Heparin use during dialysis sessions induces an increase in the antiangiogenic factor soluble Flt1

Frédéric Lavaine1,2,*, Emmanuelle Meffray1,*, Ruth J. Pepper3, Mélanie Néel1, Catherine Delcroix2, Alan D. Salama3 and Fadi Fakhouri1,2

1INSERM UMR S-1064, ITUN, Université de Nantes, Nantes, France, 2Department of Nephrology, CHU de Nantes, Nantes, France and 3Centre for Nephrology, University College London, Royal Free Hospital, London, UK

Correspondence and offprint requests to: Fadi Fakhouri; E-mail: fadi.fakhouri@univ-nantes.fr

*These authors contributed equally.

ABSTRACT

Background. Soluble Flt1 (sFlt1) is a potent inhibitor of vascular endothelial growth factor, secreted mainly by the placenta, endothelial cells and monocytes. Increased sFlt1 serum levels correlate with endothelial dysfunction and cardiovascular complications in dialysis patients. However, the impact of dialysis by itself on sFlt1 serum levels remains unknown.

Methods. We assessed sFlt1 kinetics during dialysis and the impact of different dialysis techniques [high-flux haemodialysis (HD), haemodiafiltration (HDF)] and heparinization procedures on sFlt1 serum levels in 48 patients on regular dialysis.
**Results.** sFlt1 serum levels increased as early as 1 min after the start of dialysis and peaked at 15 min before returning to baseline at 4 h [mean peak level 2551 pg/mL, versus 102 before dialysis (P < 0.0001)]. sFlt1 kinetics were similar with two different dialysis membranes. In contrast, when unfractioned heparin (UH) and low-molecular-weight heparin (LMWH) were omitted during dialysis (HD or pre-dilution HDF), no significant increase in sFlt1 levels occurred. Conversely, delayed administration of LMWH (after 30 min of a heparin-free HD) induced a sharp increase in sFlt1. Similarly, when UH and LMWH were omitted and citrate-based dialysate or a heparin-coated membrane was used, sFlt1 levels remained unchanged. When heparinization procedures were the same, no difference in sFlt1 levels was noted between HD and HDF. In vitro, UH and LMWH failed to induce sFlt1 release by monocytes from controls or HD patients. These findings suggest that priming of monocytes on the extracorporeal circuit is required for heparin-induced sFlt1 release or that endothelial cells contribute to this increase.

**Conclusions.** Our results indicate that heparin-based HD induces a major sFlt1 release, which may exacerbate the antiangiogenic state and thus endothelial dysfunction, commonly found in dialysis patients.

**Keywords:** haemodialysis, heparin, sFlt1, VEGF

**INTRODUCTION**

Cardiovascular disorders remain the leading cause of morbidity in patients with end-stage renal disease (ESRD), and account for roughly half the mortality in these patients [1, 2]. Chronic kidney disease (CKD) by itself, particularly ESRD, is an independent cardiovascular risk factor and is associated with endothelial dysfunction [3, 4]. Traditional cardiovascular risk factors explain only half of the cardiovascular mortality in ESRD patients [5]. Non-traditional risk factors have been identified in CKD/ESRD patients including various markers of inflammation (C-reactive protein, IL6, TNFα) [5–7] and asymmetric dimethyl arginine [8]. Recently, endothelial dysfunction and cardiovascular outcomes in CKD and ESRD patients have been linked to an increase in soluble Flt1 (sFlt1), an inhibitor of the vascular endothelial growth factor (VEGF) [9]. VEGF is the most potent growth factor for endothelial cells. Several types of VEGF receptors have been identified, the two main ones being VEGF-R1 and -R2. A circulating form of VEGF-R1, soluble Flt1 (sFlt1), (molecular weight 78 kDa) [10] is generated through an alternative splicing of VEGF-R1 transcript, but also, probably, from the cleavage of the membrane-bound form of VEGFR1 [11, 12]. sFlt1 is secreted mainly by monocytes, endothelial cells and placental cytrophoblasts and binds and thus inhibits VEGF. Elevated levels of sFlt1 lead to functional VEGF deficiency, and promote an effective ‘antiangiogenic state’. The clinical importance of such a sFlt1-driven ‘antiangiogenic state’ has been illustrated by the association of elevated serum sFlt1 levels with pre-eclampsia [13]. More recently, Di Marco et al. [9] showed that sFlt1 contributes to endothelial dysfunction in patients with CKD. In their study, increased sFlt1 serum levels were correlated to the occurrence of cardiovascular events, particularly myocardial infarction and stroke.

Accumulating experimental data indicate that sFlt1 release may be promoted by inflammation mainly through complement activation (anaphylatoxin C5a) [14, 15]. ESRD is perceived as a state of chronic low-grade inflammation. Besides, haemodialysis (HD) procedures per se exacerbate inflammation, mainly through the contact of blood with materials (dialysis membranes and extracorporeal circuit elements) [16]. Even though significant progress has been achieved in order to increase the biocompatibility of HD material, release of inflammatory cytokines (IL-1, -6 and -8) still occurs during HD sessions with biocompatible membranes [17]. To date, no data regarding the impact of HD by itself on sFlt1 serum levels in ESRD patients have been reported. In the present study, we aimed to assess the impact of various dialysis procedures on the serum levels of sFlt1 in patients with ESRD.

**MATERIALS AND METHODS**

**Patients and serum analysis**

Forty-eight consecutive patients who have started long-time HD for more than 1 month and followed in our institution were included in the study. Their main clinical and biological characteristics are summarized in Table 1. Patients with acute infection, recent surgery or cardiovascular event or acute and profound anaemia (haemoglobin < 8 g/dL) were excluded from the study. Blood samples were drawn from patients on chronic HD (Department of Nephrology and Immunology, CHU de Nantes, France) before and 1, 15, 30 min and 1, 2 and 4 h after the start of a regular 4-h HD or haemodiafiltration (HDF) session. Blood samples before the session were drawn from the arterial needle prior to connecting arterial blood tubing, and all the others samples were drawn from the sample site on the arterial side. Non-heparin-based solution (TAUROLOCK®) was used in the central venous catheters locks. We also included as controls 20 healthy individuals, who underwent venesection. HD sessions were performed

<table>
<thead>
<tr>
<th>Table 1. Clinical and biological characteristics of 48 patients on regular dialysis included in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n = 48</strong></td>
</tr>
<tr>
<td><strong>Female</strong></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td><strong>Time on dialysis (months)</strong></td>
</tr>
<tr>
<td><strong>Fistula/indwelling catheter</strong></td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
</tr>
<tr>
<td><strong>Myocardial infarction/cerebral ischaemia</strong></td>
</tr>
<tr>
<td><strong>Haemoglobin (g/dL)</strong></td>
</tr>
<tr>
<td><strong>CRP (mg/L)</strong></td>
</tr>
<tr>
<td><strong>Albuminaemia (g/L)</strong></td>
</tr>
<tr>
<td><strong>Phosphataemia (mmol/L)</strong></td>
</tr>
<tr>
<td><strong>Erythropoietin use</strong></td>
</tr>
<tr>
<td><strong>Renin–angiotensin system blockers</strong></td>
</tr>
<tr>
<td><strong>Statin use</strong></td>
</tr>
</tbody>
</table>

CRP, C-reactive protein.
using AK200 ULTRA S generator (GAMBRO®) and one of the following membranes: polyester-polyarylate (PEPA-FDX 210 GW, NIKKISO®) or polyacrylonitrile (AN69 ST/NEPHRAL 500 and HeprAN/EVODIAL, HOSPAL®). HDF sessions were performed with the same generator, either using post-dilution and Gambro ULTRACONTROL® system in pressure control mode, or pre-dilution with volume control mode. For haemodiafiltration, PEPA-FDX 210 GW and EVODIAL membranes were used.

Blood flow was between 300 and 350 mL/min for HD and HDF. Dialysate flow was 500 mL/min in HD, 575 mL/min in post-dilution HDF and 700 mL/min in pre-dilution HDF, respectively. In post-dilution and pre-dilution HDF, mean infusion volume/session was 21.6 L (range: 14.6–30) and 44.3 L (range: 34–48), respectively.

Heparinization procedures were different for HD and HDF sessions. During a HD session, the extracorporeal circuit was rinsed with 2 L of heparinized saline using unfractionated heparin (UH) (5000 UI/L) before the start of dialysis. In HDF, priming was on-line with ultra-pure water (no use of UH). Low-molecular-weight heparin (LMWH) was used as maintenance anticoagulation for both, HD and HDF.

The same ultrapure water was used for all dialysis modalities. Anticoagulation consisted of low-molecular-weight heparin (LMWH), enoxaparin, 2000 units injected on the arterial side during the first minute after the start of the session.

Circulating sFlt1 and C5a levels were assayed using commercial ELISA kits according to the manufacturer’s instructions (R&D systems, Minneapolis, MN, USA).

All patients gave informed consent before inclusion in the study, which was approved by the Ethics Committee of the University Hospital of Nantes.

### Human monocytes culture

Peripheral blood mononuclear cells (PBMC) were extracted from blood using Ficoll (Eurobio, Les Ulis, France) and monocytes were isolated using elutriation and cultured as previously described [18]. After stimulation for 30 min with TNFα (2 ng/mL), monocytes were incubated with serum (10%) or various reagents for a variable duration ranging from 30 min to 16 h. In some experiments, monocytes were treated for 1 h with GM 6001 (25 µM), a broad-spectrum inhibitor of the matrix metalloproteinases family (MMPinh) or with an anti-C5a human receptor (αC5aR) monoclonal antibody (10 µg/mL), before incubation with serum from HD patients.

Subsequently, cell supernatants were harvested, cell debris was removed by centrifugation and samples were stored at −20°C until further processing.

Real-time quantitative PCR for the detection of the four sFlt1 isoforms was performed as previously described [18].

### Statistics

All values are means with values range indicated. Data were analysed using one-way ANOVA, paired t-test, and Spearman rank correlation using Prism software (version 4; GraphPad, La Jolla, CA, USA). The normal distribution of the data was confirmed using the Shapiro–Wilk’s test.

### RESULTS

#### Circulating sFlt1 levels increase during a dialysis session

sFlt1 levels were slightly increased at baseline (mean 102 pg/mL; range: 32–237) compared with healthy controls (69 pg/mL; range: 28–168; P = 0.054) (Figure 1). In all patients, an additional increase in serum sFlt1 was noted at all time-points during high-flux HD or post-dilution HDF, starting as early as 1 min (1180 pg/mL; range: 71–6440; P < 0.001) and reaching a maximum at 15 min (2551 pg/mL; range: 194–6393; P < 0.0001) after the start of HD. Serum sFlt1 remained higher compared with baseline levels at 30 min, 1 and 2 h after the start of HD, but decreased to baseline levels by 4 h (Figure 1).

#### Impact of the type of dialysis membrane on sFlt1 levels during HD

To assess the impact of the type of membrane on sFlt1 levels during HD, 10 consecutive patients underwent HD using successively PEPA-FDX or NEPHRAL 500 membranes, and sFlt1 serum levels were assessed at different time-points. Serum sFlt1 levels did not differ between the two types of membranes at all time-points (P = 0.5, paired t-test) (Figure 2).

#### Impact of dialysis techniques and/or heparin on sFlt1 levels during dialysis

In order to assess the impact of dialysis procedures on sFlt1 kinetics, 27 consecutive patients underwent successively high-flux HD and post-dilution HDF using the same membrane (PEPA-FDX) and sFlt1 serum levels were assessed at different time-points (Figure 3). Serum sFlt1 levels were significantly higher during HD compared with HDF at all time-points, particularly, at 15 min [2987 pg/mL (577–5655) for HD versus 888 pg/mL (344–2807) for HDF] (P = 0.01 t-paired test, for all time-points).

**Figure 1**: sFlt1 serum levels dramatically increased during a dialysis session in 48 consecutive patients on regular haemodialysis (HD) or haemodiafiltration (HDF). Maximum levels of sFlt1 were reached 15 min after the start of dialysis. *P < 0.001 versus t0, 1, 2 and 4 h. **P < 0.0001 versus all other time-points. ***P < 0.001 versus all time-points except for 15 min. †P = 0.04 versus t0.
There were two main differences in our unit between these two dialysis modalities: on the one hand, the technique itself (HD versus HDF) and, on the other hand, the heparinization procedures. In order to test the respective roles of techniques and heparinization procedures, 11 consecutive patients underwent dialysis sessions with three different modalities: (i) ‘conventional’ HD session (extracorporeal circuit rinsed with heparinized saline, UH 5000 UI/L, and LMWH used for maintenance anticoagulation), (ii) a post-dilution HDF session (priming on line, with ultra-pure water, LMWH used for maintenance anticoagulation) and (iii) a HD session during which the dialysis circuit was not rinsed with heparinized saline but pre-filled with ultra-pure water (no use of either UH or LMWH). With modality A, sFlt1 increase was completely prevented during the first 30 min of dialysis. LMWH injection was followed by a significant and rapid increase in sFlt1 serum levels ($P < 0.01$ versus all time-points except 1 h in modality A). In contrast, with modalities B, C and D, no increase in sFlt1 levels occurred during the dialysis session.

LMWH dose was the same for each patient in the three modalities.

When the rinsing of the extracorporeal circuit with heparinized saline was avoided during an HD session (modality C), sFlt1 increase was blunted compared with ‘conventional’ HD ($**P < 0.001$). When heparinization procedures were the same, there was no difference in sFlt1 kinetics between HDF (B) or HD (C) ($P = NS$).
subsequently underwent a pre-dilutional HDF session without any use of heparin. sFlt1 serum levels remained unchanged compared with baseline levels during dialysis (Figure 4).

A heparin-coated membrane does not induce circulating sFlt1 levels increase

Finally, we assessed the effect of a heparin-coated membrane (EVDIAL+) on sFlt1 serum levels. Eight patients underwent a pre-dilutional HDF session, with standard dialysate concentrates (bicarbonate-based buffer/BiCart Select+, acetic acid-based concentrate/SelectBag+ One) but without the use of either UH or LMWH. No increase in sFlt1 levels occurred during dialysis (Figure 4). Similarly, when these patients underwent an HD session, with EVDIAL+ and a citrate-based dialysate (Citrates+) without the use of either UH or LMWH, no increase in sFlt1 levels was noted.

Change in endothelial cells markers during a dialysis session

Next, we assessed whether the increase of sFlt1 levels was associated with any change in the levels of two markers of endothelial cell activation, sVCAM and soluble E-selectin during dialysis. No significant change in serum levels of sVCAM or E-selectin was detected (Supplementary Figure S1).

Effect of UH and LMWH on sFlt1 secretion by human monocytes in vitro

Our in vivo findings prompted us to test the effect of various types of heparin on sFlt1 release by monocytes. Monocytes from healthy donors (n = 4) and monocytes from HD patients drawn at t0 before the start of a HD session (n = 5) and at 30 min after the start of a heparin-free HD (n = 5) were incubated with UH at different doses (10 and 50 IU/mL) and LMWH (0.5, 5, 25 and 50 IU/mL) for 30 min, 2, 4, 8 and 16 h. No significant and reproducible increase in the release of sFlt1 by monocytes was detected at the various time-points, regardless of the type and dose of heparin or the source of monocytes (data not shown).

Effect of HD patients’ serum on sFlt1 secretion by human monocytes in vitro

Finally, we assessed the impact of HD patients’ serum, drawn before and during a ‘conventional’ HD session, on sFlt1 release by monocytes. Monocytes from healthy donors (n = 4) and monocytes from HD patients drawn at t0 before the start of a HD session (n = 5) and at 30 min after the start of a heparin-free HD (n = 5) were incubated with UH at different doses (10 and 50 IU/mL) and LMWH (0.5, 5, 25 and 50 IU/mL) for 30 min, 2, 4, 8 and 16 h. No significant and reproducible increase in the release of sFlt1 by monocytes was detected at the various time-points, regardless of the type and dose of heparin or the source of monocytes (data not shown).

Besides, an increase in sFlt1 mRNA levels was detected in monocytes drawn from healthy donors following 4-h incubation with serum (n = 5) drawn 1 h after the start of a HD session, compared with control serum (1.52-fold increase compared with control serum (n = 5); P = 0.02, t-paired test).

DISCUSSION

Our data clearly show that heparin, and not dialysis by itself, induces a dramatic increase during dialysis in the serum levels of sFlt1, a potent antiangiogenic factor. During the first hour of a conventional heparin-based HD session, mean sFlt1 serum levels peaked at roughly 25–30 times their baseline levels, reaching values only detected in women with pre-eclampsia [13]. sFlt1 has been linked in most studies, to a worse cardiovascular outcome. For instance in the previous study by Di Marco et al. [9], markers of endothelial dysfunction and the incidence of cardiovascular events in CKD/ESRD patients were correlated to a milder (2-fold the normal range), albeit chronic, increase in sFlt1 levels. In contrast, in the acute setting of myocardial infarction, an increase in sFlt1 was associated with an improved left ventricular function [19]. However, in the setting of chronic heart failure, increased sFlt1 correlated with disease severity and adverse outcomes [20]. Besides, in the peculiar population of patients on HD, increased sFlt1 level was an independent risk factor for cardiac and all-cause mortality [21]. In a relatively limited number of dialysis patients, and in accordance with previously published data [22], we did not detect a significant increase during dialysis in two endothelial activation soluble markers, sVCAM and...
sE-selectin. However, one cannot exclude that the impressive and repeated (thrice weekly) increase in sFlt1 serum levels induced by heparin use during dialysis sessions may contribute in the short term to the impairment of VEGF-induced vasodilatation and in the long term to the impairment of endothelial repair [23] and thus to the high cardiovascular burden in ESRD patients. This point warrants further assessment in prospective long-term studies.

The introduction of UH and later of LMWH represented a key step in the improvement and thus the rapid development of dialysis [24]. HD is a unique situation that allows an in vitro assessment of the impact of heparin on sFlt1 release. Our data clearly indicate that heparin, and not dialysis by itself, is the main inducer of sFlt1 increase during HD sessions. When UFH or LMWH administration was avoided or delayed during an HD or a pre-dilutional HDF session, no significant increase in sFlt1 serum levels occurred. Our data are in accordance with recent reports on the impact of heparin on the release of sFlt1 by two of its main sites of production: the placenta and endothelium/vessel wall [25, 26]. In vitro, heparin induces sFlt1 release by placental explants through the shedding of the extracellular domain of membrane-bound Flt1. Heparins and the endogenous heparanase, an endo-β-D-glucuronidase, also induce the displacement of cell or heparan/sulphate-bound sFlt1 stored in the placenta and arterial wall [27]. In vivo, pregnant women and patients undergoing cardiac catheterization procedures, and receiving heparin, experience an increase in circulating sFlt1 levels [26]. However, this is the first demonstration of the impact of heparin on sFlt1 kinetics in dialysis patients, who unlike other types of patients, are repeatedly exposed (thrice a week) and for several years, exposed to a dramatic heparin-induced increase in sFlt1 serum levels during dialysis.

As monocytes are the main secretors of sFlt1, one could assume a major role of these cells in the sharp increase in sFlt1 during HD session. The increase in sFlt1 serum levels started as early as 1 min after the beginning of the dialysis and therefore could be due to the early contact of blood components (monocytes) with the heparinized extracorporeal circuit, before the injection of LMWH, at least during a ‘conventional’ HD session. However, in vitro, LMWH and UH did not induce sFlt1 release by monocytes isolated from healthy control or HD patients. Our in vitro experiments only assessed the effect of heparin on sFlt1 release by circulating monocytes collected during an HD session. The fact that the experimental data in vitro do not confirm the in vivo results is clearly a limitation to our study. Our hypothesis that prior activation of monocytes through adhesion to the extracorporeal circuit is mandatory for an induction of sFlt1 release by heparins remains to be tested.

In contrast, LMWH systemic administration (on the arterial line) may affect both monocytes and endothelial cells release of sFlt1. Endothelial cells may also play a central role in the increase of sFlt1 serum levels during dialysis. The impact of heparin on sFlt1 release by endothelial cells/artrial wall has been previously documented in vitro [26, 27]. Therefore, LMWH administration on the arterial line is very likely to have an effect on endothelial cells and (subsequently) on sFlt1 increase. Secondly, the absence of any increase in sFlt1 levels during dialysis with a heparin-coated membrane, EVODIAL+, also argues for a role of endothelial cells. Heparin on the surface of this membrane is irreversibly fixed by multiple ionic bonds and thus is not released in the bloodstream. The absence of any increase in sFlt1 levels with the use of this membrane suggests that the systemic administration of heparin is crucial for the induction of sFlt1 release by endothelial cells. However, one cannot exclude an effect of systemic heparin on monocytes or a reduced heparin-induced activation of monocytes at the contact of the EVODIAL membrane.

Besides, our data indicate that heparin-based dialysis not only induces a sharp increase of sFlt1 during an HD session, but also contributes to the maintenance of the previously documented low-grade chronic increase of sFlt1 in these patients [9]. Serum drawn after 1 h of HD session, but not serum drawn before a HD session, induced a late release of sFlt1 by human monocytes. This effect was mediated through an increase in the synthesis of sFlt1 but not through a cleavage of the membrane-bound Flt1 and was independent of the complement anaphylatoxin C5a. sFlt1 release was not mediated by the LMWH contained in the patients’ serum as heparin by itself did not induce any significant release of sFlt1 by monocytes. The nature of the mediators which induce this sFlt1 increase remains unknown and is currently being investigated.

Our findings have several important implications for the management of ESRD patients undergoing HD. Firstly, the evaluation of the efficiency and biocompatibility of membranes and dialysis procedures has been traditionally based on their potential to induce the activation of the coagulation and complement cascade, the release of various inflammatory cytokines and the clearance of small and medium-size molecules [28]. sFlt1 may be an additional pertinent parameter for the evaluation of dialysis biocompatibility. For instance in the present study, peak serum sFlt1 levels were significantly reduced during a HDF session compared with a HD session. This difference was ultimately linked to the different use of heparin during these two dialysis procedures. There is an on-going debate on the potentially improved survival of HDF patients compared with HD patients, due mainly to the reduction in cardiovascular complications [29–31]. The assessment of sFlt1 kinetics could be helpful in the comparison of these two techniques. Importantly, we have assessed the impact of only two high-flux biocompatible membranes on sFlt1 kinetics. The comparison of other types of dialysis membranes is therefore warranted. Due to its molecular weight (78 kDa), no dialysis removal of sFlt1 is expected whatever the membrane. Nevertheless, the potential adsorption of sFlt1 on HD membranes cannot be excluded, as evidenced by the recent report of the extracorporeal removal of Flt1 in patients with early-onset pre-eclampsia [32]. Second, our findings suggest that heparin-sparing HD techniques may be better options for ESRD patients. The impact of different modalities of heparin administration (bolus versus continuous infusion, doses), and of available alternatives to heparins (citrate-based HD or pre-dilutional heparin-free HDF) on sFlt1 kinetics and most importantly long-term cardiovascular events remain to be evaluated. In the present study, we simply used Citrasate® in association with EVODIAL®, as a way to achieve a totally heparin-free HD session. However, the concentration of Citrasate® was low, and it remains unknown whether a higher
dose of citrate (as in regional citrate anticoagulation) for a longer heparin-free session would affect sFlt1 secretion.

In conclusion, heparin-based dialysis induces a major sFlt1 release and therefore may exacerbate the sFlt1-associated antiangiogenic state in ESRD patients and thus aggravates their endothelial dysfunction and cardiovascular burden. This should be taken into account for the assessment and establishment of optimal dialysis procedures.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.

**ACKNOWLEDGEMENTS**

This study was funded by a grant from INSERM (ATIP-AVENIR).

**CONFLICT OF INTEREST STATEMENT**

None declared. The results presented in this paper have not been published previously in whole or part, except in abstract form.

(See related article by Di Marco et al. Soluble Flt-1 release response to heparin use: implications for dialysis patients. Nephrol Dial Transplant 2014; 29: 1112–1115.)

**REFERENCES**

24. Davenport A. What are the options for anticoagulation needs in dialysis for patients with heparin-induced thrombocytopenia? Semin Dial 2011; 24: 382–385

Received for publication: 13.8.2013; Accepted in revised form: 6.12.2013

Copyright © 2013 Oxford University Press. Published by Oxford University Press on behalf of NDT Publishing Group Ltd. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com