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A case-control study indicates that coagulation imbalance is associated with arteriosclerosis and markers of endothelial dysfunction in kidney failure

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ABSTRACT

Endothelial dysfunction, one of many causes of arterial changes in end-stage kidney disease (kidney failure), is a likely link between early vascular aging and the risk of thrombosis or bleeding in this condition. To evaluate this, we compared links between arterial stiffness and endothelial/coagulation factors in 55 patients receiving hemodialysis therapy and 57 age-/sex-matched control individuals. Arterial stiffness was assessed from carotid-femoral pulse wave velocity, and coagulation status from the endogenous thrombin generating potential. Markers of endothelial dysfunction (von Willebrand factor, tissue factor pathway inhibitor), neutrophil extracellular traps and tissue factor-positive extracellular vesicles were higher in patients with kidney failure. Prothrombin fragments 1 and 2, and D-dimer markers of *in vivo* coagulation activation—were also higher. However, *in vitro* in the presence of platelets, endogenous thrombin generating potential was lower and its downregulation by activated protein C impaired. Antiplatelet drugs did not affect these parameters. In multiple regression analysis, prothrombin fragments 1 and 2, D-dimer, factor VIII and monocyte-derived tissue factor-positive extracellular vesicles correlated with higher carotid-femoral pulse wave velocity. In patients with kidney failure, *in vivo* hypercoagulability occurred with reduced thrombin generation in platelet-rich plasma, likely explaining the opposing thrombotic and bleeding tendencies in patients with kidney failure. Importantly, arteriosclerosis is more closely related to a prothrombotic state. Thus, coagulation changes plus arterial stiffness highlight a major therapeutic challenge for anticoagulant and antiplatelet drug use.

Key words: End-stage kidney disease, thrombin generation, endothelium, arterial stiffness, hemostatic disorders

ABBREVIATIONS

APC: activated protein C

BP: blood pressure

DOAC: direct oral anticoagulant

ESKD: end-stage kidney disease

ETP: endogenous thrombin-generating potential

EVs: extracellular vesicles

FVIII: factor VIII

IC₅₀-APC: APC concentration that inhibits 50% of the ETP

NETs: neutrophil extracellular traps

PFP: platelet-free plasma

PRP: platelet-rich plasma

PWV: pulse wave velocity

TFPI: tissue factor pathway inhibitor

vWF: von Willebrand factor

INTRODUCTION

Cardiovascular disease is a major cause of morbidity and mortality in chronic kidney disease or end-stage kidney disease (ESKD).¹ Cardiac death is mainly attributed to arrhythmia and sudden death, while vascular death results from bleeding or ischemic/thrombotic events.²

Epidemiological and clinical evidence show that structural and functional changes of central and conduit arteries are major contributing factors. Endothelial dysfunction is one of multiple causal factors for arterial changes characterized by both atherosclerosis (atheromatous plaques and fatty depositions on the inner walls of vessels), and arteriosclerosis (thickening and stiffening of the arterial wall).

Central arterial stiffness and generalized endothelial dysfunction are key features of vascular modifications in ESKD. Arterial stiffness is an independent marker of risk,^{3,4} and a hallmark of vascular ageing in kidney disease.⁵ Arterial stiffening causes systolic hypertension, increased left ventricular afterload, left ventricular hypertrophy/stiffness, diastolic dysfunction, and concurrent dilation/hypertrophy of the left atrium. Endothelial dysfunction is one of many causal factors of arterial stiffening.⁶ Endothelial dysfunction implies impaired antithrombotic and anti-inflammatory properties, anti-proliferative actions on vascular smooth muscle cells, and regulation of vasomotor tone. Endothelial dysfunction is therefore common to both arterial stiffening and increased thromboembolic/bleeding complications in ESKD, likely explaining the associations between arterial and coagulation disorders.

The interactions between arterial stiffness, endothelial dysfunction and coagulation changes are governed by two opposing—thrombotic and bleeding—tendencies in ESKD.^{7,8}

Antiplatelet therapy is widely used for the treatment and prevention of thromboembolic and ischemic complications associated with atheromatous changes. Therapeutic decisions are more complex in patients requiring anticoagulant therapy, namely stroke prevention in the large proportion of ESKD patients with atrial fibrillation, where vitamin K antagonists are

extensively used. Evidence for the use of warfarin (vitamin K antagonist) in this situation, is essentially observational. Considerable caution is required due to the notable risk of bleeding.⁹⁻¹² Another challenge with vitamin K antagonists in ESKD could be the observed associations of warfarin use with arterial calcifications as a major feature of arterial stiffening.¹³ The approval of direct oral anticoagulants (DOAC) in the general population and moderate chronic kidney disease was based on non-inferiority data in terms of antithrombotic efficacy, less bleeding, and improved arterial stiffness.¹⁴ However, these results cannot be extended to ESKD patients who were excluded from pivotal studies. There is currently no evidence to suggest that DOAC provide greater efficacy in ESKD, and recent data in hemodialysis patients, showed that rivaroxaban with or without vitamin K2 supplementation had no effects on arterial stiffness and calcifications progression.¹⁵ The controlled clinical trials with detailed analyses of hemostatic factors and their interactions with arterial dysfunctions in ESKD are eagerly awaited. Understanding the mechanisms of thrombogenicity is crucial to assessing the benefits of anticoagulants in ESKD.¹⁶ Clinical associations between coagulation abnormalities and structural/functional changes in central/large arteries remain largely unknown. Few robust assessments of hemostasis in ESKD have been conducted,¹⁷⁻¹⁹ and yet coagulation disorders are a known feature of kidney disease.²⁰ Despite the widespread use of heparin during dialysis, and/or antiplatelet drugs for cardiovascular prevention, little is known about their safety/efficacy.

After previously establishing a link between individual coagulation factors (e.g. tissue factor pathway inhibitor [TPFI]), and arterial stiffness or arterial pulsatility in healthy women >60 years,²¹ we investigated this complex relationship in ESKD patients—where elevated arterial stiffness is a constant feature—with a particular focus on (1) pro- and anticoagulant factors, and (2) antiplatelet drugs. To apply to the complex clinical reality of these patients, the clotting cascade was evaluated using two complementary approaches. In clinical practice,

activation of the coagulation system is classically measured using *in vivo* markers such as D-dimer (a fibrin degradation product) and prothrombin fragments 1+2 (peptide released from prothrombin during thrombin formation). It explores the formation of thrombin at a given time. However, one major issue of the present study is to assess the dynamic of the thrombin generation process *in vitro* using a thrombin generation assay, a global function test providing a reliable method of estimating the thrombin-generating capacity to identify bleeding or thrombotic phenotypes.

RESULTS

Population characteristics

There were no significant clinical differences between ESKD patients and controls, except for lipid-lowering and antiplatelet therapies (Table 1). Lipid-lowering and antihypertensive treatments were more frequent in ESKD (Tables 1, 2). Twenty-six ESKD patients were on antiplatelet therapy (aspirin, n=17; clopidogrel, n=5; prasugrel, n=1; aspirin+clopidogrel combination, n=3) *vs.* none in the control group. Not all participants were on antiplatelet therapy. We therefore investigated the role of these drugs not only on platelet aggregation but also on other variables. Unsurprisingly, ESKD patients on antiplatelet drugs had lower maximum aggregation values in response to arachidonic acid compared to patients without treatment ($35\pm 8\%$ *vs.* $82\pm 2\%$; $P<0.001$). Indeed, arachidonic acid is active through cyclooxygenase receptors that are irreversibly inhibited by aspirin. None of the other differences between the ESKD patient subgroups were statistically significant. Our investigations considered ESKD patients as one single group.

Thrombin-generating potential, APC sensitivity and *in vivo* markers of coagulation activation

Circulating hemostatic factors were evaluated (Table 3). Although still within the normal range, platelet count was slightly higher in ESKD than in controls. Activation of the coagulation system *in vivo* was assessed by measuring D-dimer and prothrombin fragments 1+2: both were notably higher in ESKD (Table 3, Figure 1A-B).

Thrombin generation is complex, inducing both feedback activation and feedback inhibition. Global function tests (thrombin generation assays) provide a more reliable assessment of thrombin generation than estimates of individual contributing factors. To further understand the reactivity of the coagulation system—and not only the formation of thrombin at a given time from D-dimer and prothrombin fragments 1+2—thrombin generation was measured in PRP and PFP. The former provides information on soluble coagulation factors and the latter highlights the crucial role of platelets in the process *in vivo*. The total amount of thrombin activity (i.e. endogenous thrombin potential or ETP) is calculated as the area under the curve of thrombin generation and inhibition. Unsurprisingly, given the key role of platelets in promoting and controlling the coagulation system, ETP was higher in the presence of platelets, *versus* in the absence of platelets, in controls (1.9-fold increase) and in ESKD patients (1.7-fold increase). When we compared thrombin generation in PRP of controls and of ESKD patients, the lag time and time to peak were higher while the thrombin peak and velocity index were lower, resulting in a lower ETP in ESKD patients compared to controls (975±31 versus 1203±32 nM.min) (Table 3, Figure 1C). Thrombin generation in PFP was however comparable in both ESKD patients and controls (Table 3, Figure 1D), indicating a platelet-mediated effect on thrombin generation in ESKD.

In vivo, activated protein C (APC) is formed by the thrombin-thrombomodulin complex on the endothelium. To determine the function of the APC anticoagulant system, the thrombin generation assay includes the effect of exogenous APC, which reduces the ETP in a concentration-dependent manner. We assessed sensitivity to the APC anticoagulant pathway

of the endothelium by determining the APC concentration needed to reduce ETP by 50% (IC₅₀-APC). In contrast to PFP, the IC₅₀-APC in PRP was higher in ESKD patients than in controls (Table 3), suggesting impairment of the APC anticoagulant pathway and confirming the involvement of both platelets and the endothelium in the hemostatic profile of ESKD patients.

Procoagulant and anticoagulant soluble markers of endothelial dysfunction

We investigated the impact of endothelial damage in ESKD on plasma release of endothelial factors that affect procoagulant reactions and anticoagulant pathways. ESKD patients had higher levels of both procoagulant factor VIII (FVIII) and von Willebrand factor (vWF), and anticoagulant factors (TFPI, soluble endothelial protein C receptor and thrombomodulin) (Figure 1E-F and 1H-J), demonstrating the dual impact of endothelial dysfunction on hemostasis. Both FVIII:TFPI and vWF:TFPI ratios decreased in ESKD, indicating a major contribution of TFPI to changes in the equilibrium between circulating pro- and anticoagulant factors. Unlike controls, the correlation between TFPI and FVIII in ESKD was strongly significant ($R^2=0.07$, β coefficient: +0.05, $P=0.05$ vs. $R^2=0.32$, β coefficient: +0.27, $P=0.0001$, respectively), suggesting an endothelial-related hemostatic imbalance.

Circulating neutrophil extracellular traps and extracellular vesicles

To test whether neutrophil extracellular traps (NETs) contribute to the changes in thrombin generation observed in ESKD, we measured plasma levels of DNA-histones complexes (nucleosomes). Circulating nucleosomes were higher in ESKD than in controls (Figure 1G), confirming increased NET formation in ESKD. Circulating procoagulant extracellular vesicles (EVs) result from cell activation; circulating levels of annexin-V-positive, and/or tissue factor-bearing (TF⁺) EVs, were higher in ESKD, irrespective of cellular origin (Table 4). Nucleosomes and EVs therefore trigger a prothrombotic response in ESKD by stimulating proinflammatory cells.

Arterial stiffness and associations with coagulation in ESKD

Arterial stiffness and central pulse pressure were notably greater (higher aortic pulse wave velocity [PWV]) in ESKD (Table 2). Incremental elastic modulus was also elevated in ESKD (not measured in controls).²² These results highlight a possible critical role of arterial wall damage in thrombotic risk.

We found significant associations between age and prothrombin fragments 1+2, free TFPI, vWF, FVIII, ETP in PFP and D-dimer on one hand, and between mean BP and ETP in PRP on the other. Furthermore, mean BP was negatively correlated with prothrombin fragments 1+2, vWF, FVIII, IC₅₀-APC in PFP, and NETs (data not shown).

From the positive univariate correlations with PWV or incremental elastic modulus (Table 5), multivariate stepwise analysis of determinants of hemostatic variables showed PWV to be independently associated with procoagulant compounds: global coagulation test (prothrombin fragments 1+2 and D-dimer), procoagulant FVIII and monocyte-derived (CD14⁺) FT⁺ EVs (Table 6). Only aortic PWV was a direct independent determinant of procoagulant factors in ESKD, supporting the hypothesis that arterial stiffness is associated with procoagulant factors in ESKD patients.

DISCUSSION

Elevated arterial stiffness and endothelial dysfunction are known features of ESKD patients at high risk of thromboembolism or bleeding.

We provide compelling evidence of the contrast between increased activation of the coagulation system and decreased thrombin-generating potential in the presence of platelets in ESKD patients undergoing hemodialysis, and which is contingent on concurrent arterial stiffness and endothelial dysfunction. Antiplatelet drugs did not affect these results.²³⁻²⁵

The classic cell-based model of the coagulation cascade—thrombin generation triggering the

conversion of fibrinogen to fibrin—attributes a major role to the procoagulant surfaces of activated platelets. This concept now includes the tight interplay between hemostatic pathways, vascular cells, and the innate immune response involving NETs.^{26, 27} Measuring thrombin and fibrin levels *in vivo*, from prothrombin fragments 1+2 and D-dimer, differs fundamentally from *in vitro* monitoring of the full cycle of thrombin formation and inactivation. The thrombin-generating potential accurately reflects the likelihood of either thrombosis or bleeding—as in ESKD—whereas elevated D-Dimer or prothrombin fragments 1+2 levels suggest an intermittent physiological event, with low positive predictive value for thrombotic events.²⁸⁻³⁰ Interestingly, we found no difference in ETP in the absence of platelets whereas recent data show a lower ETP under the same conditions in middle-aged (mean age, 46 years; 18-77 years) ESKD patients.³¹ Age may well account for this discrepancy since our population was older (mean age, 71y; Table 1). Patients <52y in our study (n=7) also had a lower ETP in the absence of platelets (359±196 nM.min versus 729±347 nM.min in age-matched controls, n=5), suggesting that in the absence of platelets, ETP increases with age in ESKD. However, given the small number of young patients on hemodialysis in these two studies, no definitive conclusion can be drawn regarding an effect of age on ETP.

Although platelet count can affect thrombin generation, it was adjusted to the same value for patients and controls in our assay. Our observations of platelet-dependent lowering of thrombin generation in ESKD patients are noteworthy in light of the existing data on platelet dysfunction in primary hemostasis in ESKD.³²⁻³⁴ We also demonstrated impaired downregulation of thrombin generation by the APC anticoagulant system (increased IC₅₀-APC) in the presence of platelets, a condition known to result in an APC-resistant phenotype.³⁵ These findings highlight a role for platelets in the hemostatic profile of ESKD patients. Increased circulating levels of endothelial protein C receptor may also be deleterious to the APC pathway as previously reported in heart failure³⁶ since soluble endothelial protein

C receptor-bound APC no longer retains its ability to inactivate factor Va.³⁷ APC resistance is therefore likely attributable to endothelial dysfunction in both heart failure and ESKD.

Thrombin generation assays demonstrate the equilibrium between pro- and anticoagulant factors. As in heart failure, both procoagulant (FVIII and vWF) and anticoagulant (TFPI, soluble thrombomodulin and endothelial protein C receptor) variables related to endothelial activation were increased, thus confirming significantly impaired endothelial function in ESKD. The elevated vWF levels concur with the known increase in vWF antigen and activity levels in such patients.^{38,39} These pro- and anticoagulant factors, both determinants of thrombin generation,⁴⁰ therefore likely counteract one another. The significant decrease in FVIII:TFPI and vWF:TFPI ratios in ESKD likely confirms that changes in the equilibrium between procoagulant and anticoagulant molecules result in decreased thrombin generation, thus representing a biological hallmark to predict the risk of bleeding. Secretion of TFPI by activated platelets and endothelium in ESKD patients contributes in part to decreased thrombin generation.

Arterial stiffening, one notable hallmark of ESKD, is characterized by an increase in both aortic PWV and carotid incremental elastic modulus.⁴¹⁻⁴⁷ Predictably, elevated systolic BP was one of the typical hemodynamic characteristics even though 90% were on antihypertensive treatment. To describe the continuum from local to regional loss of arterial elasticity, arterial stiffness was assessed using various methods, including aortic PWV⁴² and carotid incremental elastic modulus.⁴³ The elevated arterial stiffness (12±4 m/s and 1106±13 kPa, for PWV and incremental elastic modulus, respectively)⁴⁷ we observed confirms the particularly high cardiovascular risk (2018 ESH/ESC guidelines).⁴⁸

Univariate and stepwise regression analyses strengthen our hypothesis that arterial stiffness and endothelial dysfunction have marked effects on hemostasis in ESKD.⁴⁹ Univariate analysis suggested that both arterial stiffness and activation of procoagulant and anticoagulant

mechanisms likely contribute to outcomes in ESKD. Regional stiffness assessed by PWV, including both structural and functional alterations of the aorta, was positively associated with several circulating procoagulant and anticoagulant factors. IC₅₀-APC in PRP was positively associated with PWV, highlighting a novel potential mechanistic relationship between the dynamic APC system and endothelial components of arterial stiffness. The single negative association between local carotid incremental elastic modulus and thrombin generation suggests that, independently of its geometry and BP, intrinsic structural alterations of the arterial wall only play a minimal role in the prothrombotic profile of ESKD patients. Given the strong co-linearities between stiffness, hemostatic parameters, age, and BP, we performed a stepwise regression analysis, which confirmed the associations between PWV and procoagulant markers, D-dimer, prothrombin fragments 1+2, FVIII and monocyte-derived FT⁺ EVs. Having previously shown an increase in endothelial EVs in ESKD patients compared to age-matched controls,⁴⁹ this analysis encompassed all cell-derived EVs, and showed that annexin-V⁺/FT⁺ EVs are elevated in ESKD. We therefore hypothesize that aortic PWV, FVIII and EVs play a novel major role in the thrombogenicity of ESKD patients. Interestingly, while we confirmed the previously reported increase in circulating NETs in ESKD patients,⁵⁰ we found no correlation with coagulation disorders or stiffness. Though surprising, this likely reflects the complex links between NETs and vWF that were not revealed by stepwise regression analysis in our cohort.

Our finding that antiplatelet drugs do not affect the hemostatic profile confirms previous data showing the absence of *in vitro* effects of platelet inhibitors on thrombin generation.⁵¹

Unsurprisingly, ESKD patients on antiplatelet drug therapy had lower maximum aggregation responses than those without this treatment; there were no other differences in coagulation parameters, with or without antiplatelet therapy. This is also consistent with the presence of impaired endothelial function in ESKD patients despite concurrent antiplatelet treatment.⁵²

Antiplatelet drugs can therefore be safely used in these high-risk patients, although to the best of our knowledge, the effects of unfractionated heparin or low molecular weight heparin on endothelial function have not been widely investigated in ESKD.

The situation is however dramatically different for warfarin-like nonspecific anticoagulant compounds, which significantly reduce thrombin generation thus significantly increasing the bleeding risk in ESKD. The delicate balance between procoagulant and anticoagulant factors in ESKD explains the controversial benefit of vitamin K antagonists, particularly in view of the associated increased vascular calcifications. A DOAC that specifically inhibits thrombin or factor Xa could therefore be an interesting alternative. We observed decreased thrombin generation in ESKD patients, highlighting clinical concerns regarding the efficacy and safety of DOACs in ESKD. Apixaban, a factor Xa inhibitor, reduced thrombin generation and fibrin formation to a lesser extent than warfarin in the recent ARISTOLE trial that excluded patients with severe renal dysfunction.⁵³ DOACs such as apixaban may therefore improve stroke prevention and reduce the risk of bleeding in ESKD. Two further recent trials on apixaban in ESKD patients have shown similar reductions in stroke/thromboembolism events to those observed with warfarin but with a significantly lower risk of bleeding.⁵³⁻⁵⁶ DOACs—factor Xa inhibitors in particular—also display pharmacodynamic activity at standard doses thus precluding drug accumulation in ESKD patients.⁵⁷ Combining anticoagulant and antiplatelet therapy can increase the propensity to bleeding since DOACs are likely to impair platelet aggregation secondary to coagulation activation.⁵⁸ Another challenge is the potential interference between DOACs and other drugs commonly used in ESKD.⁵⁵ Future studies should assess the effect of DOACs on endothelial function and arterial stiffness—the equivalent of a vascular-renal syndrome in ESKD—and ultimately on cardiovascular morbimortality.

One of the limitations of our study is the absence of investigations into fibrinolysis, the next

step up in coagulation dynamics. However, consistent with the previously reported activation of the fibrinolytic system in ESKD,⁵⁹ we did observe an increase in D-dimer in ESKD patients which in turn suggests enhanced fibrinolysis.⁶⁰ Interestingly, in line with the age-related augmentation of fibrinolysis, Gäckler et al reported a hypofibrinolytic state in younger ESKD patients.³¹ Another limitation of our study is the lack of a direct functional assessment of endothelial function. We did however observe notable activation of the endothelial secretory function—an index of endothelial disease. We previously reported on impaired endothelial vasodilatory function⁶¹ and its impact on mortality in patients with similar clinical characteristics.^{62, 63}

ESKD patients are exposed to the delicate balance between a risk of thrombosis and a risk of bleeding. Anticoagulant drugs can trigger adverse bleeding events, thus complicating treatment selection in kidney disease patients⁵⁵ who are particularly susceptible to arterial stiffening and endothelial dysfunction, two independent predictors of mortality. Evaluating the hypercoagulable status using classic tests (prothrombin fragments 1+2 and D-dimer) alone masks a paradoxical decrease in ETP in the presence of platelets. The unbalanced secretion of endothelial anticoagulant factors—another feature of endothelial dysfunction in ESKD—may at least partly explain the reduction in ETP due to the strong contribution of TFPI in the process. Our stepwise analysis suggests that aortic stiffness is more closely related to a prothrombotic state, in turn partly related to endothelial procoagulant factors. Our study highlights particularly novel features of coagulation dynamics in dialysis patients, and the urgent need to improve and individualize anticoagulant therapy in ESKD.

METHODS

Study population

This prospective cohort study (Manhes Hospital, Fleury-Merogis, France) included 55 ESKD patients on hemodialysis for >3 months, without long-term anticoagulant treatment; 26 were on antiplatelet therapy for previous cardiovascular diseases.

Cardiovascular measurements and blood samples were taken immediately before routine hemodialysis, two days after the previous session. Blood samples were centrifuged and frozen at -80°C (except those for platelet aggregation testing). Hemostasis testing and clotting factor assays were carried out at the University Hospital of Nancy (UMR_S 1116, France).

The control group comprised 57 individuals (matched for age and gender) from the ADELAHYDE-2 study.⁶⁴ Individuals with impaired renal function (eGFR [MDRD] <50 mL/min/1.73m²) or current anticoagulant therapy were excluded. Mean eGFR was 73.0±2.1 mL/min/1.73m² (95%LCL-UCL: 69-77). Platelet aggregation and carotid artery hemodynamics were not measured in controls.

This investigator-initiated study was approved by our institutional review board. All participants provided written informed consent.

Cardiovascular measurements

After a ≥10-minute rest (constant ambient temperature, 22±1°C), patients underwent brachial BP (OMRON®, Hoofddorp, The Netherlands) measurement. The average of three measurements was documented. Pulse pressure was calculated as systolic BP minus diastolic BP, and mean BP was determined by including the radial artery waveform (Sphygmocor®, AtCor Medical, Sydney, Australia). Overall arterial stiffness was determined from aortic PWV (corrected for distance x 0.80) (Complior®, ALAM Medical, Pantin, France) and local common carotid artery stiffness measurements, including carotid BP (Sphygmocor® device) and documented geometric changes (Wall Track System®, Esaote, Maastricht, The Netherlands), to calculate Young's elastic incremental modulus (index of local arterial wall stiffness). Devices and methods used have all been previously described.⁶⁵

Hemostatic variables and endothelial markers

All blood samples were taken by the same investigators, just before dialysis, then centrifuged and frozen at -80°C .

Venous blood samples were drawn into Monovette® tubes (Sarstedt) (9:1 vol/vol) and centrifuged at 190 g for 10 minutes at 20°C to obtain PRP. The remaining blood was centrifuged at 1750 g for 10 minutes at 20°C to obtain platelet-poor plasma. PFP was prepared by centrifugation of platelet-poor plasma at 4500 g for 15 minutes at 4°C . PFP was immediately frozen at -80°C . To quantify and characterize EVs, blood was subjected to two successive centrifugations at 2500 g for 10 minutes and plasma was stored at -80°C until required.

Platelet aggregation was measured in PRP adjusted to 200 G/L with platelet-poor plasma. Platelet aggregation induced by adenosine diphosphate (5 and 10 μM), collagen (2 and 20 $\mu\text{g/mL}$) and arachidonic acid (1.39 mM) was measured by turbidimetric analysis under stirred conditions (Thrombo-Aggregometer, SD Innovation, Frouard, France).

Calibrated automated thrombography was performed in PRP adjusted to 150 G/L or PFP at 37°C (Fluoroskan Ascent® fluorometer, ThermoLabsystems, Helsinki, Finland) using the dedicated software (Thrombinoscope BV, Maastricht, The Netherlands).^{36, 66} Thrombin generation curves were recorded in triplicate, ETP was assessed as the area under the curve, and the APC concentration that inhibits 50% of the ETP ($\text{IC}_{50}\text{-APC}$) was calculated.

D-dimer, FVIII, vWF antigen, total and free TFPI, soluble thrombomodulin, and soluble endothelial protein C receptor levels were quantified in PFP by enzyme-linked immunosorbent assay (Asserachrom, Stago®, Asnières, France). Prothrombin fragments 1+2 were measured (Enzygnost F1+2 micro assay, Instrumentation Laboratory).

To assess NETs, circulating levels of DNA-histone complexes were measured using the Cell Death Detection ELISA kit (Roche Diagnostics, Sigma Aldrich, USA). A positive reference control was included, and values were expressed in arbitrary absorbance units.

Cell-derived extracellular vesicles

The fluorescent antibodies, CD41-phycoerythrin, CD144-phycoerythrin, CD45-phycoerythrin, CD14- phycoerythrin, CD235- phycoerythrin and allophycocyanin-endothelial protein C receptor were purchased from BD Pharmingen™. Clone TF9-10H10, allophycocyanin-TF (the other antibody against TF) was provided by Biotechne™, and annexin-V-FITC by Fisher Scientific™. Circulating EVs were quantified by high-sensitivity flow cytometry using a standardized procedure.⁶⁷ Plasma (30 µL) was incubated in ice for 30 minutes with 30 µL of Flow Count-Fluorosphere® beads (Beckman-Coulter, Villepinte, France), 10 µL of annexin-V-FITC, and the optimal concentration of specific antibody. Stained samples were analyzed using a Gallios™ instrument (Beckman-Coulter, Miami, FL, USA).

Statistical analysis

Data are presented as mean±SEM, with lower and upper limits of confidence. We based our sample size on a 30% change in ETP in PRP from the control group (1203±240 nM.min) with an alpha risk of 5% and a beta risk of 10%. A total of 50 individuals was required: 25 per subgroup. We decided to include at least 25 ESKD patients per subgroup of antiplatelet treatment, and 50 controls. Clinical, arterial and hemostatic parameters were compared between patients with or without antiplatelet therapy, and controls. Clinical and hemostatic variables were compared between the whole study population and controls. Student's *t*-test was used to compare normally distributed continuous variables and a non-parametric Wilcoxon-Mann-Whitney test for non-normally distributed variables between controls on one hand, and ESKD patients as a single group on the other. A one-way ANOVA was performed

to compare variables between the three groups: controls, ESKD without antiplatelet therapy and ESKD with antiplatelet therapy, then a post-hoc test compared ESKD subgroups with each other and with controls (Tables 1-4). Because of outliers revealed by scatter plot analysis, univariate robust regression analysis was used to determine the associations between hemostatic parameters as dependent variables and stiffness parameters (aortic PWV and carotid incremental elastic modulus) as independent variables. Multivariable analysis was conducted through stepwise regression analysis after adjustments for potential confounders (age, mean arterial BP, antihypertensive treatment, antiplatelet treatment) separately with each hemostatic variable (8 stepwise regressions for PWV and 1 for incremental elastic modulus) as dependent variables if statistically significant after univariate analysis. We used NCSS® 2001 software (Kaysville, Utah, USA). A *P* value <0.05 was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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Table 1: Baseline characteristics of participants

Characteristics	Control Group <i>n</i> = 57	ESKD <i>n</i> = 55	<i>t</i> -test <i>P</i> -value vs controls	ESKD without APT <i>n</i> = 29	ESKD with APT <i>n</i> = 26	ANOVA vs controls <i>P</i> -value
Age, years	71 ± 1 (69-74)	71 ± 2 (67-74)	0.8525	69 ± 3 (64-75)	72 ± 2 (67-77)	0.673
Male gender, %	50.9 (38-64)	49.1 (35-63)	0.8517	28 (10-45)	73## (55-91)	<0.01
Body mass index, kg/m ²	28.5 ± 0.9 (26.6-30.3)	26.7 ± 0.8 (25-28)	0.1456	28.2 ± 1.2 (25.5-30.9)	25.0 ± 0.9 (23.2-26.9)	0.069
Dialysis vintage (months)	NA	75 ± 12 (50-99)	NA	71 ± 15 (39-102)	79 ± 20 (38-120)	NA
Cardiovascular history (n):						
<i>coronary artery disease</i>		13		1	12	
<i>myocardial infarction</i>	NA	4	NA	0	4	NA
<i>systolic left ventricular heart failure</i>		5		0	5	
<i>stroke</i>		3		2	1	
<i>peripheral artery disease</i>		19		5	14	

carotid artery endarterectomy		4		0	4	
DVT or PE for >10 years.		2		0	2	
Nephropathy etiology (n):						
<i>interstitial nephropathy</i>		3		3	0	
<i>segmental and focal hyalinosis</i>		5		3	2	
<i>other chronic glomerulopathies</i>		2		0	2	
<i>polycystic kidney disease</i>		7		4	3	
<i>angionephrosclerosis</i>		11		2	9	
<i>Berger's disease</i>	NA	2	NA	0	2	NA
<i>obstructive uropathy</i>		3		3	0	
<i>diabetes mellitus</i>		16		9	7	
<i>interferon toxicity</i>		1		1	0	
<i>postpartum hemorrhagic uropathy</i>		1		1	0	
<i>amyloidosis</i>		2		1	1	
<i>unknown</i>		2		2	0	
Antihypertensive treatment, %	86.0 (71-100)	90.9 (83-99)	0.5719	90.0 (78-100)	92.3 (81-100)	0.834
Lipid-lowering treatment, %	37	64	0.0043	48	81	<0.001

	(24-50)	(51-77)		(29-67)	(65-97)	
Current smoker, %	10.9 (2.4-19.0)	16.4 (6.3-26.0)	0.4092	10.3 (0-22.1)	23.1 (5.7-40.4)	0.281
Diabetes mellitus, %	12.8 (2.9-22.7)	27.3 (15-39)	0.0721	27.6 (10.3-44.9)	26.9 (0.8-45.2)	0.200
Glycemia, mmol/L	6.0 ± 0.4 (5.1-7.0)	5.3 ± 0.1 (5.0-5.6)	0.1841	5.2 ± 0.6 (4.7-5.3)	5.3 ± 0.7 (4.8-5.8)	0.415
Hs-CRP, mg/dL	0.50 ± 0.10 (0.28-0.72)	1.05 ± 0.3 (0.33-1.72)	0.1257	0.86 ± 0.3 (0.10-1.66)	1.28 ± 0.4 (0.14-2.43)	0.217
Platelets, 10 ³ /μL	206 ± 10 (185-227)	249 ± 12 (224-274)	0.0088	243 ± 15 (213-273)	257 ± 21* (214-299)	<0.05

Values are mean ± SEM (95% lower confidence limit-upper confidence limit).

ANOVA: Tukey-Kramer post hoc test vs controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; vs ESKD without antiplatelet therapy: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

Abbreviations: APT, antiplatelet therapy; ESKD, end stage renal disease; hs-CRP, high-sensitivity C-reactive protein; DVT, deep venous thrombosis; PE, pulmonary embolism; NA, not applicable

Table 2: Hemodynamic parameters

	Control group (n = 57)	ESKD (n = 55)	t-test P-value vs controls	ESKD without APT n = 29	ESKD with APT n = 26	ANOVA vs controls P-value
<i>Brachial measurements</i>						
• Systolic blood pressure (mm Hg)	134 ± 2 (129-139)	141 ± 3 (135-147)	0.0643	141 ± 5 (131-150)	142 ± 4 (134-149)	0.181
• Diastolic blood pressure (mm Hg)	70 ± 1 (68-72)	73 ± 2 (70-77)	0.1079	75 ± 2 (71-79)	72 ± 2 (67-77)	0.151
• Mean blood pressure (mm Hg)	91 ± 1 (89-94)	96 ± 2 (92-99)	0.0598	97 ± 2 (91-102)	95 ± 2 (90-100)	0.146
• Pulse pressure (mm Hg)	64 ± 2 (60-68)	68 ± 2 (62-73)	0.2443	65 ± 3 (58-73)	70 ± 3 (63-77)	0.345
<i>Common carotid artery measurements</i>						
• Systolic blood pressure (mm Hg)	129 ± 2 (124-133)	133 ± 3 (127-139)	0.2710	134 ± 5 (124-144)	133 ± 4 (125-140)	0.537
• Diastolic blood pressure (mm Hg)	71 ± 1 (69-74)	73 ± 2 (70-77)	0.3173	75 ± 2 (71-79)	72 ± 2 (67-77)	0.342

•						
• Pulse pressure (mm Hg)	57 ± 2 (53-62)	60 ± 3 (55-65)	0.5051	59 ± 4 (50-67)	61 ± 3 (54-67)	0.738
• Intimal medial thickness (µm)	678 ± 35 (607-749)	668 ± 15 (637-698)	0.7833	649 ± 19 (611-687)	690 ± 24 (640-740)	0.685
Heart rate, beats/min	64 ± 1 (61-66)	70 ± 2 (67-73)	0.0023	73 ± 2*** (69-80)	67 ± 2 (63-71)	0.001
Pulse wave velocity (m/s)	8.1 ± 0.2 (7.7-8.6)	12.0 ± 0.4 (11.2-12.9)	<0.0001	11.5 ± 0.4*** (10.5-12.4)	12.6 ± 0.7*** (11.1-14.1)	<0.0001
Young's incremental elastic modulus (kPa)	NA	1106 ± 139 (818-1395)		1312 ± 257 (784-1839)	877 ± 88 (695-1059)	0.132

Values are mean ± SEM (95% lower confidence limit-upper confidence limit).

ANOVA: Tukey-Kramer post hoc test vs controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; vs ESKD without antiplatelet therapy: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

Abbreviations: APT, antiplatelet therapy; ESKD, end stage renal disease

Table 3: *In vivo* coagulation markers and *in vitro* thrombin generation

	Control group (n = 57)	ESKD patients (n = 54)	<i>t</i> -test <i>P</i> -value vs controls	ESKD without APT n = 29	ESKD with APT n = 26	ANOVA vs controls <i>P</i> -value
<i>In vivo</i> coagulation markers						
Prothrombin fragments 1+2, pmol/L	190 ± 13 (163-216)	365 ± 25 (315-416)	<0.0001	393 ± 39*** (313-473)	333 ± 29*** (274-393)	<0.0001
D-dimer, ng/mL	688 ± 122 (443-933)	1519 ± 221 (1076-1963)	0.0012	1153 ± 202 (739-1566)	1944 ± 405*** (1108-2780)	<0.001
<i>Circulating pro- and anticoagulant factors</i>						
Fibrinogen, g/L	3.9 ± 0.1 (3.7-4.2)	4.1 ± 0.2 (3.8-4.5)	0.3169	4.0 ± 0.2 (3.6-4.4)	4.3 ± 0.3 (3.7-4.9)	0.3086
FVIII:TFPI ratio, a.u.	1.67 ± 0.08 (0.99-1.32)	1.17 ± 0.05 (1.06-1.28)	0.0001	1.19 ± 0.08*** (1.03-1.34)	1.15 ± 0.08*** (1.00-1.32)	<0.0001
vWF:TFPI ratio, a.u.	1.75 ± 0.12 (1.51-1.98)	1.30 ± 0.06 (1.18-1.43)	0.01	1.27 ± 0.09* (1.09-1.44)	1.35 ± 0.10 (1.15-1.55)	<0.01
<i>In vitro</i> thrombin generation parameters						
<i>Platelet-rich plasma</i>						

ETP (nM.min)	1203 ± 32 (1138-1268)	975 ± 31 (912-1037)	<0.0001	967 ± 34*** (897-1037)	984 ± 55*** (870-1097)	<0.0001
Lag-time (min)	6.3 ± 0.1 (6.0-6.6)	7.2 ± 0.2 (6.8-7.7)	0.0018	7.1 ± 0.3 (6.4-7.8)	7.4 ± 0.4* (6.6-8.1)	<0.01
Thrombin peak (nM)	207 ± 8 (192-223)	155 ± 8 (139-172)	<0.0001	157 ± 11*** (135-179)	154 ± 12*** (127-181)	<0.0001
Time to peak (min)	10.3 ± 0.2 (9.9-10.7)	11.2 ± 0.3 (10.6-12.0)	0.0160	11.1 ± 0.5* (10.1-12.1)	11.5 ± 0.5* (10.5-12.5)	<0.05
Velocity (nM/min)	58 ± 3 (51-64)	46 ± 4 (38-55)	0.0331	48 ± 6 (37-59)	45 ± 6 (33-57)	0.0955
IC ₅₀ -APC (nM)	3.4 ± 0.3 (2.8-4.0)	5.0 ± 0.6 (3.8-8.8)	0.0180	5.3 ± 0.8* (3.7-6.8)	4.6 ± 0.9 (2.8-6.4)	<0.05
ETP×IC ₅₀ -APC	4122 ± 355 (3410-4834)	5211 ± 747 (3712-6710)	0.1882	5325 ± 864 (3554-7097)	5073 ± 1298 (2388-7757)	0.4128
<i>Platelet-free plasma</i>						
ETP (nM.min)	632 ± 46 (540-725)	572 ± 30 (512-632)	0.2783	543 ± 47 (446-640)	605 ± 34 (535-676)	0.4098
Lag-time (min)	14.2 ± 0.9	13.7 ± 0.8	0.6952	13.9 ± 1.3	13.5 ± 0.9	0.9053

	(12.4-16.0)	(12.1-15.4)		(11.2-16.6)	(11.7-15.4)	
Thrombin peak (nM)	108 ± 11 (87-130)	102 ± 7 (88-116)	0.6175	97 ± 11 (75-119)	107 ± 8 (91-124)	0.7567
Time to peak (min)	15.4 ± 1.3 (12.8-17.9)	17.0 ± 0.9 (15.2-18.7)	0.2960	17.3 ± 1.5 (14.3-20.3)	16.7 ± 0.9 (14.9-18.5)	0.5602
Velocity (nM/min)	37 ± 5 (26-47)	35 ± 3 (29-42)	0.8379	34 ± 5 (23.8-44.6)	37 ± 4 (29.3-44.6)	0.9308
IC ₅₀ -APC (nM)	1.4 ± 0.2 (1.0-1.8)	1.1 ± 0.1 (0.9-1.3)	0.0785	1.0 ± 0.1 (0.7-1.3)	1.1 ± 0.1 (0.8-1.4)	0.2109
ETP×IC ₅₀ -APC	1096 ± 186 (1096-1474)	636 ± 75 (484-788)	0.0119	602 ± 109* (378-826)	672 ± 106* (452-892)	<0.05

Values are mean ± SEM (95% lower confidence limit-upper confidence limit).

ANOVA: Tukey-Kramer post hoc test vs controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; vs ESKD without antiplatelet: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

Abbreviations: APT, antiplatelet therapy; a.u., arbitrary units; ETP, endogenous thrombin potential in the absence of activated protein C (APC); IC₅₀-APC, APC concentration needed to reduce ETP by 50%

Table 4: Levels of extracellular vesicles

	Control group (n = 57)	ESKD (n = 55)	t-test P-value vs controls	ESKD without APT n = 29	ESKD with APT n = 26	ANOVA vs controls P-value
<i>Endothelium-derived (CD144+) extracellular vesicles</i>						
• annexin-V ⁺ (EVs/μL)	133 ± 23 (88-179)	317 ± 34 (246-389)	<0.0001	241 ± 35 (169-312)	403 ± 61 ^{***.#} (277-528)	<0.001
• annexin-V ⁺ /FT ⁺ (EVs/μL)	82 ± 16 (50-114)	160 ± 24 (110-211)	0.0088	127 ± 23 (81-173)	198 ± 46 ^{**} (103-293)	<0.01
<i>Platelet-derived (CD41+) extracellular vesicles</i>						
• annexin-V ⁺ (EVs/μL)	575 ± 90 (394-756)	1635 ± 285 (1064-2205)	0.0005	1315 ± 244 (815-1815)	1991 ± 534 ^{**} (890-3091)	<0.001
• annexin-V ⁺ /FT ⁺ (EVs/μL)	86 ± 18 (50-122)	151 ± 25 (102-201)	0.0334	148 ± 23 (102-195)	155 ± 46 (60-250)	0.1039
<i>Leucocyte-derived (CD45+) extracellular vesicles</i>						
• annexin-V ⁺ (EVs/μL)	466 ± 57 (351-580)	1808 ± 219 (1369-2248)	<0.0001	1541 ± 140 ^{***} (1254-1829)	2106 ± 434 ^{***} (1212-3000)	<0.0001
• annexin-V ⁺ /FT ⁺ (EVs/μL)	245 ± 27 (191-298)	625 ± 75 (475-775)	<0.0001	534 ± 71 ^{**} (388-680)	726 ± 136 ^{***} (446-1007)	<0.0001

Monocyte-derived (CD14+) extracellular vesicles

• annexin-V ⁺ (EVs/μL)	197 ± 42 (113-282)	913 ± 390 (136-1699)	0.0656	449 ± 63 (320-578)	1441 ± 817* (254-3123)	<0.05
• annexin-V ⁺ /FT ⁺ (EVs/μL)	141 ± 36 (70-212)	293 ± 25 (243-344)	0.0007	280 ± 30* (218-342)	308 ± 41** (223-393)	0.01

Erythrocyte-derived (CD235+) extracellular vesicles

• annexin-V ⁺ (EVs/μL)	1636 ± 431 (774-2499)	7629 ± 897 (5830-9428)	<0.0001	7854 ± 1265*** (5263-10445)	7379 ± 1295*** (4713-10045)	<0.0001
• annexin-V ⁺ /FT ⁺ (EVs/μL)	654 ± 148 (357-951)	1551 ± 209 (1132-1971)	0.0006	1971 ± 355*** (1243-2899)	1084 ± 161# (753-1415)	<0.001

Values are mean ± SEM (95% lower confidence limit-upper confidence limit).

ANOVA: Tukey-Kramer post hoc test: vs controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; vs ESKD without antiplatelet: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

Abbreviations: APT, antiplatelet therapy; ESKD, end stage renal disease; TF, tissue factor; EVs, extracellular vesicles

Table 5: Univariate robust regression between arterial stiffness or carotid artery elastic modulus and hemostatic variables as dependent variables

	PWV			Incremental elastic modulus		
	β	R ²	<i>P</i>	β	R ²	<i>P</i>
ETP in PFP	NS			-0.0432	0.09	0.02
IC ₅₀ -APC in PFP	0.0375	0.13	0.01	NS		
Free TFPI	1.19	0.14	0.01	NS		
vWF	6.01	0.21	0.001	NS		
FVIII	5.09	0.21	0.001	NS		
Prothrombin fragments 1+2	16	0.20	0.001	NS		
D-dimer	60.6	0.10	0.02	NS		
NETs	NS			NS		
sEPCR	-6.87	0.24	0.001	NS		
CD14 ⁺ FT ⁺ EVs	22.54	0.24	0.001	NS		

Abbreviations: PWV, pulse wave velocity; ETP, endogenous thrombin potential in the absence of activated protein C (APC); PFP, platelet free plasma; IC₅₀-APC, APC concentration needed to reduce ETP by 50%; TFPI, tissue factor pathway inhibitor; vWF, von

Willebrand factor; FVIII, factor VIII; NETs, neutrophil extracellular traps; sEPCR, soluble endothelial protein C receptor; FT, tissue factor; EVs, extracellular vesicles

Table 6: Stepwise regression of the influence of aortic pulse wave velocity on hemostatic variables

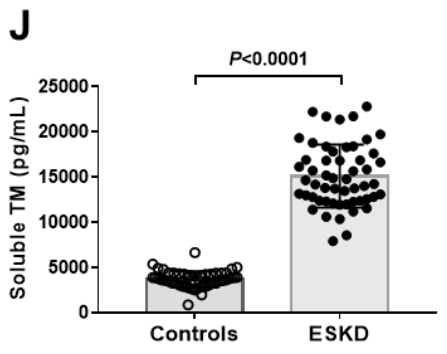
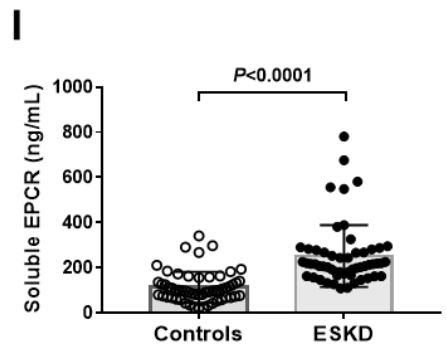
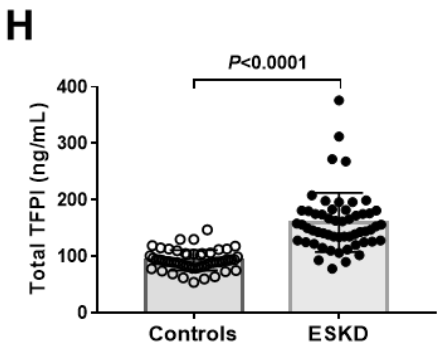
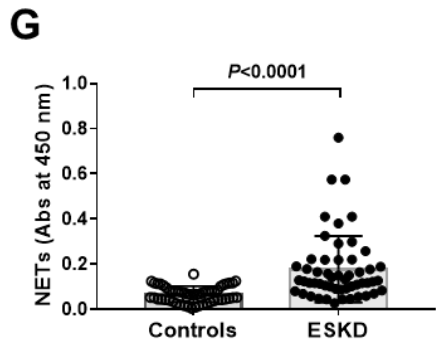
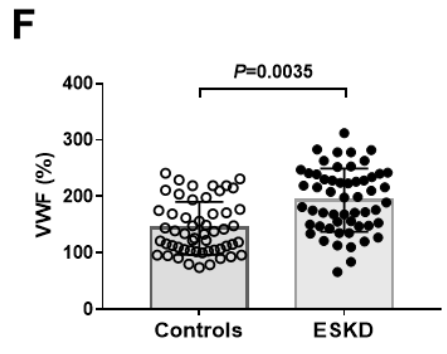
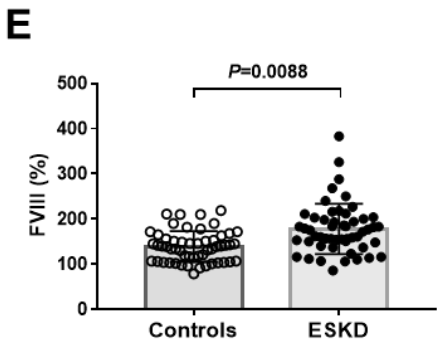
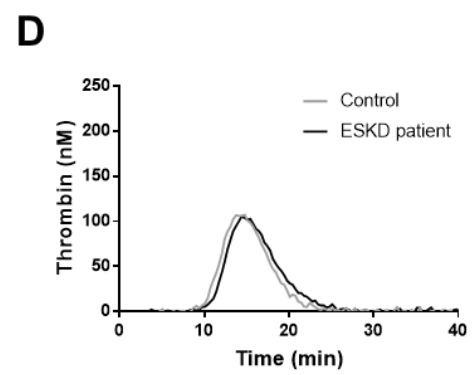
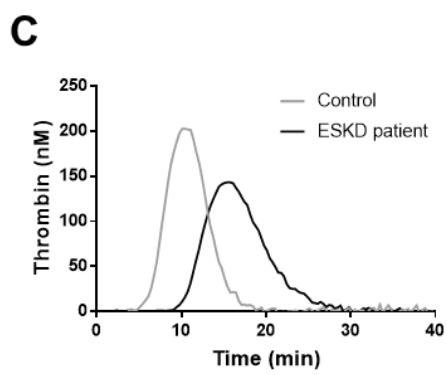
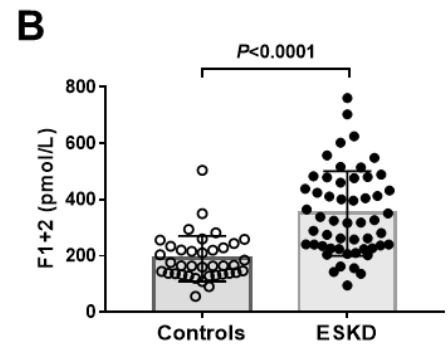
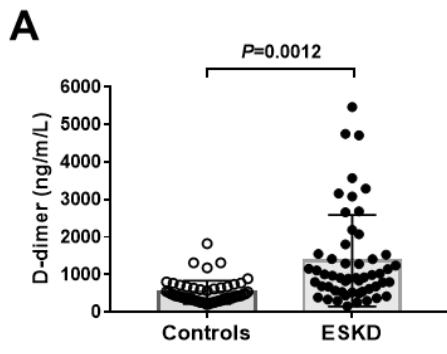
Dependent variable	R ² model	<i>P</i> value	Remaining independent variables
FVIII	0.07	0.05	0.27 × PWV
Prothrombin fragments 1+2	0.30	0.03	0.30 × PWV
D Dimer	0.36	0.006	0.37 × PWV
CD14 ⁺ FT ⁺ EVs	0.09	0.03	0.30 × PWV

Statistically significant results of multivariable analysis of pulse wave velocity plus the confounding variables (age, mean arterial pressure, antihypertensive treatment and antiplatelet treatment) as compared with each of the hemostatic factors (shown in Table 5) as individual dependent variables.

Abbreviations: FVIII, factor VIII; FT, tissue factor; EVs, extracellular vesicles

Figure legend

Figure 1. Coagulation profiles in ESKD. Plasma levels of D-dimer (A), prothrombin fragment 1+2 (B), FVIII (E), vWF (F), DNA complexed with histones (G), total TFPI (H), soluble EPCR (I) and soluble TM (J) were measured by ELISA. Each dot in a vertical scatter plot represents a single patient; the mean \pm SEM is depicted. Thrombin generation was triggered by 0.5 pM tissue factor and monitored with the Calibrated Automated Thrombogram assay. Representative curves in platelet-rich plasma are adjusted to 150×10^9 platelets/L (C) or in platelet-free plasma + 4 μ M phosphatidyl-serine/-ethanolamine/choline vesicles (D). F1+2, prothrombin fragments 1+2; FVIII, coagulation factor VIII; vWF, von Willebrand factor; NETs, neutrophil extracellular traps; TFPI, tissue factor pathway inhibitor; EPCR, endothelial protein C receptor; TM, thrombomodulin, ESKD, end-stage kidney disease.

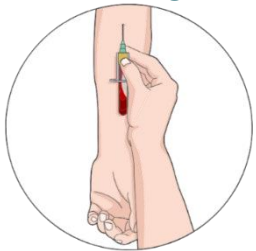


A case-control study indicates that coagulation imbalance is associated with arteriosclerosis and markers of endothelial dysfunction in kidney failure

55 ESKD patients >3 months dialysis

- no long-term anticoagulant treatment
- 29 without antiplatelet drugs
- 26 on antiplatelet therapy

Thrombotic and bleeding risk



Testing

- In-vivo markers of coagulation activation
- In-vitro thrombin generation in platelet-rich versus platelet-poor plasma
- Endothelial markers
- Neutrophil extracellular traps
- Extracellular vesicles
- Carotid artery and aorta hemodynamics

120% increase in D-dimer levels

19% decrease in thrombin generation (only in platelet-rich plasma)

48% increase in aortic stiffness

30% decrease in procoagulant/anticoagulant ratio (FVIII/TFPI)

No interaction with antiplatelet therapy

Aortic stiffness: an independent determinant of procoagulant factors in ESKD

CONCLUSION:

ESKD patients exhibit an imbalance between thrombosis risk associated with aortic stiffness, and bleeding risk associated with endothelial dysfunction and insufficient thrombin generation.

57 age-sex matched healthy control subjects