Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Morphological and functional differences in haemostatic axis between kidney transplanted and end-stage renal disease patients

Paolo Fiorina,[1] Franco Folli,[1] Elisabetta Ferrero,[1] Elena Orsenigo,[2] Giovanna Finzi,[3] Gabriella Mazzolari,[1] Claudia Placidi,[3] Lucia Perego,[1] Stefano La Rosa,[3] Marco Melandri,[4] Lucilla Monti,[1] Carlo Capella,[3] Armando D'Angelo,[5] Carlo Staudacher[2] and Antonio Secchi[1]

1 Department of Internal Medicine, San Raffaele Scientific Institute, Milan, Italy
2 Department of Surgery, San Raffaele Scientific Institute, Milan, Italy
3 Department of Pathology, Università dell'Insubria, Varese, Italy
4 Department of Nephrology, San Raffaele Scientific Institute, Milan, Italy
5 Department of Coagulation Service and Thrombosis, Research Unit, San Raffaele Scientific Institute, Milan, Italy

Keywords
end-stage renal disease, haemostasis, kidney transplant, platelets.

Correspondence
Antonio Secchi MD, Internal Medicine, Universita' Vita e Salute-San Raffaele, San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milano, Italy. Tel.: +39-02-26432575; fax: +39-02-26437788; e-mail: antonio.secchi@hsr.it

Received: 17 January 2005
Revision requested: 10 February 2005
Accepted: 29 March 2005

doi:10.1111/j.1432-2277.2005.00173.x

Summary

End-stage renal disease (ESRD) is characterized by several atherothrombotic abnormalities, and kidney transplant seems to improve most of them. However, because it is not clear which mechanism is responsible for such improvement, our purpose was to clarify that point.We conducted a cross sectional study involving 30 ESRD patients, 30 ESRD kidney-transplanted patients (Ktx) and 30 healthy controls (C) to evaluate platelet morphology and function, atherothrombotic profile, endothelial abnormalities and cytokine levels involved in the insulin resistance/endothelial dysfunction. (i) *Platelet morphology*: The ESRD group showed platelet size similar to the other two groups (ESRD = $3518 \times 10^3 \pm 549 \times 10^3$ nm^2, C = $3075 \times 10^3 \pm 197 \times 10^3$ nm^2, Ktx = $2862 \times 10^3 \pm 205 \times 10^3$ nm^2) with similar platelet granules and number. (ii) *Platelet surface glycoprotein*: The CD41 and P-Selectin were similar between groups. (iii) *Platelet intracellular calcium*: Resting intracellular calcium was statistically higher in ESRD compared to the C group (ESRD = 182.1 ± 34.5, Ktx = 126.7 ± 14.1, C = 72.0 ± 11.0 nM, $P < 0.01$). (iv) *Hypercoagulability markers and natural anticoagulants*: The Ktx and ESRD groups showed higher levels of hypercoagulability markers compared to the C group. A reduction in antithrombin activity was evident in ESRD compared to the Ktx group ($P = 0.03$). (v) *Endothelial morphology*: The ESRD group showed a thickened vessel basal membrane compared to the Ktx and C groups with more endothelial sufference. (vi) *Insulin resistance and pro-inflammatory cytokine profile*: The ESRD showed a higher homeostasis model assessment provided equations for estimating insulin resistance (HOMA-IR) compared to the Ktx and C groups (ESRD = 2.6 ± 0.3, Ktx = 1.8 ± 0.2, C = 1.1 ± 0.1, $P = 0.005$) and increased soluble tumor neurosis factor α (sTNFα) ($P < 0.05$) and soluble vascular cell adhesion molecule (sVCAM) levels ($P < 0.01$). Positive correlations were evident among HOMA-IR and sTNFα ($P < 0.001$) and sVCAM ($P = 0.01$), respectively. In a small subgroup of ESRD who underwent Ktx (five pts), our findings were confirmed at 1 year of follow-up, suggesting an improvement of almost haemostatic abnormalities. Kidney transplant is associated with a better atherothrombotic profile in ESRD, platelet intracellular calcium and cytokines seem to be most influenced by the transplant, while most morphological abnormalities are retained.

Introduction

Patients affected by end-stage renal disease (ESRD) are at high risk for several haemostatic and cardiovascular disorders [1–4]. Platelet activation, increased platelet size and a pro-thrombotic state are observed in ESRD and could contribute to increase cardiovascular risk [5–7].

Previous studies have shown that platelets' calcium homeostasis, involved in the mechanism of signal trasduction, is impaired in ESRD patients [8]. A rise in resting intracellular calcium levels $[Ca^{2+}]_i$ could be considered a sign of activation of circulating platelets [9]. Again, in ESRD a reduced expression of platelet surface glycoprotein is evident [2]. Among these surface glycoprotein the most important are: CD41/P-Selectin, which mediate platelet adhesion and aggregation and are an index of normal platelet function [10] and thrombin receptor 1 and 3, which, once activated, cause a rapid increase in intracellular platelet calcium [11,12]. Finally, abnormalities of both the clotting system and endothelium have been implicated in the pathogenesis of vascular complications in ESRD patients [13]. In particular, endothelium modulates platelet adhesion, macrophage migration, lipid transport and mitogenesis [14]. Platelets and endothelium could be activated both by insulin resistance and by enhanced pro-inflammatory cytokine levels [15].

It seems clear that kidney transplant (Ktx) improves the haemostatic balance in ESRD patients [16], particularly in those with diabetes [17], but the real mechanism for the basis of this improvement is still not completely understood. It is not clear to what extent the use of immunosuppressive drugs is deleterious for haemostatic axis [16]. Our aim is to study platelet morphology and function (platelet granule content and surface receptor), hypercoagulability markers [fibrinogen (Fg), D-Dimer, pro-thrombin fragments 1 + 2], natural anticoagulants [antithrombin (AT), protein C (PC) and protein S (PS)], endothelial morphology, insulin resistance (HOMA) and pro-atherosclerotic cytokine (IL-18, sTNFα and sVCAM) levels in ESRD and in Ktx patients. We used a morphological and functional cross-sectional study to better address which parameters are responsible for the improved haemostasis observed in kidney-transplanted patients [16,17].

Patients and methods

Patients and study designs

This is a cross-sectional study comparing three groups of patients: ESRD patients undergoing hemodialysis, ESRD patients who received a Ktx and healthy control subjects (C). The ESRD patients enrolled were on the waiting list for a Ktx. ESRD ethiology are described in Table 1. The cross-sectional approach is necessary; given that HLA matching is required for Ktx and that we have more than 200 patients on our waiting list. It is difficult to know who will be tranplanted, and it is not possible to study all the patients. Five patients of the ESRD cohort were transplanted after our study, so that we decided to study after 1 year of follow-up.

The study was conducted from June 2001 to June 2003, and all transplanted patients consecutively admitted to San Raffaele Hospital, Milan for regular check-up were

Table 1. General characteristics of patients with end stage renal disease (ESRD) and who underwent a kidney transplant (Ktx) and control subjects (C). The ESRD group has higher creatinine, insulin and triglyceride levels compared to Ktx and C groups.

	ESRD (30 pts)	Ktx (30 pts)	C (30 pts)
Age (years)	32.2 ± 0.8	31.9 ± 1.0	31.8 ± 0.6
Dialysis duration (years)	3.5 ± 0.6	3.2 ± 0.7	N/A
Transplant follow-up (months)	N/A	42.3 ± 10.6	N/A
Creatinine (mg/dl)*	11.7 ± 0.9	2.1 ± 0.7	0.8 ± 0.2
Fasting glucose (mg/dl)	84.9 ± 4.8	82.7 ± 2.5	74.3 ± 2.4
Free insulin levels (mU/L)*	13.4 ± 2.1	8.8 ± 1.3	6.6 ± 0.6
HOMA-IR*	2.65 ± 0.36	1.81 ± 0.29	1.12 ± 0.11
Triglycerides (mg/dl)*	260.7 ± 59.9	119.2 ± 11.5	90.1 ± 10.1
Cholesterol (mg/dl)	182.4 ± 16.1	164.3 ± 11.8	175.4 ± 11.1
sVCAM (ng/ml)*	1493.9 ± 74.9	1133.3 ± 86.7	759.6 ± 60.8
sTNFα (pg/ml)*	22.1 ± 8.7	8.7 ± 2.0	9.7 ± 3.6
IL-18 (pg/ml)	552.3 ± 90.3	759.6 ± 121.9	494.8 ± 156.1
Patients' etiology for ESRD	9 Glomerulosclerosis 6 Glomerulonephritis 5 Hypertension 6 IgA nephropathy 4 Undiagnosed	8 Glomerusclerosis 7 Glomerulonephritis 6 Hypertension 6 IgA nephropathy 3 Undiagnosed	N/A

HOMA-IR, homeostasis model assessment provided equations for estimating insulin resistance; sVCAM, soluble vascular cell adhesion molecule; sTNFα, soluble tuma neurosis factor α.
*$P < 0.05$.

included in the study once provided they met the inclusion/exclusion criteria. Only those transplanted patients with a follow-up greater than 1 year and with good graft function were included in the study. Patients and controls with clear signs of systemic infection, lymphoproliferative disease or urinary infection were excluded from the study. Patients taking an oral anticoagulant or an antiplatelet therapy were also excluded from the analysis. None of the enrolled subjects had impaired fasting glucose levels. Healthy volunteers were enrolled as control subjects.

All subjects provided an informed and written consent prior to study enrolment and the study was approved by the Institutional Review Board.

Clinical characteristics of the patients

All patients included in the ESRD and Ktx groups were screened for major cardiovascular risk factors. Because they were on our waiting list for a Ktx, they underwent cardiovascular assessment (i.e. ECG, radionuclide left ventriculography, myocardial perfusion scintigraphy) to evaluate the presence of coronary angiopathy. Patients with signs of reduced coronary blood reserve underwent coronary angiography and were not considered for the study. All patients were in sinus rhythmn. Regarding smoking status, 18 of 30 patients in the C group were smokers, while only four and two were smokers in the ESRD and Ktx groups, respectively (C compared to ESRD and Ktx, $P < 0.05$).

All ESRD patients were hypertensive and 25 of 30 patients in the Ktx group were hypertensive (ns), while none in the C groups ($P < 0.05$ vs. Ktx and ESRD). There were no differences between the ESRD and Ktx groups, in term of cardiovascular medications, except for ACE inhibitors, which are more common between ESRD groups (25/30) as compared to the Ktx group (10/30) ($P < 0.05$). None of the Ktx patients considered were on statin therapy, while 10 patients in the ESRD were on statin treatment ($P < 0.05$). Finally, all ESRD patients were on erythropoietin therapy according to new treatment regulations, while only two patients in the Ktx group were on the same treatment.

Transplantation and immunosuppression

Organs for transplantation were obtained from cadaver donors through Nord Italia Transplant (NITp, Milan, Italy). Immunosuppression was maintained using cyclosporine (CyA) (therapeutic range between 100 and 250 ng/ml), mycophenolate mofetil (500–2000 mg/day) and methylprednisolone (10 mg/day). Steroids were withdrawn after 6 months following transplant. None of the enrolled patients were on steroids or FK506 at the moment of the study.

Intracellular calcium in platelets

Blood was drawn by clean puncture from an antecubital vein and collected into plastic tubes containing 1 ml of ACD-solution (0.065 mol citric acid, 0.085 mol sodium citrate), 2% glucose monohydrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 220 *g* for 15 min at room temperature and the supernatant was collected. Platelet $[Ca^{2+}]_i$ was evaluated according to the method previously described [18].

$[Ca^{2+}]_i$ was evaluated fluorometrically under resting conditions and after the addition of human thrombin at 0.05, 0.1 and 0.5 U/ml (final concentration). The three doses of thrombin were added in a randomized fashion as described [8,9]. The parameters analysed were resting calcium $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ curve, $[Ca^{2+}]_i$ plateau and $[Ca^{2+}]_i$ peak.

Flow cytometry analysis (FACS)

Expression of GpII/IIIa (CD41), P-Selectin (CD63) and PAR-1 and PAR-3 receptors was examined by flow cytometry analysis of PRP aliquots as previously described [19], using the following antibodies: anti-PAR-1 and anti-par-3 polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-P-Selectin (CTB201) monoclonal antibody (Santa Cruz Biotechnology Inc.) and antihuman CD-41a (BD Biosciences, San Diego, CA). Results are expressed as mean log fluorescence intensity versus number of cells.

Electron microscopy (EM) of platelets

Aliquots of PRP were fixed in 2% Karnovsky solution for electron microscopy of platelets to evaluate platelet size, morphology and granule content [20]. Ultrastructural analyses were carried out on four cases for each group.

Measurements were performed using the 'Measure arbitrary area' tool of AnalySIS Image Processing 3.0 software (Soft Imaging System GmbH, Münster, Germany), which permits measurement of the areas of platelets and granules.

Laboratory analysis

Platelet-poor plasma was obtained within 2 h by centrifugation at room temperature for 10 min at 1500 *g*. Determinations of prothrombin time (PT), activated-partial thromboplastin time (aPTT), Fg, AT, D-Dimer fragments (D-Dimer), PC and PS were carried out on fresh plasma samples as previously reported [21,22].

To monitor changes in *in vivo* thrombin generation, plasma levels of prothrombin fragments 1 + 2 (F_{1+2}) were

measured with a commercial enzyme-linked immunoabsorbent assay (ELISA; Enzygnost by F_{1+2}; Dade-Behring, Milan, Italy). Human sVCAM-1 levels were assayed with specific ELISA kits (Bender MedSystems GmbH, Vienna, Austria) with no cross-reactivity for members of the immunoglobulin superfamily. Human sVCAM-1 had a minimum detectable dose of 0.9 ng/ml. Human IL-18 levels were assayed with an ELISA kit (Bender MedSystems GmbH). The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample without detectable cross-reactivity. The limit of detection was determined to be 55 pg/ml. Human TNFα levels were assayed with an enzyme-linked immunosorbet assay kit (Bender MedSystems GmbH) with a minimum detectable dose of 2.5 pg/ml.

Homeostasis model assessment provided equations for estimating insulin resistance (HOMA-IR)

$$\text{HOMA} - \text{IR} = G_0 \bullet I_0/22.5$$

where I_0 (μU/ml) is the fasting insulin concentration, G_0 (mmol/l) is the fasting glucose concentration and 22.5 represents a constant applied to correct the value to unity as previously described [23].

Skin biopsy

Patients underwent skin punch biopsy on the internal surface of the arm [24,25]. Microvessel lesions and endothelial injury were evaluated at ultrastructural examination.

For this analysis six specimens for each group were analysed. A mean of 15 vessels was considered for each case in order to evaluate the basal membrane thickness, the collapse of the lumen, the microvillar ramification, the dilatation of the endoplasmic reticulum, the presence of vimentin-like filaments and Weibel–Palade granules and the nuclear aspect (chromatin condensation and irregular nuclear coutour). These morphological aspects were scored on a scale from 0 (normal) to 3 (highly pathological). The measurement of the basal membrane thickness and the collapse of the lumen were performed using the 'Measure arbitrary distance' and the 'Measure arbitrary area' tool of AnalySIS Image Processing 3.0 software (Soft Imaging System GmbH, Münster, Germany).

Statistical analyses

Data were analysed using SPSS statistical package for Windows 10.1 (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as mean ± standard error and were tested for normal distribution with the Kolmogorov–Smirnov test and for homogeneity of variances with Levene's test. Two-sided paired Student's *t*-test (for parametric data) and Wilcoxon test (for nonparametric data) were used to compare pretransplant parameters versus 1-year follow-up data. When more than two groups were compared cross-sectionally, ANOVA (for parametric data) or the Kruskal–Wallis test (for nonparametric data) was used according to distribution. When ANOVA was used, multiple *post hoc* comparison analysis was performed with a Tukey test. Correlations were assessed with a multivariate model. A P value of less than 0.05 (by two-tailed testing) was considered an indicator of statistical significance.

Results

Patient characteristics

This cross-sectional study included 30 ESRD, 30 ESRD Ktx and 30 healthy controls (C). Table 1 displays demographics and clinical characteristics of the study population. Cardiovascular conditions were similar between the Ktx and ESRD groups, with no differences for previous myocardial infarctions, lipid status and smoking habit. All the patients showed a normal platelet count.

Platelets morphology

Electron microscopy for the C group showed numerous platelets with many granules (mean 35.5 granules/platelet) (mainly α type), with some tubules and vesicles and very little glycogen β particles (Fig. 1, Panel a). In the ESRD group platelets have an irregular shape, numerous granules (mean 35.7 granules/platelet) (mainly α type), some tubules and vesicles, almost no nearly glycogen particles (Fig. 1, Panel b). In the Ktx group platelets have numerous granules (mean 33.0 granules/platelet) (mainly α type), some tubules and vesicles; the glycogen β particles are present as little agglomerates (Fig. 1, Panel c). The ESRD group showed a nonstatistical higher platelet size than the other two groups (ESRD $= 3518 \times 10^3 \pm 549 \times 10^3$ nm^2, C $= 3075 \times 10^3 \pm 197 \times 10^3$ nm^2, Ktx $= 2862 \times 10^3 \pm 205 \times 10^3$ nm^2) with similar number and size of platelet granules (Fig. 1, Panels a–f).

Platelets intracellular calcium

The three groups showed a different behaviour of calcium curve profile (Fig. 2, Panels a–d). Resting $[Ca^{2+}]_i$ was statistically higher in ESRD compared to C groups (ESRD $= 182.1 \pm 34.5$, Ktx $= 126.7 \pm 14.1$, C $= 72.0 \pm 11.0$ nM, $P < 0.01$) (Fig. 2, Panels d). After stimulation with thrombin both ESRD and Ktx groups retained high calcium levels at the plateau, without a normal refilling of calcium storage

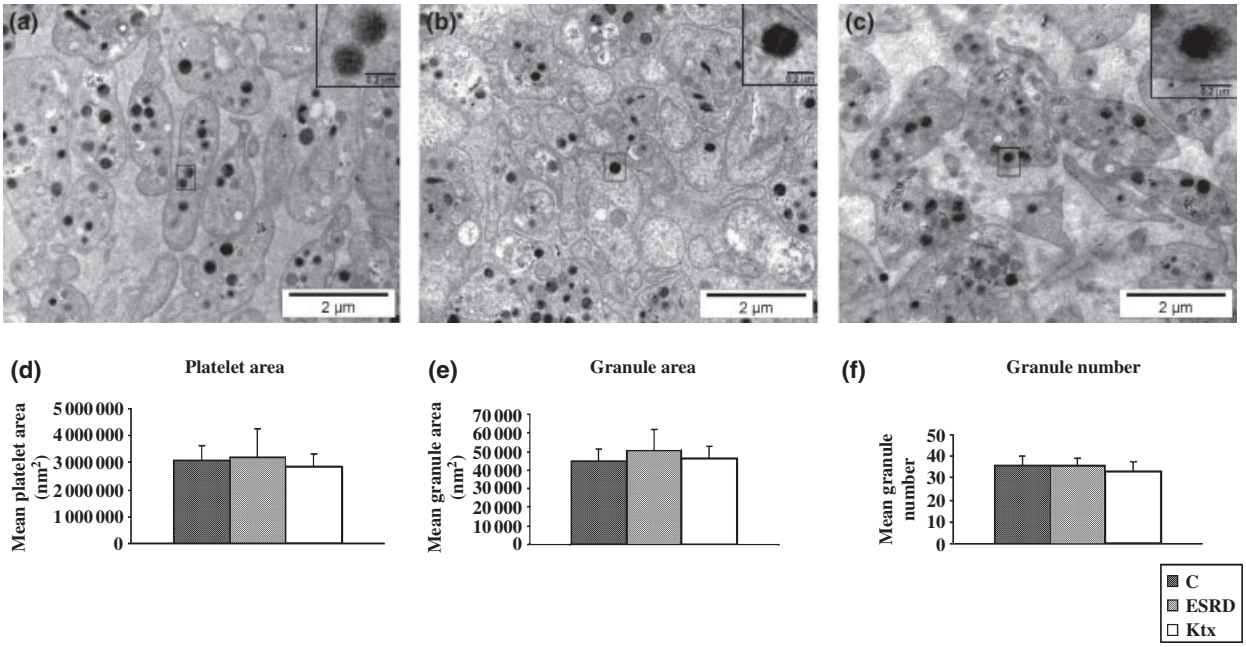

Figure 1 Electron microscopic view of human platelets. Platelets of a healthy subject (C group) (Panel a) (original magnification 11.000×). As shows the shape is extended, the granules are numerous (mean 35.50 granules/platelet) (mainly α type), some tubules and vesicles remain with very little glycogen β particles. The insert shows granules that have a mean area of 44 580 nm^2. Platelets of patients with end-stage renal disease (ESRD) (Panel b) (original magnification 11.000×). We demonstrated that the platelets show an irregular shape, numerous granules (mean 35.75 granules/platelet) (mainly α type), some tubules and vescicles and almost no granules of glycogen. The insert shows the granules that have an area with a mean of 50 100 nm^2. Platelets of a kidney transplant patient (Ktx group) (Panel c) (original magnification 11000×). In this picture we show the main characteristics of this patient: irregular platelet shape, numerous granules (mean 33 granules/platelet) (mainly α type), some tubules and vesicles and glycogen β particles are present as little agglomerates. The insert shows the granules that have a mean area of 46 400 nm^2. Score of electron microscopic characteristics of human platelets of healthy subjects (C), of ESRD patients and Ktx patients. Platelets of the ESRD group showed nonstatistical increase in platelet size (Panel d), while similar values for granule area (Panel e) and granules number (Panel f) were evident.

(ESRD = 294.4 ± 32.6, Ktx = 293.3 ± 33.5, C = 173.5 ± 14.3 nM, $P = 0.01$) (Fig. 2, Panels a–c). The Ktx group showed a higher peak of calcium compared to both the C and ESRD groups at each of the three thrombin stimuli (Fig. 2, Panels a–c).

Platelez surface glycoprotein

The groups showed similar levels of CD41 and P-Selectin platelet expression, even if a trend toward a reduction in both receptors' expression is evident in ESRD compared to the Ktx and C groups (Fig. 3, Panels a and b). The three groups showed similar levels of expression for both thrombin receptors (Fig. 3, Panels c and d).

Hypercoagulability markers

The Ktx and ESRD groups showed higher levels of hypercoagulability markers (Fg, D-Dimer and F_{1+2}) compared to the C group. Fg and F_{1+2} appeared to be higher in ESRD and Ktx compared to C groups ($P < 0.01$ and <0.05, respectively) (Fig. 4, Panels a–c).

Natural anticoagulants

A reduction in AT anticoagulant activity was evident in ESRD compared to the Ktx group ($P < 0.05$). A trend toward an increase in PC and S activity was evident in the Ktx and C groups compared to ESRD (Fig. 4, Panels d–f).

Endothelial morphology

At immunohystochemistry, the vessel lumen of the C group is correctly expanded and the basal membrane is thin, while the skin biopsy specimen of the ESRD group showed that the vessel lumen is much collapsed and the basal membrane is quite thick. In the Ktx group the vessel lumen is moderately collapsed and the basal membrane is fairly thin. These features were confirmed with electron microscopy. The ESRD group showed a thickened vessel basal membrane compared to the Ktx and C groups [ESRD = 1186 (range 652–2608) nm, Ktx = 1042 (range 688–1380) nm and C = 505 (range 178–1015) nm] (Fig. 5, Panels a–f). The endothelial cells of cutaneous capillaries of the ESRD group showed more cell injury

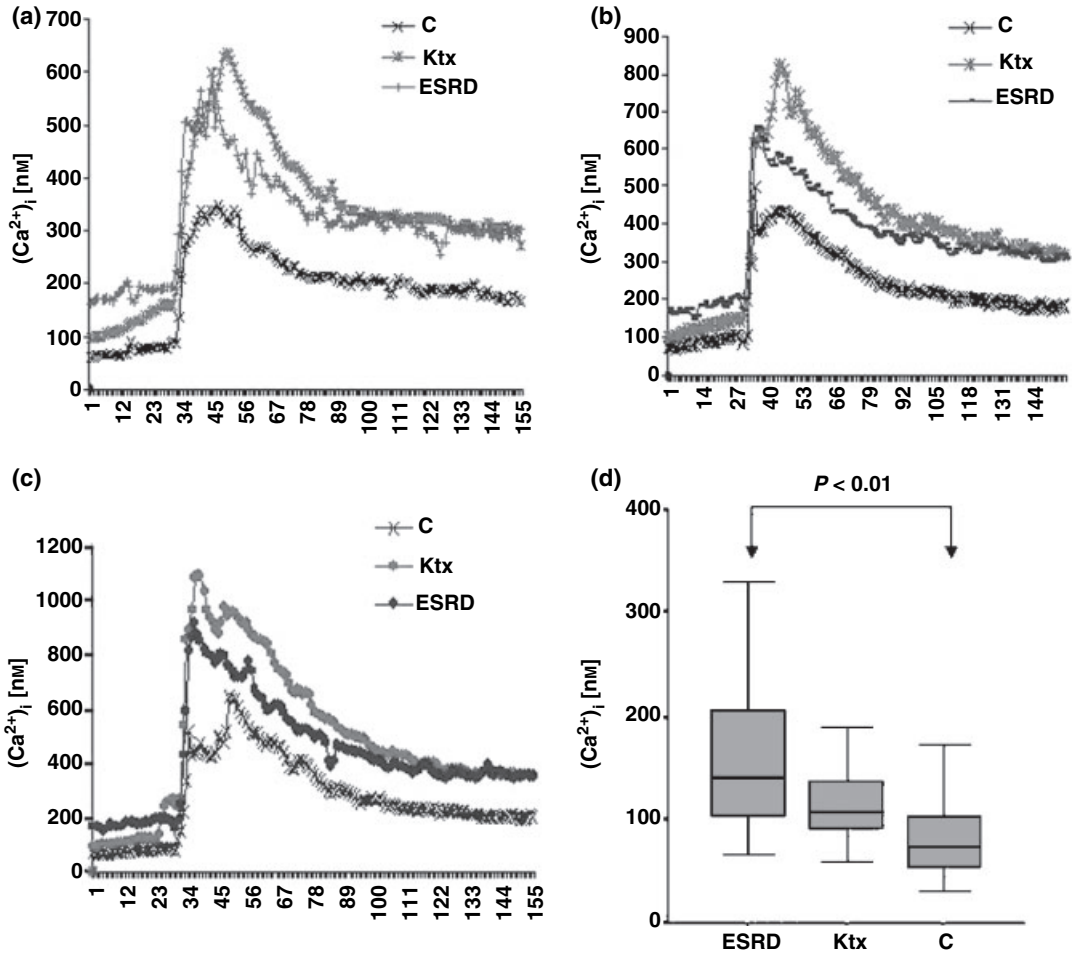

Figure 2 Platelet calcium curve profile after thrombin stimulus (0.05 U) (Panel a), (0.1 U) (Panel b), (0.5 U) (Panel c) and resting calcium ($P < 0.01$) (Panel d) in control subjects (C), in patients with ESRD and with ESRD who underwent a kidney transplant (Ktx). Ktx group showed a near normal fashion of resting calcium but retained higher calcium plateau levels, as did the ESRD group.

signs compared to capillaries of the Ktx and C groups (Fig. 5, Panels a–f). Particularly, in the ESRD group more collapsed lumen ($P = 0.05$), dilated endoplasmic reticulum, condensation of vimentin filaments and irregular nuclear contour with chromatin condensation were evident compared to Ktx and ESRD groups (Fig. 5, Panels a–f). A progressive increase of basal membrane thickening and collapsed lumen is evident from C to Ktx and to ESRD (Fig. 5, Panels g and h), while Ktx retained signs of endothelial sufference (expressed as microvilli ramification) (Panel i).

Insulin resistance and pro-inflammatory cytokine profile

The ESRD showed higher insulin levels ($P < 0.01$) and HOMA-IR values (ESRD = 2.6 ± 0.3, Ktx = 1.8 ± 0.2, C = 1.1 ± 0.1, $P < 0.01$) compared to Ktx and C groups and higher sVCAM ($P < 0.01$) and sTNFα ($P < 0.05$), but not IL-18 levels (Table 1). Positive correlations were evident among HOMA-IR and sTNFα ($\beta = 0.55$, $P < 0.001$) and sVCAM ($\beta = 0.34$, $P = 0.01$) in all populations.

Sub-analysis after 1 year of follow-up in the ESRD who underwent kidney transplant

Five patients underwent Ktx according to HLA allocation after our haemostatic studies, so we recalled them after 1 year of follow-up to revaluate their haemostatic axis.

In this cohort of patients, despite the short-term follow-up, a significant improvement of many haemostatic parameters was evident (Table 2). Particularly, a statistical reduction of platelet intracellular calcium and hypercoagulability markers was evident (Table 2). Furthermore, some morphological features seem to improve, even with-

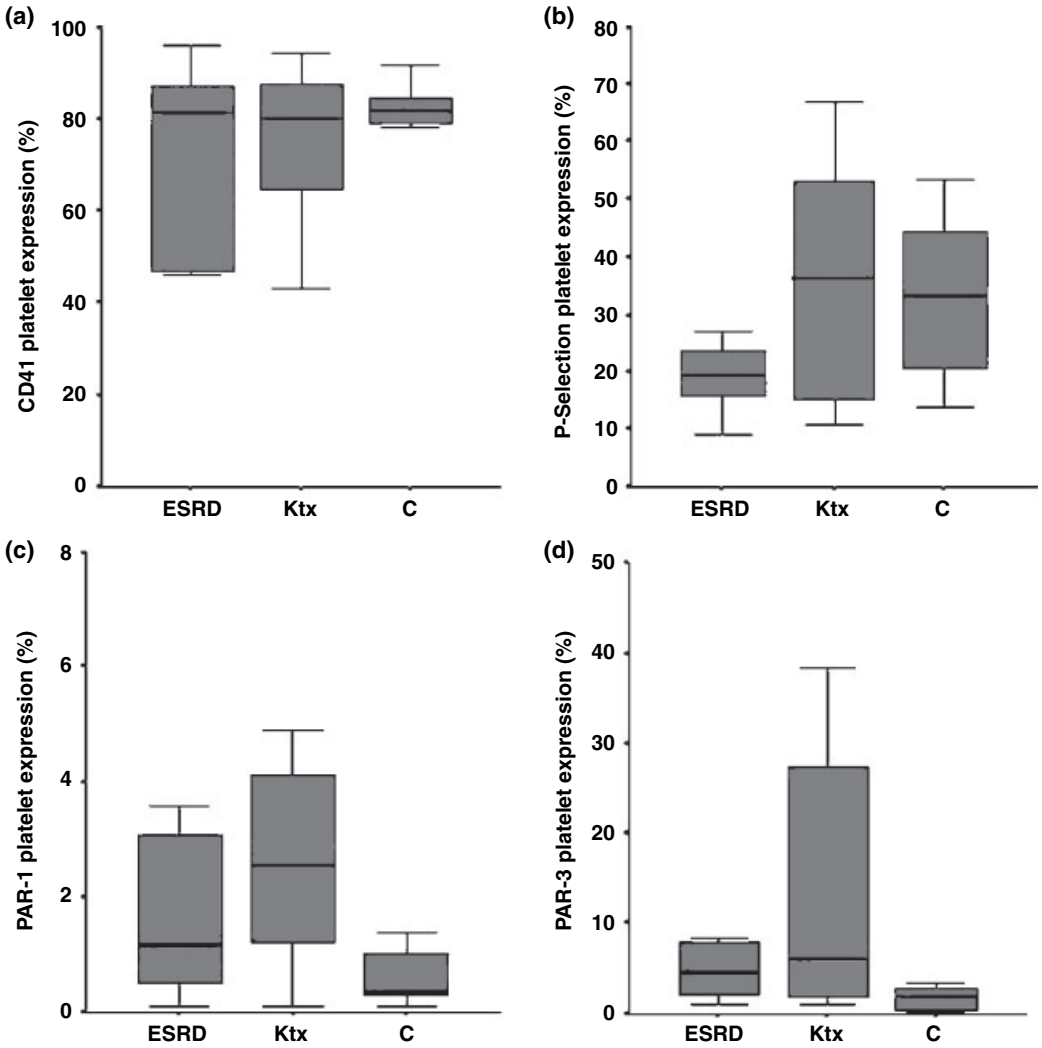

Figure 3 CD41 (Panel a), P-Selectin (Panel b), PAR-1 (Panel c) and PAR-3 (Panel d) platelet expression in control subjects (C), in patients with end-stage renal disease (ESRD) and with ESRD who underwent a kidney transplant (Ktx). The Ktx group did not normalize CD41, PAR-1 and PAR-3 platelet expression.

out reaching statistical significance (i.e. platelet area and lumen basal membrane thickness).

Discussion

Kidney transplant in ESRD is associated with improved microvascular function and atherothrombotic profile. However, morphological abnormalities of the platelet and endothelium cannot be reversed completely. An important improvement from a functional point of view (i.e. intraplatelet calcium profile, pro-thrombotic markers, cytokine levels and insulin resistance) can be achieved, suggesting the importance of shortening the waiting list time to reduce the cardiovascular events in ESRD population. This tendency was confirmed in the small group of ESRD who underwent Ktx according to HLA after our studies. It is possible that immunosuppressive drugs do not allow a complete normalization of haemostatic abnormalities. The major differences in our papers are between ESRD and the control group. This probably means that transplant cannot normalize all the haemostatic abnormalities or simply that a longer follow-up is required.

A not statistically significant lower platelet volume is evident in the Ktx group, with a near-normal calcium homeostasis, particularly for resting calcium levels. On the contrary, an abnormal calcium refilling is evident in the Ktx and ESRD groups, as shown by higher calcium plateau. This could be due, at least in the Ktx group, to the usage of CyA.

Cyclosporine has been implicated in increasing cardiovascular morbidity and mortality following renal transplantation. Impairment of the fibrinolytic system is one

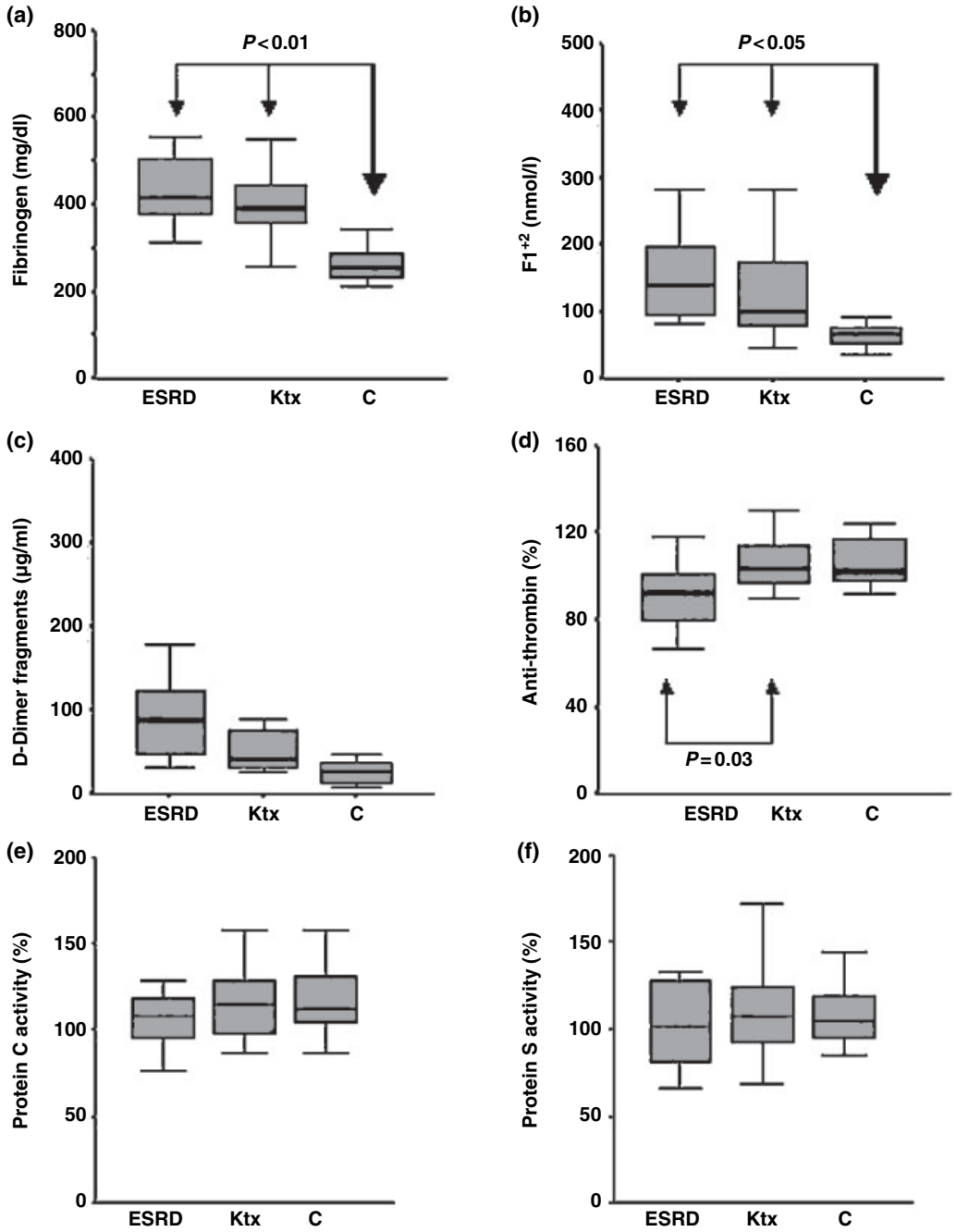

Figure 4 Fibrinogen (Fg) (Panel a), D-Dimer fragments (D-Dimer) (Panel b) and pro-thrombin fragments (F_{1+2}) (Panel c) appeared to be higher in patients with end-stage renal disease (ESRD) and with ESRD who underwent a kidney transplant (Ktx) compared to C groups. A reduction in anti-thrombin anticoagulant activity (Panel d) was evident in ESRD compared to the Ktx group ($P = 0.03$). A trend toward an increase in Protein C (Panel e) and S (Panel f) activity was evident in Ktx and C compared to ESRD groups.

factor involved in the development of thrombotic complications. A work by Malyszko J *et al.* showed that patients on triple immunosuppressive treatment displayed longer PT and aPTTs; higher Fg, platelet counts and fibrinolytic activity index and lower thrombin generation markers [26]. Two additional *in vitro* studies showed a significant increase in platelet intracellular calcium mobilization in the presence of CyA [27,28]. Finally, an enhanced platelet aggregation in CyA-treated kidney allograft recipients may have clinical implications in regard to the reported

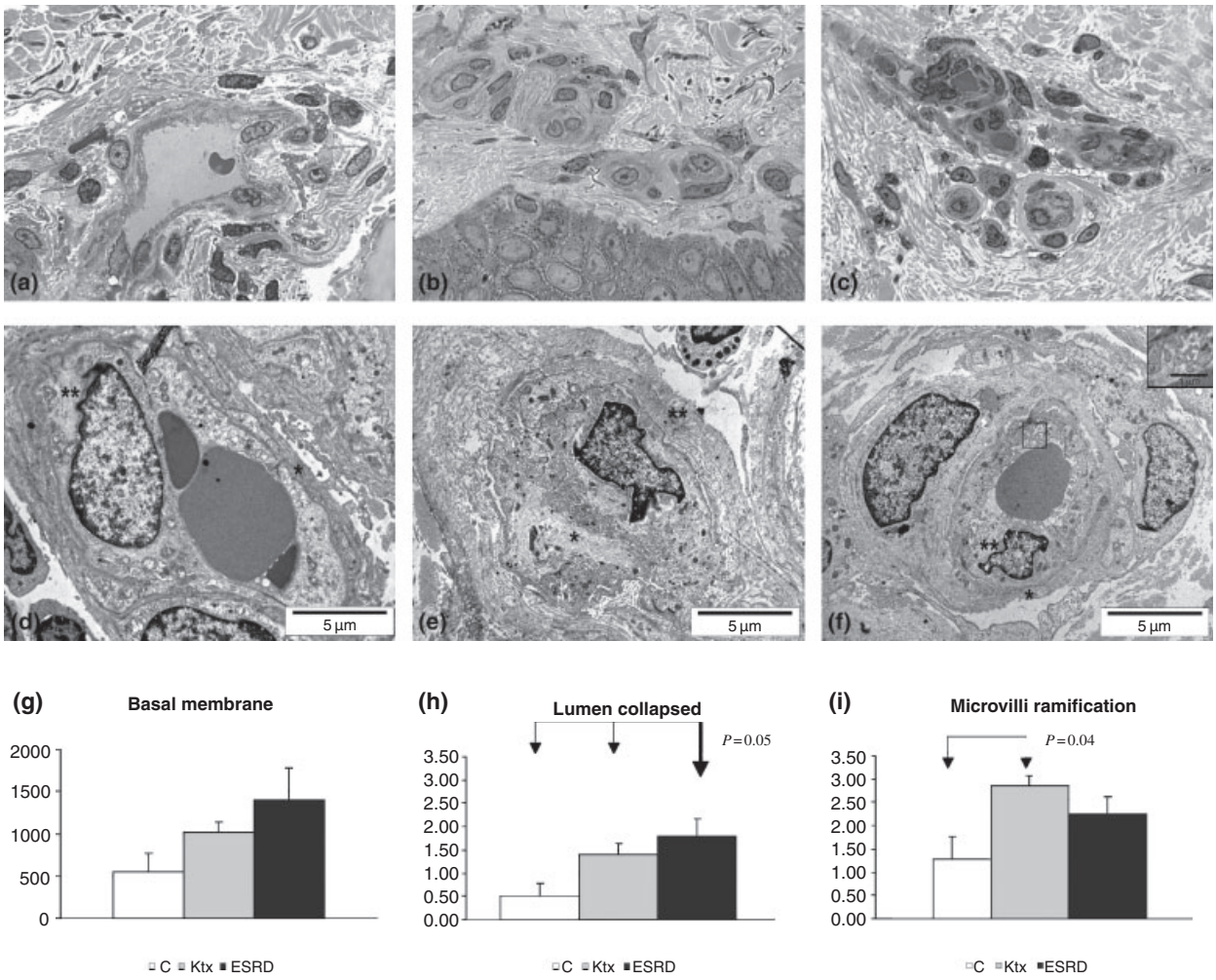

Figure 5 Histochemical analysis of skin biopsy stained with toluidine blue. Skin biopsy specimen of a healthy subject (C group) (Panel a). The vessel lumen is correctly expanded and the basal membrane is thin (original magnification 1000×). Skin biopsy specimen of a patient with end-stage renal disease (ESRD) (Panel b). The vessel lumen is much collapsed and the basal membrane is quite thick (original magnification 1000×). Skin biopsy specimen of a patient with ESRD who underwent a kidney transplant (Ktx group) (Panel c). The vessel lumen is moderately collapsed and the basal membrane is fairly thin (original magnification 1000×). Ultrastructural examination of skin biopsy at electronic microscope. Skin biopsy specimen of a healthy subject (C group) (Panel d) (original magnification 4400×). As shown the vessel lumen is correctly expanded, the endothelial cells are well preserved and basal membrane is thin (A*). Moreover it is possible to see the presence of some intermediate filaments in the cytoplasm (A**). Skin biopsy specimen of ESRD patient (Panel e) (original magnification 4400×). For these patients, the presence of a much collapsed lumen ($P < 0.05$ versus control) is observable, as there are also numerous Weibel–Palade bodies and more intermediate filaments (B*). The basal membrane is quite thick (B**). Skin biopsy specimen of a Ktx patient (Panel f) (original magnification 4400×). For these patients a collapsed moderate lumen is evident, the basal membrane is fairly thin (C*) and filaments bundles are present (C**). The more important characteristic ($P < 0.05$ versus control) observable for these cases is the presence of branched microvilli (see insert). A progressive increase of basal membrane thickening and of collapsed lumen is evident from C to Ktx and to ESRD (Fig. 5, Panels g and h), while Ktx retained signs of endothelial sufference (expressed as microvilli ramification) (Panel i).

tendency of thrombosis in those patients, and to CSA-induced nephrotoxicity. Thus, inhibition of platelet activity in these patients might be of clinical benefit [29]. While substantial in vitro data suggest CyA is prothrombotic, an independent clinical association with thrombosis is unproven [30]. Interventions to reduce thrombotic risk, including heparin, warfarin and aspirin, have been evaluated. In unselected patients at low clinical risk, aspirin (75–150 mg/day) with or without a short period of unfractionated heparin appears to reduce the risk of renal allograft thrombosis significantly with a low risk of bleeding [30].

The increased platelet size and activation seen in the ESRD group could lead to reduced platelet function, as

Table 2. Subanalysis in the small cohort of ESRD, who underwent kidney transplant at 1-year post-transplant.

Parameters	Pretransplant	Post-transplant	*P*-value
Platelet resting calcium (nM)	133.9 ± 8.6	94.8 ± 5.3	0.004
VCAM (ng/ml)	1642.4 ± 166.0	980.8 ± 65.5	0.04
D-Dimer (μg/ml)	94.8 ± 25.4	40.8 ± 10.4	0.04
F_{1+2} (mmol/l)	172.8 ± 36.6	83.2 ± 3.5	0.05
Fg (mg/dl)	424.6 ± 30.4	359.8 ± 42.5	Ns
TNF (pg/ml)	10.8 ± 4.8	4.0 ± 2.4	Ns
IL-18 (pg/ml)	656.2 ± 170.2	1052.8 ± 205.5	Ns
IRI (μU/ml)	12.8 ± 3.9	8.6 ± 0.9	Ns
HOMA	2.1 ± 0.5	1.7 ± 0.1	Ns
Platelet area (nm^2)	3 409 229 ± 625 539	2 614 963 ± 511 275	Ns
CD41 platelet expression (%)	77.8 ± 7.7	82.7 ± 5.4	Ns
PAR-1 platelet expression (%)	4.6 ± 3.3	1.0 ± 0.4	Ns
Lumen basal membrane thickness	2255.0 ± 353.0	1211.0 ± 168.0	Ns

ESRD, end-stage renal disease; VCAM, vascular cell adhesion molecule; D-Dimer, D-Dimer fragments; Fg, fibrinogen; IRI, insulin; HOMA, homeostasis model assessment; Ns, nonsignificant.

shown by low CD41 and P-Selectin platelet expression, leading to the bleeding diathesis in the ESRD group. Furthermore, the clotting system appeared to be impaired in the ESRD group, with high levels of hypercoagulability markers in the directions of a pro-thrombotic state. This pro-thrombotic state is partially no longer evident in the Ktx group. Reduced levels of natural anticoagulants are evident in the ESRD group, while this is no longer observed in the Ktx group. Finally, a better endothelial morphology is evident in Ktx and C groups compared to the ESRD group. All these features are associated with lower insulin resistance and pro-atherosclerotic state in Ktx. In the long term this can lead to platelet malfunctioning, which also produces bleeding tendencies.

Cardiovascular and cerebrovascular events account for the majority of the excess death seen in ESRD patients [8,26]. These two causes account for more than half the death and most occur within 2 years from the beginning of dialysis [8,31]. Haemostatic abnormalities previous studies and we have found, could partially explain the high cardiovascular risk observed in ESRD patients [8,31]. However, a certain degree of haemostatic abnormalities is retained in the Ktx group as well, this could partially explain the proneness of transplanted patients towards atherothrombotic and cardiovascular disease [31]. A pro-inflammatory and pro-atherosclerotic state is evident in ESRD and it increases the risk of cardiovascular events in these patients [15]. However, we did not evaluate in our cohort the rate of cardiovascular events.

$[Ca^{2+}]i$ is involved in the mechanism of signal transduction that leads to platelet activation [8,9]. In particular, $[Ca^{2+}]i$ seems to play a key role in many events that characterize platelet response to stimuli [8,9]. Evaluation of the role of $[Ca^{2+}]i$ as an intracellular messenger requires quantitative measurements of cytosolic-free calcium [8,9]. It is likely that prolonged platelet activation leads to an altered thrombocytopoiesis, which prevent full platelet maturation resulting in their malfunction [32,33]. This is well demonstrated in our study by a reduced expression of CD41 and P-selectin in the ESRD group. In the long-term, low CD41 expression induces an inability to link von Willebrand factor and Fg, leading to platelet malfunction. Our study shows that hypercoagulability markers (D-Dimer fragment, Fg and F_{1+2}) are lower in kidney-transplant compared to ESRD patients. Furthermore, anticoagulant activity is reduced in the ESRD group and this could explain the higher incidence of thromboembolic event in the ESRD group [34]. The incomplete normal haemostasis in the Ktx group may explain the higher incidence of occlusive arterial disease in transplant patients [35].

Histology and immunohistochemistry of skin biopsies did not show any major morphological differences. The alterations most frequently reported in electron microscopy in the ESRD group include thickening of the capillary basement membrane [36], collapsed lumen, cell swelling and dilated endoplasmic reticulum in endothelial cells and separations of interendothelial junctions [17,37–39]. Basal membranes were thicker in the ESRD group than in the Ktx group, perhaps because of the continuous process of endothelial cell death and regeneration [38–39]. The presence of nuclear chromatin condensation, indicating apoptosis in the endothelial cells of the ESRD group, is consistent with previous studies showing a role of ESRD in the induction of apoptosis in endothelial cells [40]. Interestingly, a lower insulin resistance and pro-inflammatory state is evident in the Ktx group as compared to ESRD. It is well know that insulin resistance has deleterious effects on the endothelium and can exacerbate inflammation; this resistance is evident in ESRD and

could contribute to worsen on cardiovascular conditions [41–46]. Insulin resistance in ESRD has been studied previously [43] and seem sto be related to a defect(s) distal to the insulin receptor kinase. Interestingly, the withdrawal of uremia with the Ktx can ameliorate insulin resistance.

The IL-18 has a different behaviour in our casuistry and so it is possible that drugs, which interfere with the IL-2 pathway (e.g. calcineurin inhibitors) could induce a switch in cytokine production [47]. The increase in pro-inflammatory cytokines we showed, has been reported by other authors [48–50] and could be partially related to a reduction in kidney clearance.

The major limitation of this study is the use of a cross-sectional approach. Nevertheless, the homogeneity of the groups in terms of clinical conditions counterbalances this methodological limitation. Furthermore, the perspective approach in a limited number of ESRD patients who underwent Ktx after our studies and were revaluated at 1-year post follow-up confirm our data.

In conclusion, Ktx in ESRD patients is associated with a better microvascular and atherothrombotic profile than in ESRD on the waiting list.

Acknowledgements

We thank Mollie Jurewicz for Editing and Shirine Dada for her helpful suggestions.

References

1. Zwaginga JJ, Iisseldijk MJW, de Groot PG, Vos J, de Bos Kuil RLJ, Sixma JJ. Defects in platelet adhesion and aggregate formation in uraemic bleeding disorder can be attributed to factors in plasma. *Arterioscler Thromb* 1991; **11**: 733.
2. Moal V, Brunet P, Dou L, Morange S, Sampol J, Berland Y. Impaired expression of glycoprotein on resting and stimulated platelets in uraemic patients. *Nephrol Dial Transplant* 2003; **18**: 1834.
3. Mezzano D, Tagle R, Panes O, *et al.* Hemostatic disorders in uremia: the platelet defect, main determinant of the prolonged bleeding time, is corrected with indices of activation of coagulation and fibrinolysis. *Thromb Haemost* 1996; **76**: 312.
4. Gralnick HR, McKeown LP, Williams SB, Shafer BC, Pierce L. Plasma and platelet von Willebrand factor defects in uremia. *Am J Med* 1988; **85**: 806.
5. Rauch U, Ziegler D, Piolot R, *et al.* Platelet activation in diabetic cardiovascular autonomic neuropathy. *Diab Med* 1999; **16**: 848.
6. Winkler J, Fuchs J, Morduchowicz G, Boner G, Sulkiss J, Weinberger I. Circulating aggregated platelets, number of platelets per aggregate and platelet size in chronic dialysis patients. *Nephron* 1997; **77**: 44.
7. Bath PM, Butterworth RJ. Platelet size: measurement, physiology and vascular disease. *Blood Coagul Fibrinolysis* 1996; **7**: 157.
8. Vicari AM, Taglietti MV, Pellegatta F, *et al.* Deranged platelet calcium homeostasis in diabetic patients with end-stage renal failure. A possible link to increased cardiovascular mortality? *Diab Care* 1996; **19**: 1062.
9. Vicari AM, Monzani ML, Pellegatta F, Ronchi P, Galli L, Folli F. Platelet calcium homeostasis is abnormal in patients with severe arteriosclerosis. *Arterioscler Thromb* 1994; **14**: 1420.
10. Tschope D, Schwippert B, Schettler B, *et al.* Increased GPIIB/IIIA expression and altered DNA-ploidy pattern in megakaryocytes of diabetic BB-rats. *Eur J Clin Invest* 1992; **22**: 591.
11. Ishihara H, Connolly AJ, Zeng D, *et al.* Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; **386**: 502.
12. Ishihara H, Zeng D, Connolly AJ, Tam C, Coughlin SR. Antibodies to protease-activated receptor 3 inhibit activation of mause platelets by thrombin. *Blood* 1998; **91**: 4152.
13. Kario K, Matsuo T, Yamada T, Nakao K, Shimano C, Matsuo M. Factor VII hyperactivity in chronic dialysis patients. *Thromb Res* 1992; **67**: 105.
14. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; **340**: 115.
15. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int* 1999; **55**: 648.
16. Amir Kazory, Didier Ducloux. Acquired hypercoagulable state in renal transplant recipients. *Thromb Haemost* 2004; **91**: 646.
17. Fiorina P, Folli F, D'Angelo A, *et al.* Normalization of multiple haemostatic abnormalities in uremic type 1 diabetic patients after kidney-pancreas transplantation. *Diabetes* 2004: **53**: 2291.
18. Rink TJ, Sage SO. Calcium signaling in human platelets. *Annu Rev Physiol* 1990; **52**: 431.
19. Wyant TL, Smith PC, Brown B, Kantor AB. Whole blood microvolume scanning cytometry for monitoring resting and activated platelets. *Platelets* 2001; **12**: 309.
20. Zucker-Franklin D, Kaushansky K. Effect of thrombopoietin on the development of megakaryocytes and platelets: an ultrastructural analysis. *Blood* 1996; **88**: 1632.
21. Vigano D'Angelo S, Comp PC, Esmon CT, D'Angelo A. Relationship between protein C antigen and anticoagulant activity during oral anticoagulation and in selected disease states. *J Clin Invest* 1986; **77**: 416.
22. Viganò D'Angelo S, Tombesi S, Marcovina S, *et al.* Monoclonal antibody-based enzyme-linked immunosorbent assays (ELISA) for measurement of vitamin K-dependent protein S: the effect of antibody immunoreactivity on

plasma protein S antigen determinations. *Thromb Haemost* 1992; **67**: 631.
23. Matthews DR, Hosker JP, Rudenski AS, *et al.* Homeostasis model assessment: insulin resistance and ß-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412.
24. Properzi G, Terenghi G, Gu XH, *et al.* Early increase precedes a depletion of endothelin-1 but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. *J Pathol* 1995; **175**: 243.
25. Fiorina P, Folli F, Bertuzzi F, *et al.* Long-term beneficial effect of islet transplantation on diabetic macro-/microangiopathy in type 1 diabetic kidney-transplanted patients. *Diab Care* 2003; **26**: 1129.
26. Malyszko J, Malyszko JS, Hryszko T, Mysliwiec M. Some aspects of hemostasis in kidney transplant recipients maintained on cyclosporine, azathioprine and prednisone in comparison to patients treated with cyclosporine and prednisone. *Transplant Proc* 2003; (Dec) **35**: 2940.
27. Fox SC, Judge HM, Allen BR, Heptinstall S. Platelet aggregation and intracellular calcium mobilisation responses are enhanced by cyclosporin A but not by pimecrolimus (SDZ ASM 981). *Platelets* 2002; (Jun) **13**: 213.
28. Reis F, Tavares P, Rito LC, *et al.* Platelet activation is increased in cyclosporin A-induced hypertensive rats. *J Cardiovasc Pharmacol* 2000; (Jul) **36**: 56.
29. Malyszko J, Malyszko JS, Takada A, Mysliwiec M. Effects of immunosuppressive drugs on platelet aggregation in vitro. *Ann Transplant* 2002; **7**: 55.
30. Irish A. Hypercoagulability in renal transplant recipients. Identifying patients at risk of renal allograft thrombosis and evaluating strategies for prevention. *Am J Cardiovasc Drugs* 2004; **4**: 139.
31. Dimeny EM. Cardiovascular disease after renal transplantation. *Kidney Int Suppl* 2002; **80**: 78.
32. Tschoepe D, Roesen P, Esser J, *et al.* Large platelets circulate in an activated state in diabetes mellitus. *Semin Thromb Hemost* 1991; **17**: 433.
33. Cagliero E, Porta M, Cousins S, Kohner EM. Increased platelet volume in diabetic retinopathy. *Hemostasis* 1982; **12**: 293.
34. Folsom AR, Aleksic N, Lu Wang, Mary Cushman, Wu KK, White RH. Protein C, antithrombin, and venous thromboembolism incidence. A prospective population-based study. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1018.
35. Ibels LS, Stewart JH, Mahony JF, Sheil AG. Deaths from occlusive arterial disease in renal allograft recipients. *Br Med J* 1974; **3**: 552.
36. Ahonen RE, Makitie J, Kock B. Striated muscle capillaries in uremic patients and in renal transplant recipients. *Arch Intern Med* 1981; **141**: 867.
37. Properzi G, Terenghi G, Gu XH, *et al.* Early increase precedes a depletion of endothelin-1 but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. *J Pathol* 1995; **175**: 243.
38. Mompeo B, Ortega F. Immunohistochemical and ultrastructural study of microvessels in diabetic veins. *Ultrastruct Pathol* 1999; **23**: 25.
39. Helmke BP, Goldman RD, Davies PF. Rapid displacement of vimentin intermediate filaments in living endothelial cells exposed to flow. *Circ Res* 2000; **86**: 745.
40. Matsumoto J, Yanagisawa N, Konoma T, Haizuka H, Nakashima Y, Sato M. Increased Fas antigen in uremia accelerates adhesion of mononuclear cells to endothelial and sinovial cells via stimulated hyaluronan production. *Am J Kidney Dis* 2001; **38** (Suppl. 1): S54.
41. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 2004; **291**: 1978.
42. Lehrke M, Lazar MA. Inflamed about obesity. *Nat Med* 2004; (Feb) **10**: 126.
43. Folli F, Sinha MK, Brancaccio D, Caro JF. Insulin resistance in uremia: in vitro model in the rat liver using human serum to study mechanisms. *Metabolism* 1986; **35**: 989.
44. Caro JF, Lanza-Jacoby S. Insulin resistance in uremia. Characterization of lipid metabolism in freshly isolated and primary cultures of hepatocytes from chronic uremic rats. *J Clin Invest* 1983; **72**: 882.
45. Cecchin F, Ittoop O, Sinha MK, Caro JF. Insulin resistance in uremia: insulin receptor kinase activity in liver and muscle from chronic uremic rats. *Am J Physiol* 1988; **254**: E394.
46. DeFronzo RA, Tobin JD, Rowe JW, Andres R. Glucose intolerance in uremia. Quantification of pancreatic beta cell sensitivity to glucose and tissue sensitivity to insulin. *J Clin Invest* 1978; **62**: 425.
47. Escobar-Morreale HF, Botella-Carretero JI, Villuendas G, Sancho J, San Millan JL. Serum interleukin-18 concentrations are increased in the polycystic ovary syndrome: relationship to insulin resistance and to obesity. *J Clin Endocrinol Metab* 2004; **89**: 806.
48. Macdougall IC. Could anti-inflammatory cytokine therapy improve poor treatment outcomes in dialysis patients? *Nephrol Dial Transplant* 2004; **19** (Suppl. 5): V73.
49. Pertosa G, Grandaliano G, Gesualdo L, Schena FP. Clinical relevance of cytokine production in hemodialysis. *Kidney Int Suppl* 2000; **76**: S104.
50. Chiang CK, Hsu SP, Pai MF, *et al.* Interleukin-18 is a strong predictor of hospitalization in haemodialysis patients. *Nephrol Dial Transplant* 2004; **19**: 2810.