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Endothelial injury is closely related to osteopontin and TNF receptor-mediated inflammation in end-stage renal disease

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ABSTRACT

Background: Endothelial dysfunction, inflammation and active mineralization are key processes involved in cardiovascular burden in end stage renal disease (ESRD). Serum (soluble) thrombomodulin (sTM) is an established marker of endothelial injury.

Patients: 80 patients in ESRD were recruited consecutively. Baseline distribution of sex, age, main comorbidities and Framingham score was similar. A biochemical panel including sTM, intact PTH (iPTH), interleukin-6 (IL-6), pentraxin 3 (PTX3), fibroblast growth factor 23 (FGF-23), osteopontin (OPN), osteoprotegerin (OPG), osteocalcin (OC), osteonectin (ON), soluble tumor necrosis factor receptor type 2 (TNFR2), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), vascular endothelial growth factor receptor type 2 (sVEGFR2) and stromal cell-derived factor 1α (SDF1α) was investigated in each patient. Samples obtained while establishing haemodialysis (HD) access were stained for radial artery calcifications (RACs) with Alizarin red and examined histologically.

Results: After adjustment for HD status, sTM showed a significant positive correlation with serum creatinine, TNFR2, OPN, HGF, SDF1α, sVEGFR2, Pi, iPTH, FGF-23, OPG, OC and ON. In forward stepwise multiple regression, serum creatinine, TNFR2, and OPN were identified as significant, independent predictors of sTM. Grades 1–3 of RACs correlated with sTM ($R = 0.50$, $p = 0.017$), while grade 3 RACs were significantly associated with higher sTM ($p = 0.02$) than less advanced lesions.

Conclusion: Among novel renal and cardiovascular biomarkers, OPN and TNFR2 are closely related to sTM. This may link endothelial damage, vascular remodeling and inflammation. Progression of RAC parallels a presumed compensatory rise in sTM, reflecting endothelial injury. sTM has an intricate role in endothelial function and potential clinical and prognostic applications.

1. Introduction

In atherosclerotic lesions, the expression of thrombomodulin (TM), a cell membrane glycoprotein, has been demonstrated on the surface of endothelium, intimal and medial smooth muscle cells (SMC) and macrophages. The expression of TM in SMC depended on lesion type and patient age [1]. A soluble form of TM (sTM) is released from endothelial cells in response to endothelial damage [2], or TNFα-stimulated inflammatory settings [3]. TNFα was previously shown to display cytotoxic effects on microvascular endothelium [4], whereas serum TM

Abbreviations: AVF, arteriovenous fistula; CKD, chronic kidney disease; CV, cardiovascular; Ca, calcium; EPC, endothelial progenitor cells; ESRD, end stage renal disease; FGF, 23 fibroblast growth factor; HD, hemodialysis; HGF, hepatocyte growth factor; IL, interleukin; hs CRP, high sensitive C-reactive protein; iPTH, intact parathormone; MAC, medial artery calcification; OC, osteocalcin; ON, osteonectin; OPG, osteoprotegerin; OPN, osteopontin; Pi, phosphate; PTX3, pentraxin 3; SMC, smooth muscle cells; sTM, soluble thrombomodulin; TNFR2, soluble tumor necrosis factor receptor type 2; RAC, radial artery calcification TGF,β -transforming growth factor-β; sVEGFR2, vascular endothelial growth factor receptor type 2; SDF1α, stromal cell-derived factor 1α

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has been correlated with measures of microvascular dysfunction [5]. Transgenic mice with endothelial TNFα expression, designed to represent chronic endothelial activation, developed inflammation which improved after exogenous sTM treatment [6]. The Atherosis Risk in Communities (ARIC) study revealed that incidence of coronary heart disease in healthy individuals was inversely correlated with sTM [7]. It was suggested that anti-coagulant function of TM, as a cofactor of protein C, and modulation of thrombin activity may be responsible for this protective effect. Conversely, incidence of carotid atherosclerosis was higher in the group characterized by higher sTM quintiles, which might reflect a compensatory, physiological response to endothelial dysfunction and inflammation [7]. Results of the Hoorn study indicate that endothelial dysfunction contributes to cardiovascular (CV) mortality, even in mild renal insufficiency [8]. Indeed, a recent meta-analysis has shown that endothelial function tests are able to significantly predict CV events [9]. In patients with a history of vascular incidents, higher sTM levels have been associated with increased mortality [10]. Elevated sTM in hemodialysis (HD) patients was found to rapidly decline after kidney transplantation, reflecting restoration of the endothelial function [11]. The aim of this study was to assess correlations of sTM with various parameters of cardiovascular and renal pathology in patients with end stage renal disease. In a cross-sectional analysis, we investigated a wide panel of novel biomarkers implicated in cardiovascular comorbidity and chronic kidney disease.

2. Methods

Eighty patients in stage 5 CKD were consecutively recruited using a convenience sample from the Department of Nephrology, Jagiellonian University Medical College. Patients qualified for hemodialysis (HD) (n = 30) and undergoing HD (n = 50) were included. Following medical examination, blood samples were collected for the panel of laboratory investigations. In patients requiring surgical creation of arteriovenous fistula (AVF) for HD access, radial artery samples were collected for histopathological examination.

2.1. Histology

Small fragments of radial artery were processed as reported previously (for details see [12]). Briefly, the sections were stained with alizarin red to identify calcium deposits and examined using Olympus BX-50 microscope (Olympus, Tokyo, Japan) in bright field mode. Olympus DP-71 digital CCD camera with Olympus AnalySIS FIVE software was used to capture images. Degree and advancement of vascular calcification was evaluated blindly by an experienced histopathologist, using a semiquantitative 0–4 scoring system: 0 - no mineral content, 1 - a few small dispersed concretions, 2 - numerous small dispersed concretions, 3 - larger granular concretions, 4 - large areas occupied by fused mineral deposits. The calcifications were found exclusively in the vascular media (Fig. 1).

2.2. Biochemistry

Fasting venous blood samples were collected from patients into ethylenediaminetetraacetic acid (EDTA) tubes, in the morning prior to surgical AVF procedures, and stored at −70 °C. Biochemical tests were then performed using automatic biochemical analyzers: Hitachi 917 (Hitachi, Japan) and Modular P (Roche Diagnostics, Mannheim, Germany).

The predefined biochemical panel included: serum TM, creatinine, calcium, phosphate, intact PTH (iPTH), interleukin-6 (IL-6), pentraxin 3 (PTX3), soluble tumor necrosis factor receptor type 2 (TNF R2), hepatocyte growth factor (HGF), stromal cell-derived factor 1α (SDF1α), vascular endothelial growth factor receptor type 2 (sVEGFR2), transforming growth factor-β (TGF-β), fibroblast growth factor 23 (FGF-23), osteopontin (OPN), osteoprotegerin (OPG), osteocalcin (OC), osteonectin (ON).

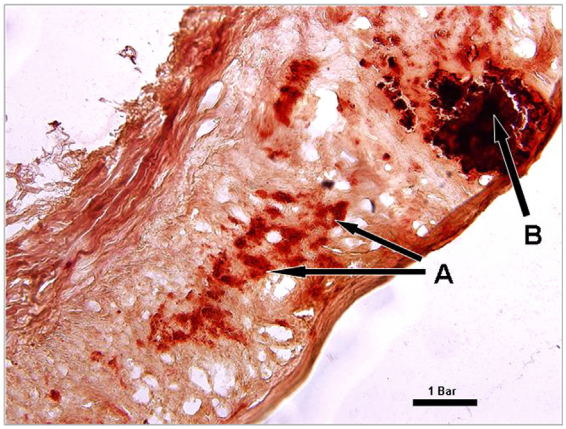

Fig. 1. Radial artery calcification of high grade - stage 4 (alizarin red staining). A. larger granular concretions, B. large areas occupied by fused mineral deposits Bar = 100 μm.

sTM levels in platelet-poor EDTA plasma samples were measured using commercially available ELISA (Human Thrombomodulin/BDCA-3 Immunoassay-R&D Systems, Minneapolis, USA (Human CXCL12/ SDF1α Immunoassay-R&D Systems, Minneapolis, MN, USA). SDF1α levels in platelet-poor EDTA plasma samples were measured using commercially available ELISA (Human CXCL12/ SDF1α Immunoassay-R&D Systems, Minneapolis, MN, USA). The reference range for sTM is 2.86–5.31 ng/ml, mean – 4.03 ng/ml, standard deviation- 0.637 ng/ml.

Endothelial injury, inflammation and mineralization markers were assessed using ELISA micro-plate immunoassays and ELX808 automatic reader (BIO-TEK® Instruments Inc., Vermont, VT, USA). The following kits were applied: OPG (BioVendor, Brno, Czech Republic); OPN, SDF1α, sVEGFR2, HGF, IL-6, TGF-β, sTNF R2, PTX3, ON (R&D Systems, Minneapolis, USA); OC (Metra/Quidel, CA, USA), FGF-23 (Immunotopics Int., San Clemente, CA, USA)

2.3. Statistical analysis

The number and respective percentage of patients is reported for categories. Quantitative variables are expressed as median (lower-upper quartile) due to a lack of normal distribution for the majority of parameters. Differences between groups were assessed using the Mann-Whitney test. Right-skewed variables were log-transformed before correlation and regression analysis. We used Pearson correlation coefficient, except for the correlation between sTM and the grade of alizarin red staining (the ordinal variable) where the Spearman coefficient was used. Linear regression was used to assess whether the simple correlations of log (sTM) were independent of HD status. Multiple forward stepwise model was built to identify independent predictors of log (sTM). The tests were two-tailed and the results were considered significant at $p < 0.05$. We used Statistica 12.0 (StatSoft, Tulsa, OK, USA) for computations.

2.4. Ethical considerations

The study is in accordance with the Declaration of Helsinki and was approved by the Bioethics Committee of the Jagiellonian University. All patients signed an informed consent for participation prior to their inclusion into the study.

3. Results

3.1. Pre-dialysis and haemodialysis patient characteristics

The patients did not differ with respect to sex, age, main

Table 1
Characteristics of the studied patients.

Characteristic	Pre-dialysis patients (N = 30)	HD patients (N = 50)	p-value
Age, years	62 (57; 75)	62 (50; 74)	0.09
Male sex, N (%)	19 (63)	29 (58)	0.6
Dialysis therapy duration, months	not applicable	14 (4; 38)	–
BMI, kg/m^2	25.6 (23.8; 28.9)	24.2 (21.5; 27.5)	0.06
Diabetes, N (%)	10 (33)	17 (34)	0.9
Hypertension, N (%)	29 (97)	41 (82)	0.054
Hyperlipidemia, N (%)	18 (60)	25 (50)	0.4
Active smoking, N (%)	8 (27)	12 (24)	0.8
Framingham risk score, %	11 (8; 18)	11 (6; 22)	0.3
Serum creatinine, μmol/l	366 (284; 434)	478 (392; 628)	< 0.001
sTM, ng/ml	14.6 (13.4; 17.3)	18.5 (14.9; 22.5)	< 0.001
PTX3, ng/ml	0.76 (0.46; 1.59)	1.76 (1.14; 2.67)	0.001
IL-6, pg/ml	2.81 (1.86; 4.98)	5.86 (2.81; 8.44)	0.008
TNFR2, μg/ml	10.18 (8.93; 13.15)	16.57 (13.70; 20.67)	< 0.001
HGF, pg/ml	1.98 (1.66; 2.53)	2.61 (2.02; 3.76)	0.003
SDF1α, ng/ml	2.94 (2.66; 3.39)	3.19 (2.84; 3.58)	0.2
sVEGF R2, ng/ml	9.84 (8.79; 10.76)	10.53 (9.17; 11.51)	0.2
TGF-β, μg/ml	6.17 (4.22; 7.84)	5.16 (4.09; 6.45)	0.2
Ca, mmol/l	2.21 (2.09; 2.33)	2.23 (2.08; 2.33)	0.6
Pi, mmol/l	1.39 (1.28; 1.47)	1.66 (1.37; 2.15)	0.002
Ca × Pi, mmol^2/l^2	2.94 (2.77; 3.37)	3.64 (2.96; 4.74)	< 0.001
iPTH, pg/ml	291 (174; 457)	279 (169; 476)	0.9
FGF-23, RU/ml	476 (289; 981)	2278 (1020; 5859)	< 0.001
OPG, pmol/l	5.94 (4.98; 7.70)	8.17 (6.21; 11.36)	0.017
OPN, ng/ml	210 (154; 307)	336 (225; 587)	< 0.001
OC, ng/ml	40.2 (29.3; 49.0)	51.1 (35.1; 84.52)	0.031
ON, ng/ml	113 (74; 139)	107 (88; 144)	0.8

comorbidities and cardiovascular risk factors. However, serum creatinine concentrations were higher among those under HD, as were the concentrations of the studied inflammatory markers, inorganic phosphate, FGF-23, OPG, OPN and OC. Of note, sTM concentrations were also significantly higher in patients treated with HD (Table 1).

3.2. Serum thrombomodulin and traditional cardiovascular risk factors

sTM did not differ between patients with and without diabetes (p = 0.6), hypertension (p = 0.8), hyperlipidemia (p = 0.5), as well as between smokers and non-smokers (p = 0.3). Log-transformed sTM concentrations did not correlate significantly with age (R = 0.22; p = 0.056) and log (Framingham risk score) (R = −0.12; p = 0.3). sTM positively correlated with serum creatinine, the markers of inflammation and endothelial dysfunction, and the markers of mineral and bone metabolism (Table 2). In forward stepwise multiple regression, serum creatinine, TNF-R II, and OPN were identified as significant independent predictors of sTM concentrations (Table 3).

3.3. Radial artery calcifications in ESRD

Radial artery fragments were available in 50 patients. No calcifications were detected in 20 patients (40%), while 30 patients (60%) had calcifications of various grades (Fig. 2A). Patients with grade 3 calcifications (Fig. 2B) had higher concentrations of sTM than patients with less advanced lesions. Moreover, there was a significant correlation between calcification grades 1 to 3 and the concentrations of sTM (R = 0.50; p = 0.017).

4. Discussion

The salient finding of the present study is that inflammatory and mineralization cytokines TNFR2 and OPN independently predict sTM concentrations. Moreover, medial radial artery calcifications (RAC) closely correlate with sTM levels.

To our knowledge, this is the first study to investigate the relationship between sTM and an extensive panel of novel inflammatory, tissue remodeling and mineral/bone biomarkers in the setting of pre-dialysis and HD.

Previous studies reported that among other hemostatic parameters, sTM correlated with highly sensitive C-reactive protein (hsCRP) and carotid intima-media thickness, a surrogate marker of atherosclerosis, providing a potential link between coagulation abnormalities and

Table 2
Correlations of log (sTM) with studied markers of inflammation and mineral/bone homeostasis. Standardized beta coefficients were calculated in simple linear regression adjusted for HD status.

Variable	Univariate		Adjusted for HD status	
	R	p	beta ± SE	p
log (creatinine)	0.68	< 0.001	0.64 ± 0,09	< 0.001
log (PTX3)	0.26	0.025	0.15 ± 0.12	0.2
log (IL-6)	0.27	0.020	0.20 ± 0.12	0.09
log (TNFR2)	0.70	< 0.001	0.69 ± 0.09	< 0.001
log (HGF)	0.41	< 0.001	0.32 ± 0.11	0.003
SDF1α	0.39	< 0.001	0.34 ± 0.10	0.001
sVEGF R2	0.26	0.020	0.23 ± 0.0.10	0.032
log (TGF-β)	0.16	0.2	Not analyzed	
Ca	−0.02	0.9	Not analyzed	
log (Pi)	0.43	< 0.001	0.32 ± 0.11	0.004
log (iPTH)	0.29	0.014	0.27 ± 0.10	0.012
log (FGF-23)	0.58	< 0.001	0.55 ± 0.11	< 0.001
log (OPG)	0.40	0.001	0.34 ± 0.11	0.003
log (OPN)	0.42	< 0.001	0.35 ± 0.11	0.003
log (OC)	0.41	< 0.001	0.34 ± 0.11	0.003
log (ON)	0.47	< 0.001	0.45 ± 0.09	< 0.001

Table 3
Multiple forward stepwise regression model to predict log (sTM).

Variable	Multiple forward stepwise model (R^2 = 0.55)	
	beta ± SE	p
log (creatinine)	0.43 ± 0.10	< 0.001
log (TNF R2)	0.29 ± 0.11	0.014
log (OPN)	0.22 ± 0.10	0.032

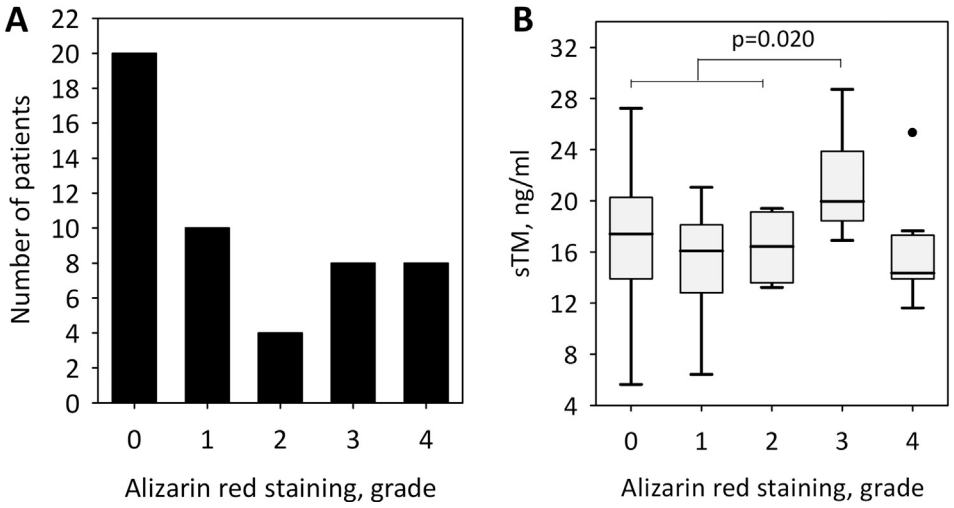

Fig. 2. Prevalence and grade of radial artery calcifications stained with alizarin red in predialysis patients (A) and correlations between sTM concentrations and calcification grade (B). In panel B, data are shown as median, interquartile range (box), non-outlier range (whiskers), and outliers (points); p-value is shown for the difference between grade 3 and less advanced calcifications.

systemic inflammation in incident atherosclerosis in CKD patients [13]. More recently, Drożdż et al. underscored the potential of sTM as a marker of endothelial dysfunction in CKD, reporting correlations with hsCRP, oxidative stress markers, hypertension and left ventricle hypertrophy in the pediatric population [14]. Our study compliments and extends these findings, as it has been previously suggested that CRP is only an intermediary to antecedent pathways of TNFα-mediated inflammation in CKD [15]. There is accumulating evidence of circulating TNFRs as biomarkers in CKD progression and comorbid CV risk prediction [15–18]. In a population cohort of 4962 persons followed for 15 years, TNFR2 had the highest predictive value for CKD incidence [15]. We add on to that evidence by associating sTM and endothelial damage with TNFR2 levels in ESRD.

Different signalling pathways involving TNFRs are the subject of many studies, however, the exact role of TNFRs in CKD still remains unclear [19]. TNFR2 has high affinity for transmembrane TNFα and is shed to a soluble form under inflammation [20]. TNFR2-knockdown in mice prevents glomerulonephritis and in the nephritic kidneys TNFR2 expression is initially localized to glomerular endothelium [21]. In animal models of proliferative nephritis, TNF induced chemokine expression, while TNFR2 was central for monocyte recruitment [22]. Indeed, Omote et al. demonstrated that an anti-TNF agent, etanercept, decreased TNFR2, albuminuria and macrophage infiltration in the kidneys [23]. Coronary artery lesions have also been firmly associated with TNFR2 [24]. TNFα induces expression of procoagulant molecules, suppresses TM in human microvascular endothelial cells, while TNFR2 is necessary for *in vivo* TNFα-accelerated arteriolar thrombus formation [25]. TNFR2 seems to be involved in renal and vascular pathology. Our findings indicate TNFR2 and sTM share a close relationship in CKD. This may occur due to a link between endothelial injury and TNF-mediated inflammation. Correlation of sTM with features of systemic and vascular inflammation, namely IL-6 and PTX-3, in univariate analysis may further suggest that they contribute to endothelial injury as downstream inflammatory intermediaries.

HGF is a pleiotropic cytokine thought to induce anti-inflammatory processes, modulate macrophage recruitment and tissue regeneration [26,27]. In animal models of ischemic injury, it was observed to prevent endothelial neutrophil infiltration, tubular apoptosis and renal dysfunction [28]. In a previous study, we demonstrated that in patients on peritoneal dialysis, HGF is a predictor of long-term total and CV mortality [29]. The correlation of HGF with sTM might represent a physiological “feedback” response to endothelial injury, albeit insufficient to counteract the offending stimuli in ESRD. Hence, HGF and sTM should be considered in models of comorbid CV risk in CKD.

Osteopontin, a pleiotropic cytokine, is thought to promote fibrosis through inflammatory pathways associated with recurring tissue injury [30]. Its role in CKD is not well understood. In murine models assessing Pi intake and uremia, OPN and FGF-23 were strongly associated, and intercorrelated, with development of medial arterial calcifications (MAC) [31]. Of note, it was observed without indicators of atherosclerosis and inflammation, therefore tissue remodeling associated with OPN can occur without evident inflammatory stimuli. London et al. studied ESRD and observed MAC in younger patients without traditional atherosclerotic risk factors, who thus may represent a less “symptomatic” population with more difficult CV risk stratification [32]. However, MAC was associated with all-cause and CV mortality. Our findings indicate that endothelial injury and successive sTM release correlated with OPN, FGF-23 and other mineralization contributors, which may reflect the processes involved in MAC. The sudden decrease in sTM concentrations associated with the highest grade of MAC may indicate that with a certain severity of vascular lesions the endothelial response to injury (sTM release) is subdued. An osteogenic phenotype shift of SMC with increased OPN expression may occur early in MAC, as observed in initial calcification foci [31]. OPN deficiency has also been reported to reduce the effect of uremia on SMC reverse-differentiation, while in macrophages it reduced pro-inflammatory gene expression [33]. Pedersen et al. thus discussed the potential of plasma OPN as a biomarker, justified by a positive link with atherosclerosis progression in a uremic milieu, which itself also increases plasma OPN [33]. TM is thought to promote mitogenic changes to vascular SMC [34]. It may also regulate Pi-induced vascular SMC differentiation and apoptosis via the ERK pathway in active calcification [35]. Indeed, the Multi-Ethnic Study of Atherosclerosis previously showed that high sTM was correlated with vascular calcification [36]. The nature and mechanism of the relationship between OPN and TM is still unclear, though Pi may indirectly regulate OPN and TM genes in SMC [37]. Barretto et al. observed that OPN aids in prediction of mortality and CV risk in CKD, however its value seems to be tied to other inflammatory markers [38]. Our findings suggest that the relationship between sTM and OPN is complex. Whether this reflects their role as pathogenetic mediators, or simply markers of concurrent processes involved in CKD is unknown. They may serve as biomarkers and improve the early prediction of vascular lesions in ESRD.

Expression of inflammatory and remodelling molecules, including OPN, was enhanced in a transgenic model of chronic endothelial activation through transmembrane TNF overexpression, and down-regulated following sTM treatment [6]. In ESRD the abundance of inflammatory triggers, as well as lasting uremic and oxide stress may overcome the physiological capacity of endothelium-derived sTM to reduce these pathological processes. Earlier, Bao et al. demonstrated that sTM increased with advancing CKD and suggested that sTM could represent an endogenous countermeasure to the diseased state [39]. In

line with the previous research, we observed sTM levels to be closely and independently correlated with renal function [5,13,39]. By analogy to the ARIC study, the significance of sTM concentrations seems to depend on patient morbidity [7], and would explain the "conflicting" concentrations of sTM in patients with ESRD, in which sTM levels might mirror the severity of associated disease processes.

Previous studies suggested that in CKD patients with vascular calcifications, recurrent endothelial injury leads to increase in microparticles recruiting endothelial progenitor cells (EPCs), which may be skewed into osteogenic differentiation with osteocalcin expression, and thus lead to calcification [40]. Migration of EPCs seems to be promoted by angiogenic factors such as VEGF and SDF1, perceived as "feedback" molecules counteracting the shortage of EPC due to recurring endothelial damage [41]. We observed SDF-1 and VEGFR correlated with sTM, a putative marker of endothelial injury, which lends support to this hypothesis. It has also been suggested that mobilization of EPCs into circulation can be blunted by inhibitory effects of TNFα [42]. In this context, the association between endothelial injury and TNFR2 reported at present may indicate that the endothelial reparative response is limited in ESRD, and the use of TNF-inhibiting agents can amplify both endogenous and exogenous sTM effects.

Clinical applications of sTM are emerging with an improvement of glomerulonephritis and ischemic injury in animal models [43,44], and implications of direct anti-inflammatory effects, independent of activating protein C [45,46]. Li et al. observed that recombinant TM attenuated the proliferation of SMC induced by thrombin [47]. Another study showed that sTM reduced atherosclerosis and neointimal formation by inhibiting thrombin activity [48]. In short, the therapeutic potential of sTM in ESRD is noteworthy.

Importantly, we did not find sTM to correlate with age, or Framingham CV risk score. Unexplained CV comorbidity in CKD [49] requires the establishment of a new model comprising biomarkers defining CKD, for which independence from traditional risk factors is important to discriminate and account for specific renal pathology. We conclude that sTM, OPN and TNFRs merit further study, and may be key molecules involved in processes shaping ESRD.

4.1. Limitations of the study

Due to cross-sectional design we are unable to draw causal relationships between the studied markers and to formulate definite conclusions, only on the basis of the statistical analysis performed. Our study was not oriented toward a mechanistic investigation, but rather toward the evaluation of an extensive panel of biomarkers. Following this preliminary evidence, future specifically-designed studies are warranted. Our results could benefit from a larger study sample, a weakness of many cross-sectional investigations. Radial artery fragments were collected only in patients awaiting dialysis, therefore we were not able to conclude how sTM relates to RAC in patients undergoing HD. Despite histological evaluation being an advantage of this study, the correlation between morphology and biochemical assessments, should be interpreted with caution. The comparison of biochemical findings, which may not reflect localized, lesion specific microenvironments, with histological findings, which are more focused on the "micro" scale is a natural limitation, to which it is difficult to adapt methodology. Blood sampling at only one point in the study is defined by cross-sectional character, however, longitudinal confirmation of the trends described should shortly follow.

4.2. Conclusion

Previous studies have demonstrated that sTM reflects endothelial injury. Among several novel biomarkers of kidney and cardiovascular pathology, sTM is particularly related to OPN and TNFR2 in ESRD. Whether this implicates a causal relationship between chronic TNF-mediated inflammation, vascular remodeling and injury of endothelial cells or rather reflects an overlapping of these processes in CKD, as followed indirectly through biomarkers, remains to be established. Medial calcification in radial arteries may parallel the ongoing endothelial damage, as expressed by sTM levels. Further studies should thus examine whether sTM may aid in the assessment of vascular risk associated with medial artery calcification, which often remains asymptomatic.

Ethics approval and consent to participate

The study is in accordance with the Declaration of Helsinki and was approved by the Bioethics Committee of the Jagiellonian University. All patients signed an informed consent for participation prior to their inclusion into the study.

Disclosure statement

The manuscript has not been published elsewhere.

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Authors' contributions

KB and KK conceived the study, were the major participants in its design, coordination, interpretation of results and statistical analysis, they also prepared draft manuscript. MG carried out histological examinations. PD performed statistical analysis. KW, DF and MK participated in the design of the study. JAL participated in data analysis and in preparation of the final manuscript version. MAK and WS participated in study design and coordination. All authors were involved in data collection, draft manuscript modifications and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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