/DataTM Sodium Citrate, Horsham, USA). The tubes were centrifuged at 860 rpm for 10 min using an Eppendorf™ Centrifuge 5810 (Eppendorf AG, Germany) and platelet rich plasma (PRP) was collected. The tubes were re-centrifuged at 2500 rpm for 10 min to obtain platelet poor plasma (PPP) which is required for blanking. Platelet aggregation was performed using the gold standard light transmission aggregometry (PAP-8E Platelet Aggregation Profiler V 2.0, Bio/Data Corp, USA) after respectively stimulating PRP aliquots with different platelet agonists: (Adenosine diphosphate (ADP) final concentration 5 μM; Arachidonic Acid (AA) final concentration 0.75 mM; Collagen (Coll) final concentration; 95 μg/mL, Epinephrine (Epin) final concentration; 5 μM, Ristocetin (Risto) final concentration; 1.2 mg/mL (Bio/Data Corp, USA) and Thrombin (Throm); final concentration 1.25 IU/mL (Siemens

Table 1
Study population demographics (controls and patients).

	Healthy controls n = 20	Aspirin controls n = 20	Haemodialysis patients n = 21	Haemodiafiltration patients n = 20
Males (n, %)	9 (45)	11 (55)	10 (48)	10 (50)
Females (n, %)	11 (55)	9 (45)	11 (52)	10 (50)
Age males (mean, range)	40 (20–73)	64 (50–77)	67 (46–81)	46 (20–73)
Age females (mean, range)	31 (21–47)	64 (31–78)	64 (57–74)	56 (38–71)
eGFR (mL/min/1.73 m2) (mean, range)	104 (85–139)	94 (62–147)	7 (4–13)	5 (4–7)
Haemoglobin males (g/dL) (mean, range)	15.3 (13.5–17.2)	14.1 (12.6–16.2)	11.1 (8.8–12.5)	11.2 (10.4–12.3)
Haemoglobin females (g/dL) (mean, range)	13.5 (12.6–14.3)	13.1 (8.5–14.4)	10.9 (9.2–12.3)	10.9 (8.7–13.6)
Platelet Count ($x10^9$/L) (mean, range)	258 (156–396)	254 (129–383)	242 (114–469)	187 (119–257)
Platelet Mass Index Platelet Count/MPV (mean, range)	2790 (1560–3960)	2728 (1306–4519)	2541 (1402–4409)	1983 (1261–2778)
Aspirin intake (n, %)	0 (0)	20 (100)	19 (95)	16 (80)
Reported thrombotic events (n, %)	0 (0)	0 (0)	3 (14.29)	6 (30)
Reported bleeding episodes (n, %)	0 (0)	0 (0)	1 (4.76)	3 (15)

Healthcare Diagnostrics, Germany). Saline final concentration 0.09 % (Versylene® NaCl Fresenius Kabi, France) was used as a negative control. A curve of percentage aggregation (%) against time (minutes) was automatically constructed by the platelet aggregometer. The maximum aggregation (MA), i.e., the highest aggregation point in the graph was used to assess platelet aggregation.

2.3. Factor assays

Three whole blood samples were collected in evacuated tubes (Vacuette® Tube 2 mL, 9NC Coagulation Sodium Citrate 3.2 %, Greiner Bio- One, Kremsmünster, Austria) and were centrifuged at 2500 rpm for 10 min. The obtained plasma was pooled and was re-centrifuged, aliquoted and frozen at −80 °C. The patient and control samples were then thawed at 37 °C using a water bath (FALC® SB 24, Treviglio, Italy) and analysed accordingly using Sysmex® CS-2500 analyser (Sysmex Corporation, Japan). FXIII activity levels were quantified using the chromogenic method by BIOPHENTM FXIII (HYPHEN BioMed, France). The VWF activity was measured using the INNOVANCE VWF: Ac kit (Siemens Healthcare Diagnostic, Germany) while the VWF antigen was measured using the LIAPHENTM VWF: Ag kit (HYPHEN BioMed, France). Fibrinogen activity was measured using the Clauss method [25] using Siemens Healthcare Diagnostic, Germany reagents.

2.4. Soluble P-selectin

One blood sample was collected in an evacuated tube containing a clotting activator (Vacusera 3.5 mL CAT serum gel and clot activator, Izmir, Turkey) and was centrifuged at 3000 rpm for 10 min. The obtained serum was aliquoted and stored at -80 °C. On the day of analysis, the sera were thawed at room temperature and sP-Sel levels were measured using enzyme linked immunosorbent assay (ELISA) by Invitrogen (ThermoFisher Scientific, Massachusetts, USA).

2.5. Accounting for post-dialysis water volume changes

Water retention is an inevitable consequence of renal failure; therefore, one major role of dialysis is to remove excess water. Since this 'dehydrating' effect is likely to increase the concentration of clotting factors, water volume changes were accounted for using changes in albumin concentration. Albumin was used as the reference parameter because of its large molecular weight of 66.5 kDa, since the sieving coefficient of the Diacap® Pro (Braun, Germany) used in this study was <0.001, with the information-for-use leaflet specifying negligible losses of albumin. Accounting for this water loss was necessary to determine whether post-dialysis factor elevation was due to this water-removal effect or due to a true pathophysiological effect. A formula was established to mathematically account for water losses:

$$Adjusted\ factor = Factor\ post\ dialysis - \left(Factor\ pre\ dialysis - \left(\frac{albumin\ post\ dialysis}{albumin\ pre\ dialysis}\right)\right) x\ Factor\ pre\ dialysis$$

However, to confirm the accuracy of the equation, a proportion of post-dialysis samples were manually adjusted, and the results of the mathematical and manual methods were statistically compared. Manual adjustment involved diluting post-dialysis samples with saline (0.9 %, Versylene® NaCl Fresenius Kabi, France) and re-performing the factor assays. The appropriate adjustment volume of diluent for each post-dialysis sample was calculated using the simple formula $C_1V_1 = C_2V_2$ with 'C' being the concentration of albumin. No difference emerged between the two adjustment methods (mathematical adjustment and the manual adjustment), for all factor assays ($p > 0.05$) (Supplement Table 1). Therefore, the less laborious and more cost-effective mathematical method was used.

2.6. Statistical analysis

Statistical analysis of the data was performed using IBM® SPSS® Statistics software Version 27 (IBM Corporation, Armonk, New York). Outliers in each dataset were identified using boxplots and nonsensical influential outliers were excluded from the data. Distribution of data was assessed using the Shapiro-Wilk test where parametric (mean-based) statistical tests were subsequently used for normally distributed data while non-parametric (median-based) analogues were used for data with abnormal distribution. Comparison of the manually adjusted and mathematically adjusted water-loss results were performed using the paired samples *t*-test. This test or its non-parametric analogue (Wilcoxon signed-rank test) was also used to compare pre- versus post-dialysis results. The independent samples t-test or its non-parametric analogue (Mann-Whitney *U* test) was used to compare the results between two

cohorts, whereas the one-way analysis of variance (ANOVA) or its non-parametric analogue (Kruskal-Wallis test) was used to compare results between three or more cohorts. The homogeneity of variance between two variables was first assessed using the Levene's test. This was done to determine the appropriate statistical value of the Independent samples t-test as well as to determine whether the ANOVA or Kruskal-Wallis test should be used. A cut-off of 0.05 level of significance was used for all statistical analyses.

3. Results

3.1. Study population demographics and outliers

Study population demographics are found in Table 1. Through the obtained data it was found that 9 out of 41 kidney failure patients (21.95 %) reported at least 1 episode of thrombosis and 4 out of 41 (9.76 %) reported at least 1 bleeding episode. None of these patients reported experiencing both events. Influential outliers were removed accordingly.

3.2. Platelet aggregation

3.2.1. Comparison of HD versus HDF

No statistically significant difference emerged between the platelet mass index (Platelet Count x Mean Platelet Volume) of HD and HDF treated patients (pre-dialysis: $p = 0.077$ | post-dialysis: $p = 0.375$). No difference in MA emerged in platelet aggregation between 19 pre-HD and 15 pre-HDF patients and between 18 post-HD and 15 post-HDF patients (taking aspirin) as well as between 2 HD and 4 HDF patients (not on aspirin), both pre- and post-dialysis. Therefore, HD and HDF patients were grouped together and categorised as renal patients taking aspirin (pre-dialysis taking aspirin; $n = 34$ | post-dialysis taking aspirin; $n = 33$) and renal patients (not taking aspirin; $n = 6$). Statistical analysis of these data can be found in Table 2 of the Supplement.

3.2.2. Comparison of dialysis patients versus healthy controls

The mean platelet aggregation against time graph points were computed at 0.5 s intervals for 6 groups: the healthy controls, the aspirin controls, the pre- and post-dialysis renal patients taking aspirin, and the pre- and post-dialysis renal patients not taking aspirin. Six graphs were generated (A - F) which depict the platelet aggregation response after stimulating the platelets with 6 respective agonists (Fig. 1). Since aspirin is known to have a direct effect on platelet function, the platelet aggregometry results of kidney failure patients who were not taking aspirin were compared solely to those of the healthy cohort. On the other hand, the results of kidney failure patients taking aspirin were compared to both healthy controls as well as to aspirin controls. Statistical analysis of these data can be found in Tables 3 and 4 of the Supplement.

Since aspirin is a direct platelet inhibitor, the MA of the aspirin cohort, compared to the healthy cohort, was significantly reduced for ADP, AA, Coll, Epin and Risto ($p < 0.05$) but remained similar for thrombin ($p = 0.211$). This showed that thrombin is impervious to the effects of aspirin. Graphs C and D of Fig. 1 show a significant reduction in MA in kidney failure (pre-dialysis) with thrombin being the only stimulant exceeding the 45 % cut-off. The MA of kidney failure patients not taking aspirin (C) was similar to the MA of kidney failure patients taking aspirin (D) for all agonists except for AA ($p = 0.001$) and Coll ($p = 0.037$), where the MA was more diminished in patients taking aspirin. This shows that the platelet receptors of AA and Coll are strongly affected by aspirin. The similar results obtained for the other agonists (ADP, Epin, Risto and Thrombin) shows that aspirin is not the sole contributor to impaired platelet function in kidney failure. The MA of kidney failure patients, both pre- and post-dialysis was significantly

Table 2
Comparison of HD versus HDF pre- and post-dialysis.

Factor	Time of collection	Dialysis type	Sample size (n)	Mean	Standard deviation	Shapiro-Wilk	Independent samples T-Test or Mann Whitney *U* test	Statistical difference in Aspirin intake	Result interpretation
FXIII (%)	Pre-dialysis	Haemodialysis	20	134.63	24.05	0.001	0.620	No	No Significant difference in FXIII levels between HD and HDF-treated patients (both pre- and post-dialysis)
		Haemodiafiltration	20	149.67	45.04	0.076			
	Post-dialysis	Haemodialysis	20	168.12	42.04	0.231	0.779	No	
		Haemodiafiltration	20	172.26	50.29	0.261			
Fibrinogen Activity (g/L)	Pre-dialysis	Haemodialysis	20	3.88	0.74	0.911	0.293	No	No Significant difference in Fibrinogen levels between HD and HDF-treated patients (both pre- and post-dialysis)
		Haemodiafiltration	20	3.53	1.06	0.154			
	Post-dialysis	Haemodialysis	20	4.16	0.73	0.809	0.506	No	
		Haemodiafiltration	20	3.98	0.99	0.224			
VWF Antigen (Males) (%)	Pre-dialysis	Haemodialysis	10	161.46	53.54	0.792	0.067	No	No Significant difference in VWF Antigen levels between HD and HDF-treated male patients (both pre-and post-dialysis)
		Haemodiafiltration	9	125.08	17.57	0.249			
	Post-dialysis	Haemodialysis	10	199.02	74.99	0.578	0.056	No	
		Haemodiafiltration	9	144.82	28.60	0.752			
VWF Antigen (Females) (%)	Pre-dialysis	Haemodialysis	11	214.67	82.85	0.294	0.123	No	No Significant difference in VWF Antigen levels between HD and HDF-treated female patients (both pre- and post-dialysis)
		Haemodiafiltration	9	157.74	71.90	0.059			
	Post-dialysis	Haemodialysis	11	259.15	84.94	0.986	0.094	No	
		Haemodiafiltration	9	190.02	89.35	0.177			
VWF Activity (Males) (%)	Pre-dialysis	Haemodialysis	10	133.68	42.15	0.689	0.181	No	No Significant difference in VWF Activity levels between HD and HDF-treated male patients (both pre- and post-dialysis)
		Haemodiafiltration	9	113.00	17.69	0.778			
	Post-dialysis	Haemodialysis	10	164.00	55.89	0.921	0.057	No	
		Haemodiafiltration	9	121.88	28.06	0.840			
VWF Activity (Females) (%)	Pre-dialysis	Haemodialysis	11	149.02	41.92	0.037	0.412	No	A Significant difference in VWF Activity levels between HD and HDF-treated female patients (post-dialysis)
		Haemodiafiltration	9	130.83	67.79	0.396			
	Post-dialysis	Haemodialysis	11	188.55	55.45	0.780	**0.038**	Yes	
		Haemodiafiltration	9	128.21	64.83	0.128			
sP-Selectin (ng/mL)	Pre-dialysis	Haemodialysis	13	144.41	28.12	0.239	0.620	No	No Significant difference in sP-Selectin levels between HD and HDF-treated patients (both pre- and post-dialysis)
		Haemodiafiltration	14	138.30	34.47	0.221			
	Post-dialysis	Haemodialysis	13	164.96	25.89	0.546	0.969	No	
		Haemodiafiltration	14	164.34	52.00	0.361			

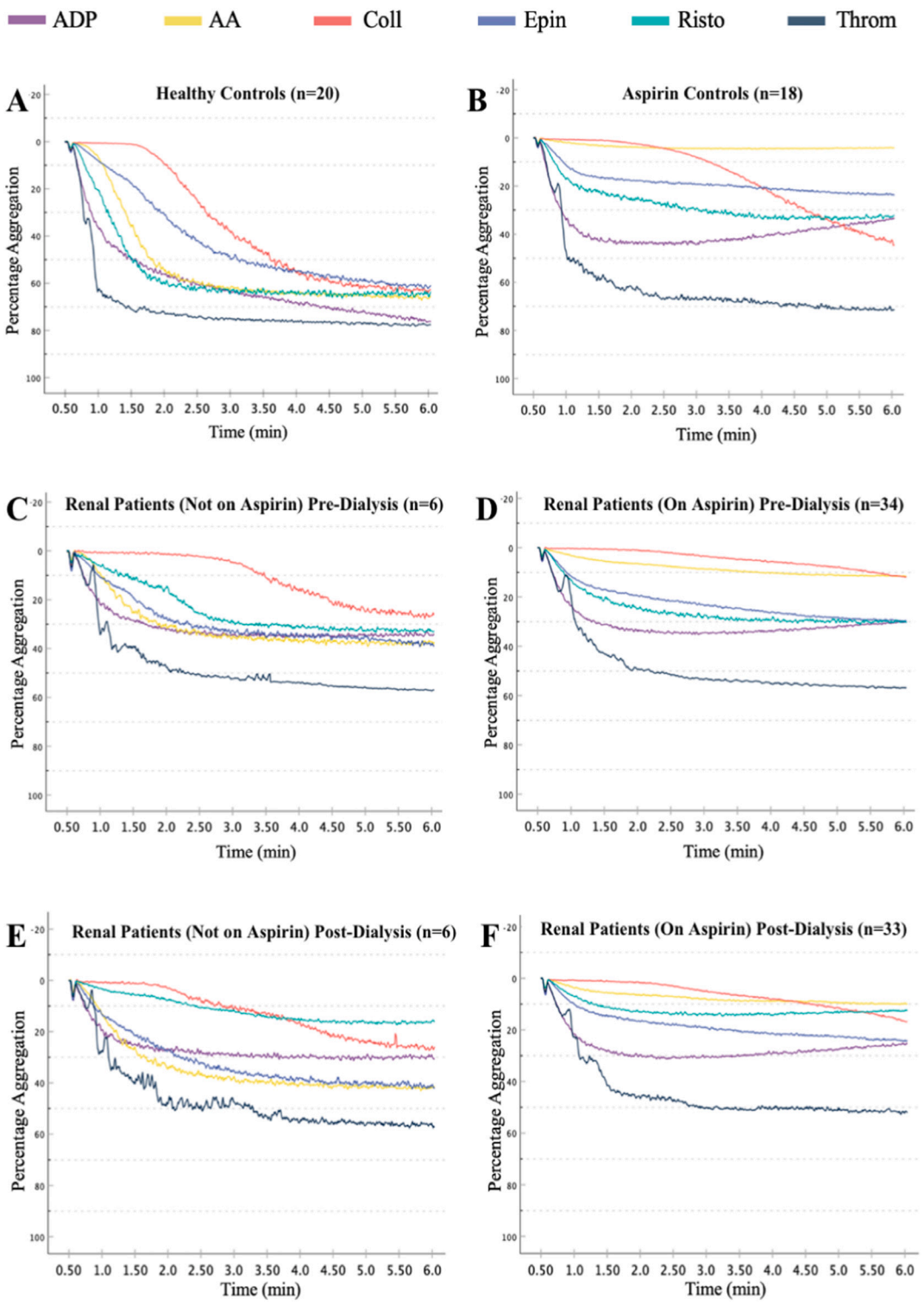


Fig. 1. Platelet aggregation versus time curves.
Graph (A) represents the healthy cohort ($n = 20$) and shows normal platelet aggregation where a negative curve with a maximum aggregation (MA) greater than the 45 % cut-off was obtained for all agonists. Graph (B) shows the MA of the aspirin controls ($n = 18$). Graph C represents the MA of pre-dialysis patients taking aspirin ($n = 34$) while graph D represents the MA of pre-dialysis patients not taking aspirin ($n = 6$). Graph E represents the MA of post-dialysis patients taking aspirin ($n = 33$) while graph F represents the MA of post-dialysis patients not taking aspirin (n = 6).

smaller than that of the healthy controls (A) for all agonists (including thrombin), irrespective of aspirin intake ($p < 0.05$). Furthermore, the MA of kidney failure patients taking aspirin was weaker than that of the aspirin control cohort (B) for all agonists, except for AA and Epin (both pre- and post-dialysis) and for Risto (pre-dialysis). This further suggests that aspirin is not the only platelet aggregation suppressor in kidney failure.

3.2.3. *Comparison of pre- versus post-dialysis*

When comparing the pre- and post-dialysis MA, a significant

suppression in platelet aggregation was seen. The MA was in fact weaker for all agonists with the exception of Collagen, where a small yet significant mean increase of 2.72 % was obtained. Statistical analysis of these data can be found in Table 5 of the Supplement.

3.3. Clotting factors

3.3.1. Comparison of HD versus HDF

As shown in Table 2, no difference in factor levels was found in kidney failure treated with HD and HDF (both pre- and post-dialysis) with the exception of VWF Activity in female patients (post-dialysis) ($p = 0.038$). Therefore, HD and HDF-treated patients were grouped together and categorised as renal patients for statistical analysis on FXIII, fibrinogen, VWF Antigen, VWF Activity (males) and sP-Selectin. Aspirin intake did not seem to affect any of the factor levels (Supplement Table 6).

3.3.2. Comparison of patients versus healthy controls and comparison of pre- versus post-dialysis

Figs. 2–6 below depict box-plots each of which showing 3 important statistical findings on the measured parameters. Part A shows comparison of the results between renal patients (both pre- and post-dialysis) and the healthy controls (long-term assessment) as well as shows comparison of the pre- versus post-dialysis results (short-term assessment). Accounting for water loss is shown in part B of each figure.

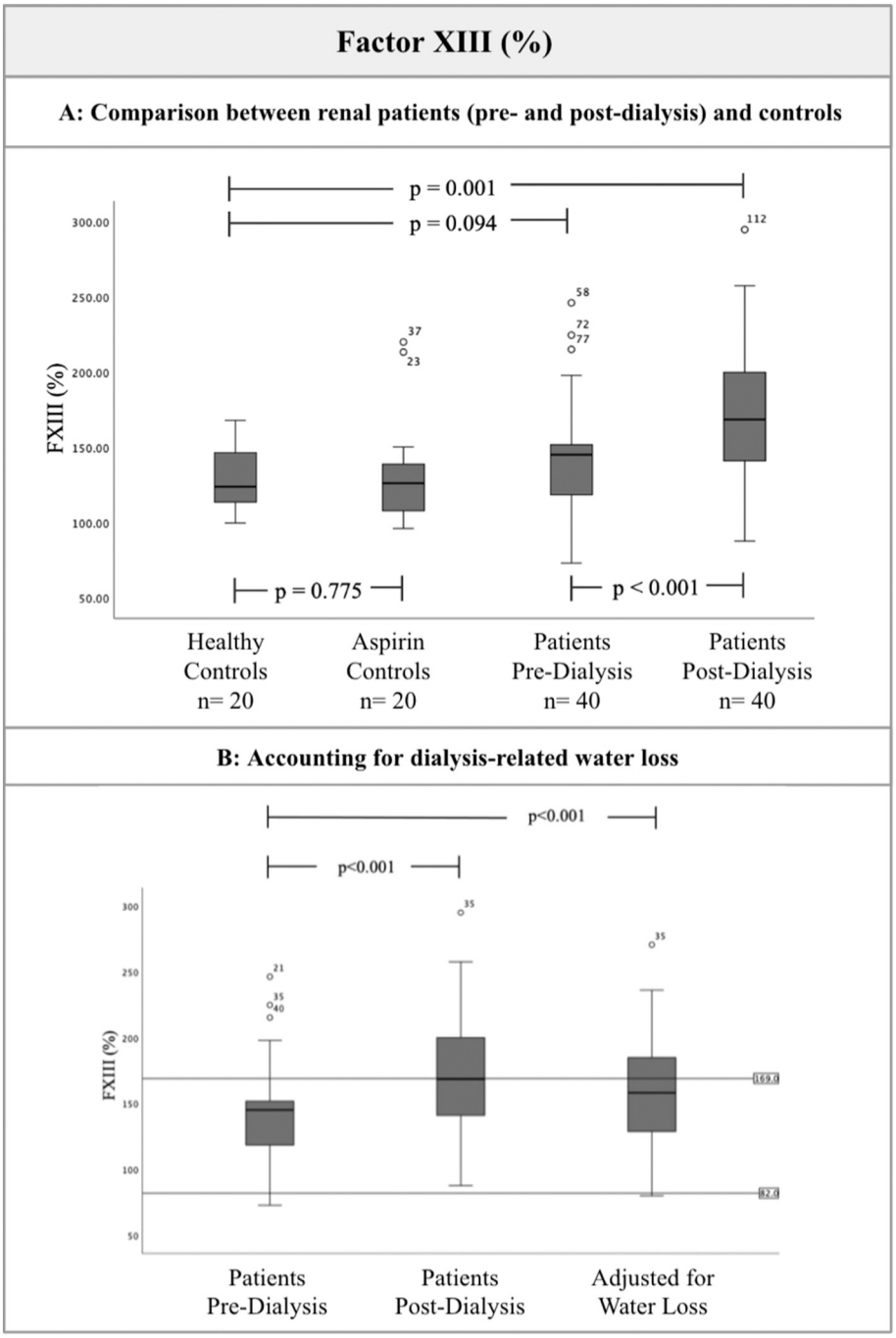


Fig. 2. FXIII levels in pre-dialysis kidney failure patients were similar to those of the healthy cohort ($p = 0.094$; mean pre-dialysis 142.15 %). Post dialysis, a significant elevation was identified ($p = 0.001$; mean post-dialysis 170.19 %) and this remained so after adjustment for the relative decrease in water (Fig. 2).

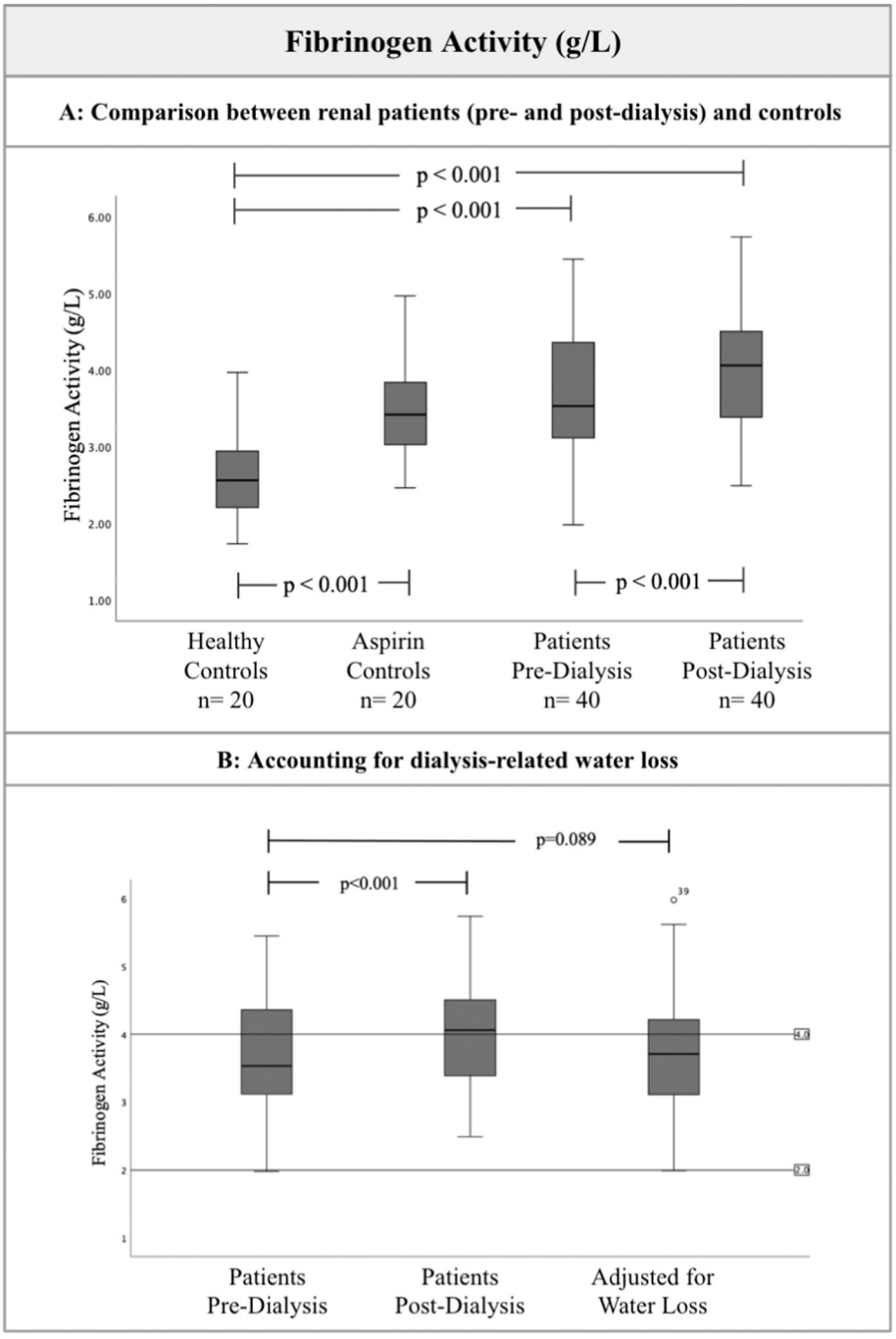


Fig. 3. Pre-dialysis, Fibrinogen activity was greater than that of the healthy cohort ($p < 0.001$; mean pre-dialysis 3.70 g/L). Post-dialysis, fibrinogen activity increased significantly ($p < 0.001$; mean post-dialysis 4.07 g/L) This elevation was however attributed to the relative decrease of water by dialysis.

3.3.3. Function of VWF

To assess whether VWF is dysfunctional in renal failure patients, the VWF Antigen to Activity ratio was calculated for each renal patient. A 0.7 ratio cut-off was used where values lower than 0.7 suggest type 2 Von Willebrand disease [26]. Pre-dialysis, 2 male patients and 6 female patients treated with HD were found to have ratios below the 0.7 cut-off. Post-HD, the ratios remained lower than the cut-off. Pre-dialysis, only 2 female patients treated with HDF were found to have ratios below the cut-off, however post-HDF, the ratios of 3 male patients and an additional 3 female patients became lower than the cut-off. This suggested that HDF itself may increase the risk of acquired type 2 VWF disease.

4. Discussion

The aim of this study was threefold: to determine if the two dialysis modalities (HD and HDF) have a different effect on the haemostatic system of kidney failure patients. Secondly, to assess the long-term effects of kidney failure with respect to primary haemostasis by comparing the results of dialysis patients to individuals with preserved kidney function. Finally, to assess the short-term effects of dialysis-treated kidney failure by comparing pre-dialysis versus post-dialysis results, whilst taking dialysis-related water loss into account. The tests performed in this study are important in primary haemostasis and included platelet function testing (PFT) and measurement of the coagulation components: Fibrinogen, FXIII, VWF and sP-Selectin. These components

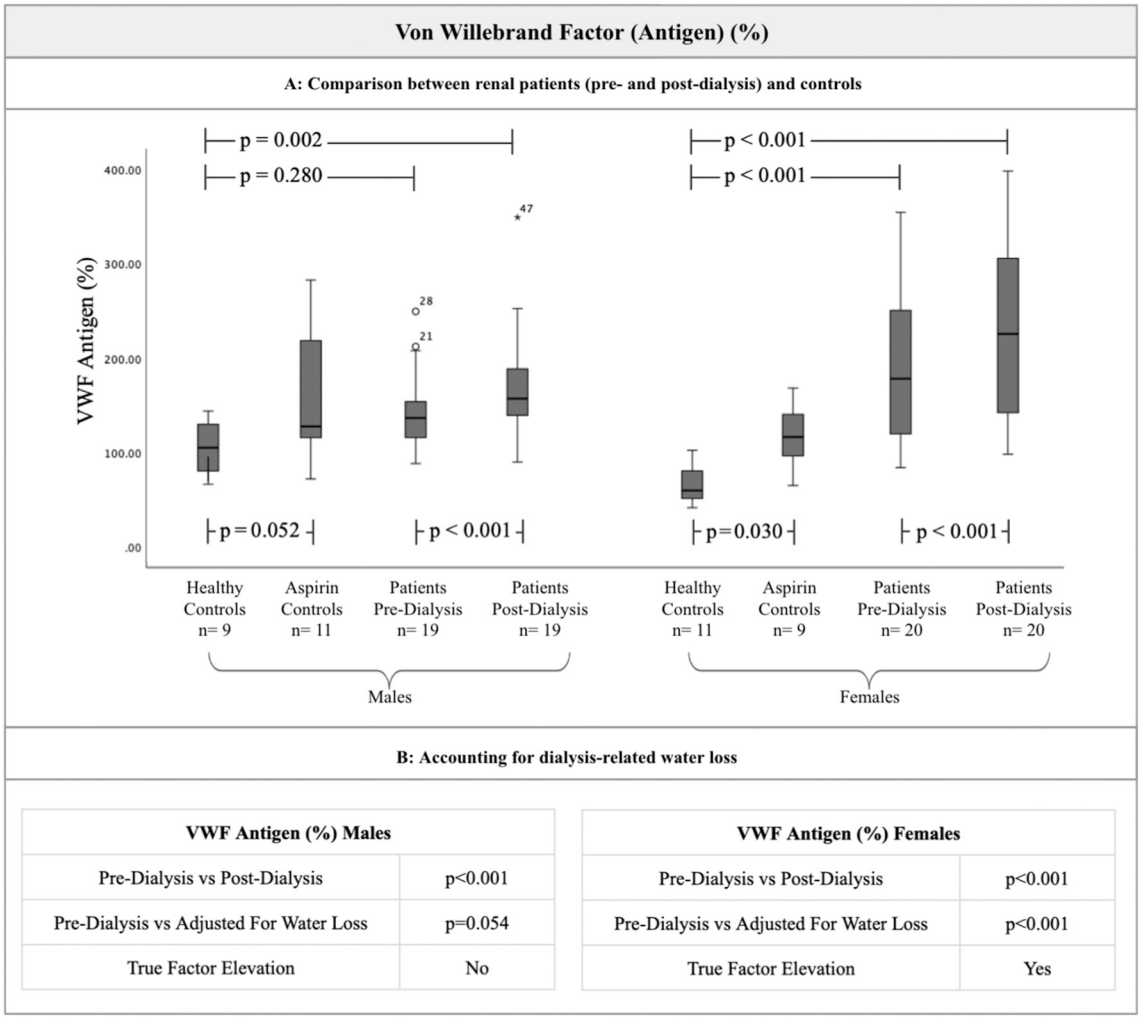


Fig. 4. Pre-dialysis, VWF Antigen (Ag) of male patients was similar to that of the male control cohort ($p = 0.238$; mean male healthy controls 107.10 %, mean male pre-dialysis 144.23 %) whilst the VWF Ag of female patients was greater than that of the female healthy cohort ($p < 0.001$; mean female healthy controls 66.13 %, mean female pre-dialysis 189.06 %). Post dialysis, a significant increase in VWF Ag as compared to the healthy controls was seen in both genders (males $p = 0.002$; mean males post-dialysis 173.35 % | females $p < 0.001$; mean females post-dialysis 228.04 %). The post-dialysis VWF Ag level increase was attributed to post-dialysis water loss, whereas for females, this elevation was due a true physiological effect.

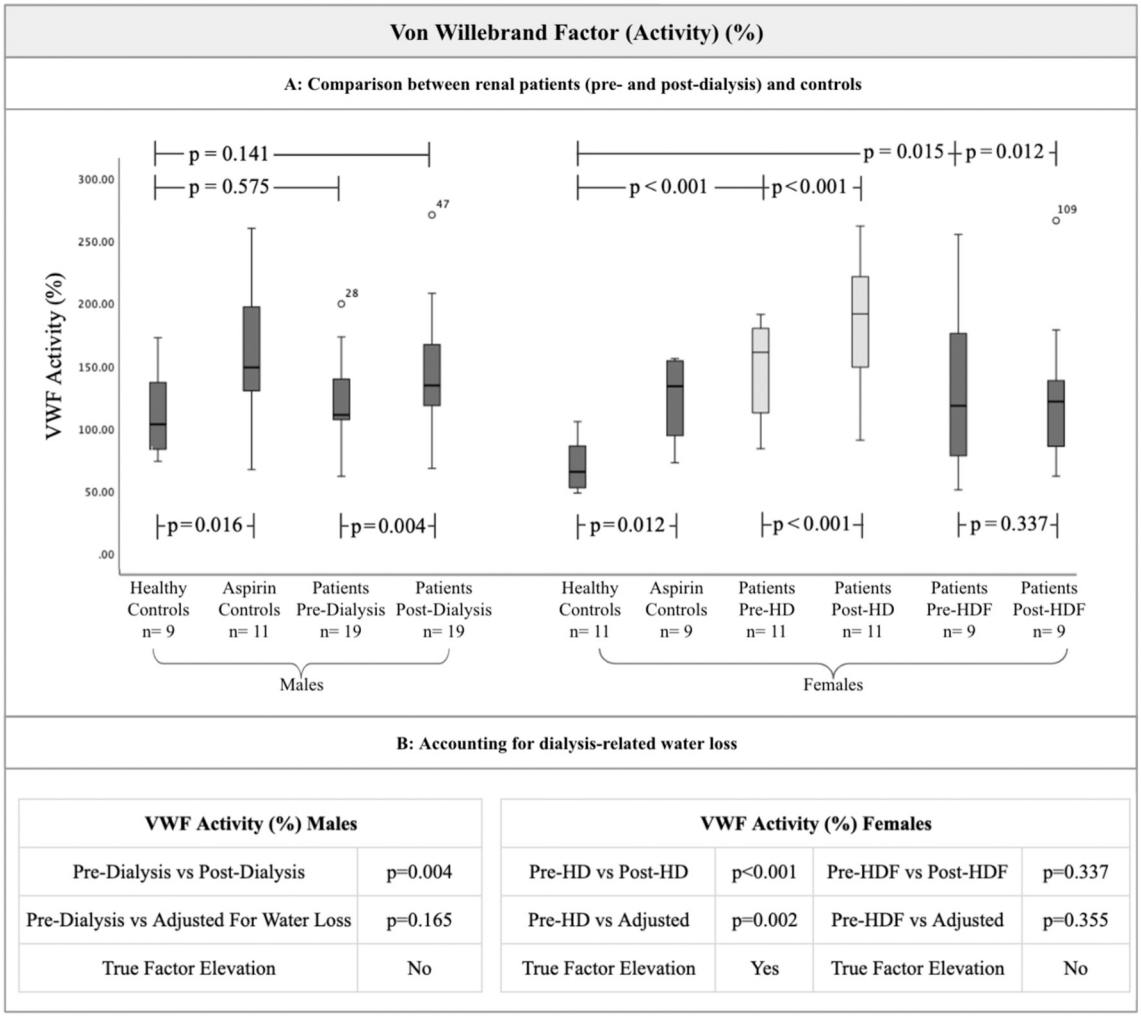
are not only found in blood plasma but a proportion is also stored in platelet alpha granules, and are released upon platelet activation [27]. FXIII is mostly found within the platelet cytoplasm and is externalised to the platelet cell membrane upon activation [28,29].

Platelet surface receptors include GPIb, GPIIb/IIIa, GPVI, P2Y12, PAR 1 and PAR 4, and GPIb-IX-V complex [30,31]. These receptors mediate platelet activation, adhesion, and aggregation. Aspirin hinders platelet function by inhibiting thromboxane A2 generation via COX-1 [32]. Platelet receptor function was assessed by light transmission aggregometry using specific agonists that are specific to platelet receptors: ADP (P2Y1, P2Y12), Epin (endogenous ADP release), AA (GPIIb/IIIa), Coll (GPIa/IIa, GPVI), Risto (GPIb-IX-V), and thrombin (PAR 1, PAR 4) [33,34].

Aspirin's effect on platelet function was assessed by comparing healthy and aspirin control cohorts. Aggregation was diminished for all agonists except thrombin, indicating thrombin's resistance to aspirin. In renal patients not taking aspirin, aggregation was significantly reduced for all agonists compared to healthy controls, including thrombin (Supplement Table 3). This suggests that uraemic thrombocytopathy, not just aspirin, impairs platelet function in renal patients. Possible causes include poor receptor expression, storage pool defects, and fibrinogen degradation products interfering with GPIIb/IIIa binding [35,36]. A study by Zwaginga et al. (1991) [37] found hindered platelet function in uremic plasma which was not improved after adding washed uraemic platelets into normal plasma. This indicated that the defect was within the platelets themselves, likely due to uremic toxins or changes in platelet membrane glycoproteins.

Our results corroborate those of Malyszko, Malyszko et al. (2001) [38] who found the MA of 24 HD treated patients to be lower than that of healthy controls when induced by AA, Coll, ADP and Risto. An earlier study by Di Minno, Martinez et al. (1985) [16] also found decreased platelet sensitivity towards Epinephrine and Collagen. In contrast, Sabovic, Salobir et al. (2008) [39] found no significant difference in ADP induced aggregation of HD treated patients when compared to healthy controls. Two more recent studies evaluated platelet function using closure-time based PFT; Gäckler, Rohn et al. (2019) [8] and Pavlou, Georgatzakou et al. (2021) [40]. The closure times induced by Coll-Epin and Coll-ADP in both studies were found to be prolonged in renal patients when compared to healthy controls, however, statistical significance was only reached in one of these studies [40].

When comparing the MA results of renal patients not taking aspirin to those taking aspirin, a few differences emerged. The MA of renal patients taking aspirin was lower than that of those not taking aspirin when induced by AA (pre-dialysis $p < 0.001$; post-dialysis $p = 0.003$),



B: Accounting for dialysis-related water loss

VWF Activity (%) Males	
Pre-Dialysis vs Post-Dialysis	p=0.004
Pre-Dialysis vs Adjusted For Water Loss	p=0.165
True Factor Elevation	No

VWF Activity (%) Females			
Pre-HD vs Post-HD	p<0.001	Pre-HDF vs Post-HDF	p=0.337
Pre-HD vs Adjusted	p=0.002	Pre-HDF vs Adjusted	p=0.355
True Factor Elevation	Yes	True Factor Elevation	No

Fig. 5. Pre-dialysis, VWF Activity (Act) of male patients was similar to that of the male control cohort ($p = 0.575$; mean male healthy controls 114.27 %, mean male pre-dialysis 123.88 %) whilst the VWF Act of female patients was greater than that of the female control cohort; mean 71.36 % (HD $p < 0.001$; mean females pre-HD 149.02 % | HDF $p = 0.015$; mean females pre-HDF 114.96 %). Post-dialysis an increase in the VWF Act of male patients was seen; mean 144.05 %, however this did not reach a level of statistical significance ($p = 0.141$). In HD treated females, a significant elevation in VWF Act was seen ($p < 0.001$; mean females post-HD 188.55 %) which could not be explained by dialysis-related water loss ($p = 0.002$). On the other hand, in HDF treated females, no statistically significant difference was obtained when comparing pre- and post-dialysis results ($p = 0.337$; mean females post-HDF 110.58 %).

Coll (pre-dialysis $p = 0.037$) and Epin (post-dialysis $p = 0.047$). No other significant differences emerged, again suggesting that aspirin is not the sole contributor to diminished platelet function in these patients.

Comparing AA and Epin-induced MA in renal patients on aspirin to the aspirin control group showed no significant difference, indicating strong aspirin effect. Pre-dialysis, Risto-induced aggregation was similar to controls ($p = 0.608$), but significantly reduced post-dialysis ($p = 0.001$). ADP, Coll, and thrombin-induced MA were significantly weaker in renal patients than in the aspirin control cohort ($p < 0.001$). This again indicated that impaired platelet function in t is not solely due to aspirin intake, especially since thrombin induced MA was also diminished in spite of its resistance towards aspirin. Pluta et al. (2020) [40] also found diminished thrombin-induced MA in uraemic patients, attributed to GPIIb/IIIa defects.

Comparing pre- and post-dialysis MA in renal patients showed a 2.72 % increase post-dialysis with Coll ($p = 0.026$), but significant reductions with AA, ADP, Risto, Epin, and Throm ($p < 0.05$), indicating higher bleeding risk. This partially aligns with van Bladel et al. (2012) [36], who found impaired platelet reactivity in renal patients but found no pre- and post-dialysis differences in ADP and Throm aggregation. Our results also align partially with Pavlou et al. (2021) [40] and Chen et al. (2022) [41], who reported diminished aggregation with Coll-Epin and Coll-ADP in HD patients. Pavlou et al. (2021) [40] found no pre- and post-dialysis difference, while Chen et al. (2022) [41] noted prolonged Coll-Epin closure times in Low Flux HD patients.

Pre-dialysis fibrinogen levels were significantly higher than those of the healthy controls, likely due to a pro-inflammatory state from uraemic toxicity and other comorbidities. Post-dialysis, fibrinogen levels increased further, however this increase was attributed to the relative decrease of water by dialysis. Brophy et al. (2014) [42] and Pénzes et al. (2020) [13] also reported elevated pre-dialysis fibrinogen levels and significant post-dialysis increases due to water loss. High fibrinogen levels are linked to a prothrombotic state and cardiovascular complications [43].

Pre-dialysis FXIII levels were similar to those of the healthy controls, but post-dialysis FXIII levels rose significantly. This elevation could not be explained by the relative decrease of water with dialysis but was associated with a true physiological effect. Pénzes et al. (2020) [13] and Pavlou et al. (2021) [40] reported higher pre-dialysis FXIII levels as well as a significant post-dialysis increase that could not be explained by water loss. This could be hypothesised by the release of FXIII from platelet cell membrane after platelets become activated and possibly

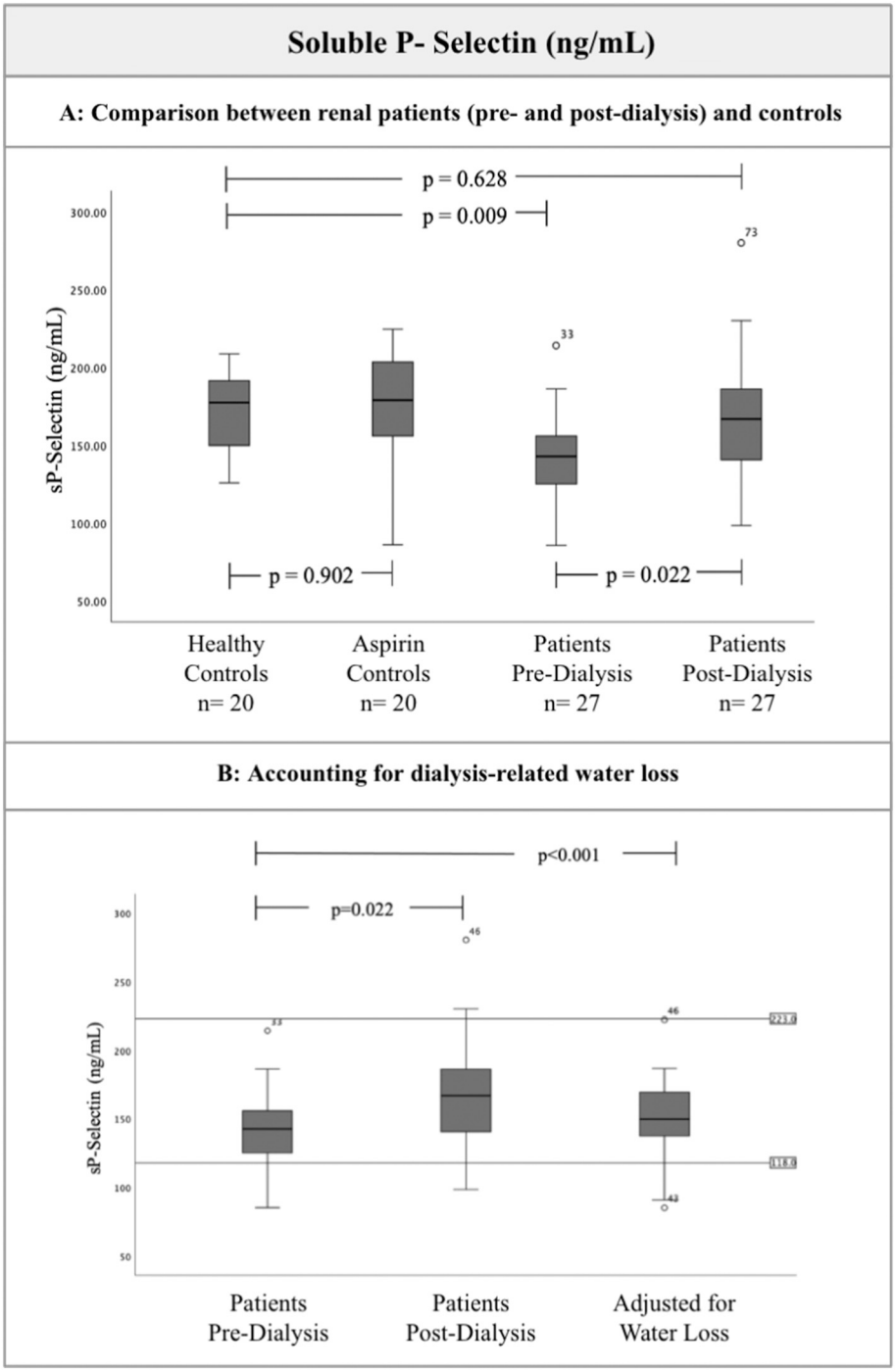


Fig. 6. Pre-dialysis, sP-Sel levels were lower than those of the healthy cohort ($p = 0.009$; mean healthy controls 170.54 ng/mL, mean pre-dialysis 141.24 ng/mL). Post-dialysis, sP-Sel levels increased significantly and the results became similar to those of the healthy cohort ($p = 0.628$; mean post-dialysis 164.63 ng/mL). This elevation persisted after accounting for the relative decrease of water by dialysis ($p < 0.001$). This confirmed that the elevation was due to a physiological effect.

disintegrated during the dialysis process as a result of platelet interaction with the extracorporeal surface of the dialyser [28,44]. It is not yet clear whether elevated FXIII levels pose a pro-thrombotic risk; however activated FXIII promotes clot stabilisation and prevents clot dissolution by crosslinking α2-antiplasmin to fibrin [28].

sP-Selectin (sP-Sel), stored in endothelial Weibel–Palade bodies and platelet α-granules, promotes proinflammatory and prothrombotic responses upon dimerization, making it a useful biomarker for platelet activation [45]. Pre-dialysis, renal patients had significantly lower sP-Sel levels than healthy controls. Post-dialysis, sP-Sel levels increased significantly but remained below healthy control levels. This elevation could not be explained by the relative decrease in plasma water. A possible explanation for this increase is the release of sP-Sel from alpha-granules after platelet activation during treatment. Brophy et al. (2014) [42] found higher pre-dialysis sP-Sel levels using ELISA, but did not measure post-dialysis levels. Becs et al. (2016) [46] reported similar pre-dialysis levels to controls and a significant post-dialysis increase.

VWF plays a crucial role in haemostasis by carrying FVIII, protecting it from cleavage by Protein C, and mediating platelet adhesion and aggregation by binding to GPIb and GPIIb/IIIa complexes [47]. Elevated VWF levels are associated with a prothrombotic state, contributing to thromboinflammatory diseases and increasing the risk of venous

thromboembolism [48].

In this study, VWF antigen levels showed no significant variation between HD and HDF modalities but varied by sex. Pre-dialysis, male patients had VWF antigen and activity levels comparable to healthy males, and a significant post-dialysis increase attributed to a relative decrease in water volume was found. Female patients exhibited three-fold higher pre-dialysis VWF antigen levels than healthy females, with a further significant post-dialysis increase unexplained by water volume changes. Post-dialysis, VWF activity in female patients varied by modality, with HD showing significantly higher activity levels than HDF (Mean VWF activity in HD-treated females: 188.55 %; HDF-treated females: 110.58 %, $p = 0.007$).

Pre-dialysis, both HD and HDF treated female patients had elevated VWF activity levels compared to healthy females. Post-HDF, VWF activity showed a slight (4 %) decrease, not significantly different from pre-HDF levels. In contrast, a significant post-HD elevation in VWF activity was found suggesting a true physiological effect such as enhanced inflammation and VWF release from platelet granules during dialysis. Studies by Brophy et al. (1991) [42] and Pavlou et al. (2021) [40] found elevated pre-HD VWF activity, with Pavlou et al. (2021) [40] also observing a significant post-dialysis increase, similar to our findings in HD-treated female and male patients.

A study by Zwaginga et al. (1990), [49] explored platelet adhesion in uremic patients and found that increased VWF levels might help compensate for adhesion defects. In our study, ristocetin-induced aggregation did not improve post-dialysis, despite a significant increase in VWF antigen and activity. This suggested that elevated VWF levels do not fully compensate for impaired platelet aggregation, possibly due to premature platelet activation during dialysis leading to platelet exhaustion. Additionally, some renal patients showed dysfunctional VWF, with VWF antigen to activity ratios below 0.7, indicating a possible type 2 acquired von Willebrand disease.

A limitation of this study is the lack of measurement of ADAMTS-13 activity and inhibition, which would provide further insight into VWF behaviour in uremia, as ADAMTS-13 is a key regulator of VWF function (Pavlou et al., 2021) [40].

Another limitation in this study was the lack of evaluation of the pathophysiology of uraemic thrombocytopathy. In this study, we evaluated several haemostatic parameters and managed to show the paradoxical risk of bleeding and thrombosis in these patients and provided possible explanations on the cause of factor elevation and the paradoxical cause of impaired platelet function after treatment. However, we did not provide evidence on the quantity and/or quality of platelet granules and platelet GPIb, GPIIb/IIIa and PAR receptors. In vitro experiments that assess platelet receptors in uraemia were performed during the late 1980s and to our knowledge no other studies have been performed beyond the early 2000s. Therefore, we suggest future studies to re-assess these receptors and granules and correlate them with uraemic toxins and interfering factors using more advanced technology.

Nonetheless, it is also important to consider the possibility that defective pre-dialysis platelet function may be linked to the effects of the dialysis treatment itself. Platelets possess a lifespan of approximately 10 days, whereas the necessity for dialysis is approximately 3 days. This hence limits the crucial time-frame for platelet regeneration, resulting in defective pre-dialysis platelet assessment. Therefore, a more accurate assessment of the pathophysiology of uraemic thrombocytopathy may be obtained by evaluating platelet function in chronic kidney disease patients in the pre-treatment phase, right before commencing the first dialysis session.

Another limitation is the small sample size of kidney failure patients not taking aspirin. This can be mitigated by increasing the number of recruited participants, albeit most of these patients are prescribed this anti-platelet drug to prevent vascular access thrombosis.

5. Conclusion

In this study we provided insight on the haemostatic dysregulation between the procoagulant and anticoagulant mechanisms in uraemic patients treated with dialysis. Our findings demonstrate that renal patients exhibit markedly impaired platelet aggregation, which is not solely attributed to aspirin intake but is also indicative of uraemic thrombocytopathy. Furthermore, we observed that dialysis itself exacerbates haemostatic disturbances, increasing bleeding risks, particularly post-dialysis when platelet aggregation is notably reduced. Post-dialysis elevation of fibrinogen, FXIII and VWF levels suggest heightened thrombogenicity complicating clinical management in patients treated with either HD or HDF. These findings align with previous research and highlights the necessity for revising anticoagulant strategies during dialysis. Furthermore, future research should delve into the mechanisms behind these haemostatic alterations to provide deeper insights into how uraemia and dialysis disrupt this haemostatic balance. Regular assessment of the haemostatic status of renal patients should be considered to facilitate tailored therapeutic interventions, enhancing patient safety and improving clinical outcomes in this vulnerable population.

CRediT authorship contribution statement

Caruana Jessica: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Vella Kevin:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Vella Amy Marie:** Writing – review & editing, Supervision, Investigation. **Borg Marica:** Writing – review & editing, Supervision, Investigation. **Cini Masini Maria:** Writing – review & editing, Supervision, Investigation. **Farrugia Emanuel:** Writing – review & editing, Project administration, Conceptualization. **Camilleri Liberato:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Riva Nicoletta:** Writing – review & editing, Supervision, Formal analysis. **Gatt Alexander:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No more data to share. All relevant data have been provided in the manuscript and the Supplement.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2024.109120.

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