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ORIGINAL ARTICLE

Endotrophin, a collagen type VI-derived matrikine, reflects the degree of renal fibrosis in patients with IgA nephropathy and in patients with ANCA-associated vasculitis

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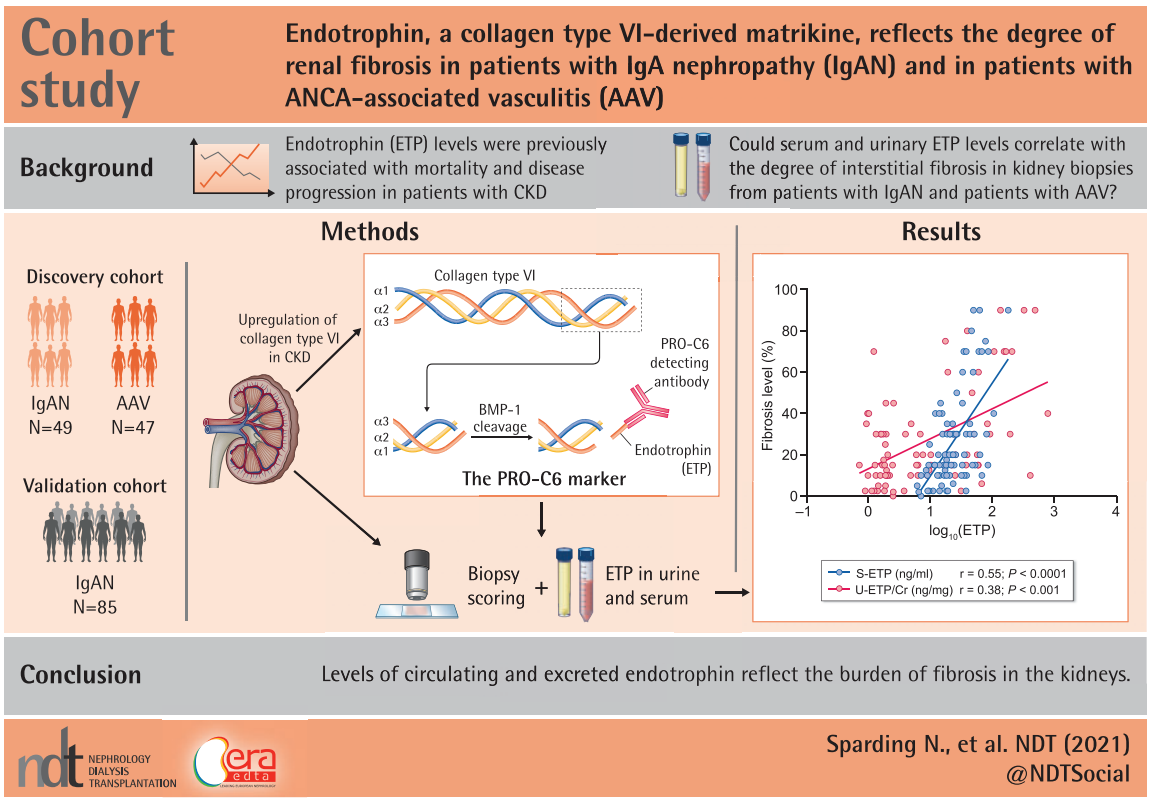
GRAPHICAL ABSTRACT



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KEY LEARNING POINTS

What is already known about this subject?

- non-invasive biomarkers for assessment of kidney fibrosis burden and progression in patients with chronic kidney disease (CKD) are lacking;
- endotrophin (ETP) is a signalling molecule released from collagen type VI and this molecule is highly present in fibrotic kidneys; and
- previously, ETP was associated with adverse outcomes, such as mortality and disease progression, in different populations of CKD patients; however, the level of ETP has not been directly linked to the degree of interstitial fibrosis in patients with Iimmunoglobulin A nephropathy (IgAN) and patients with anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV).

What this study adds?

- this study shows that serum levels and urinary excretion of ETP reflect the degree of interstitial fibrosis in kidney biopsy (ETP measured in samples taken just before kidney biopsy) from patients with IgAN and patients with AAV;
- based on multiple regression analysis including serum levels of ETP and serum creatinine (sCr), only ETP and not sCr was independently associated with the degree of fibrosis; and
- ETP outperformed the known fibrosis marker Dickkopf-3 for discrimination of patients with advanced fibrosis.

What impact this may have on practice or policy?

- this study highlights the potential of ETP as a non-invasive biomarker of interstitial fibrosis that might supplement kidney biopsies and may aid in the evaluation of anti-fibrotic compounds.

ABSTRACT

Background. Renal fibrosis is the hallmark of chronic kidney disease (CKD) and is characterized by an imbalanced extracellular matrix remodelling. Endotrophin (ETP) is a signalling molecule released from collagen type VI (COL VI). ETP can be measured by the PRO-C6 assay, which quantifies the levels of COL VI formation. ETP levels were previously associated with mortality and disease progression in patients with CKD. We hypothesized that serum and urinary ETP levels correlate with the degree of interstitial fibrosis in kidney biopsies from patients with immunoglobulin A nephropathy (IgAN) and patients with anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV).

Methods. We examined a cohort of 49 IgAN and 47 AAV patients. A validation cohort of 85 IgAN patients was included. ETP was measured in serum (S-ETP) and urine (U-ETP/Cr) samples, taken on the same day before renal biopsy was performed, using the enzyme-linked immunosorbent assay PRO-C6. The biopsies were evaluated for interstitial fibrosis and tubular atrophy according to the Banff and MEST-C scores.

Results. S-ETP and U-ETP/Cr levels correlated with kidney function, increased CKD severity, correlated with the extent of interstitial fibrosis and gradually increased with increasing degree of interstitial fibrosis and tubular atrophy. ETP outperformed the known fibrosis biomarker Dickkopf-3 for discrimination of patients with high fibrotic burden. The association of S-ETP and U-ETP/Cr with the level of kidney fibrosis was confirmed in the validation cohort.

Conclusions. We demonstrated that high levels of circulating and excreted ETP are not only indicative of lower kidney function, but also reflect the burden of fibrosis in the kidneys.

Keywords: ANCA-associated vasculitis, biomarkers, chronic kidney disease, IgA nephropathy, interstitial fibrosis

INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is one of the most common primary diagnoses leading to glomerulonephritis worldwide [1], representing ∼20% of all biopsy findings [2]. It is caused by an increased level of poorly O-galactosylated IgA1 glycoforms resulting in the deposition of IgA1-containing immune complexes in the glomerular mesangium causing glomerular injury [3, 4]. IgAN is a slowly progressive disease [5] characterized by onset at young age with apparent benign course during short-term follow-up; however, 20–40% of patients progress to end-stage kidney disease (ESKD) within 20 years [4].

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) occurs in only 5% of renal biopsies [2] and is a group of heterogeneous diseases associated with ANCAs directed against proteinase 3 (PR3) or myeloperoxidase (MPO) in 95% of the patients [6, 7]. This group of diseases is usually characterized by severe and partly reversible renal injury at presentation with putative progression to ESKD due to non-

immunologic mechanisms and/or renal relapses [8–10]. Renal involvement is found in up to 90% of the patients with AAV [11]. In contrast to IgAN, AAV is a rare disease with a much later median age at disease onset. AAV is more progressive than IgAN and up to 20–40% of AAV patients develop ESKD within 5 years [4, 12].

Renal fibrosis is the hallmark of chronic kidney disease (CKD) [13, 14] and fibrosis is caused by an imbalance in the turnover of extracellular matrix (ECM) components such as collagens. Seminal studies have shown that collagens are not merely structural entities but can release fragments with paracrine and endocrine properties, termed matrikines [15, 16]. Collagen type VI (COL VI) is an important structural collagen found in the interface between the interstitial matrix and the basement membrane in the kidney, where it is produced by fibroblasts [17]. COL VI is expressed at low levels in healthy adult kidney [18], and is markedly upregulated in patients with renal fibrosis [19, 20]. The C-terminal of the α3 chain of COL VI is cleaved off the mature molecule upon secretion from the producing cell and can therefore be used as a biomarker of COL VI formation [21, 22]. Interestingly, the released fragment, called endotrophin (ETP), has important biological effects such as attracting macrophages, increasing transforming growth factor β expression, promoting epithelial–mesenchymal transition, adipose tissue fibrosis and metabolic dysfunction [22, 23]. The PRO-C6 enzyme-linked immunosorbent assay (ELISA) detects levels of ETP. High levels of ETP in serum have previously been associated with an increased risk of mortality and disease progression in patients with CKD and diabetic kidney disease [19, 24, 25].

Dickkopf (DKK)-3 is a stress-induced tubular epithelia-derived glycoprotein, which has shown pro-fibrotic activity in kidney disease [26], and high levels in urine have been shown to be associated with higher risk of kidney function loss. Urinary DKK-3 also improved the prediction of kidney function loss compared with that of estimated glomerular filtration rate (eGFR) or albuminuria alone [27].

In this study, we investigated for the first time whether levels of ETP in serum and urine samples collected on the day of renal biopsy correlated with the degree of histological fibrosis in patients with IgAN (in a discovery and a validation cohort) and in patients with AAV, and we compared the ability of ETP and DKK-3 to reflect kidney fibrosis.

MATERIALS AND METHODS

Study subjects

The examined study cohort consisted of patients with biopsy-proven IgAN ($n = 49$) and patients with AAV ($n = 47$) diagnosed at the General University Hospital in Prague, Czech Republic. All consecutive patients with AAV diagnosed between 2011 and 2016 and with IgAN diagnosed between 2011 and 2013, with a representative biopsy sample, available specimens in local biobank and who were willing to participate in this research study were included. A validation cohort of 85 patients with IgAN, consisting of patients with available urine and serum samples and representative biopsy diagnosed between 2012 and 2015, was added. Blood and urine samples were taken on the same day before renal biopsy was performed. Ten healthy volunteers were enrolled as controls, and blood and urine samples were collected in the same hospital following the same procedures.

Follow-up data were available for IgAN patients 1 year after diagnosis ($n = 32$) and during the follow-up patients received either steroids alone, steroids combined with other immunosuppressive drugs or renin–angiotensin system blockade only. The AAV patients all received corticosteroids plus cyclophosphamide at entry, and were treated with corticosteroids combined with other immunosuppressive drugs (mostly azathioprine) or (less commonly) corticosteroids only at 1 year ($n = 45$) and at 3 years ($n = 31$) follow-up.

The study was conducted in compliance with the declaration of Helsinki principles. Informed consent was obtained from the participants.

Data collection

Clinical and laboratory data were collected or measured in the laboratories of the General University Hospital, including sex, age, serum creatinine (sCr), proteinuria (PU), C-reactive protein (CRP), haemoglobin, ANCA levels and ANCA type (all routinely measured). eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation [28], and patients were stratified based on eGFR according to the CKD classification.

Renal biopsy

Ultrasound-guided renal biopsies were collected for diagnostic purposes. All biopsies were classified according to official international classifications, such as MEST-C and Berden's in combination with Banff classification, while glomerular morphology and interstitial fibrosis with tubular atrophy were evaluated in both groups.

Evaluation of fibrosis. Inasmuch as Berden's classification does not include evaluation of interstitial fibrosis, the Banff classification was used in both groups (IgAN and AAV) for direct comparison of interstitial fibrosis. T-score of MEST-C score was used in patients with IgAN as well. Quantitative criteria for T-score in IgAN were used: T0: ≤25%, T1: 26–50% and T2: >50% [29]. Similarly, the Banff criteria were as follows: interstitial fibrosis in cortical area; ci0: in up to 5%, ci1: in 6–25% (mild), ci2: in 26–50% (moderate) and ci3: in >50% (severe) [30]. All biopsies were also evaluated for the percentage level of fibrosis (continuous variable).

Evaluation of glomeruli. MEST-C score was used to describe and classify morphology in IgAN. The glomerular morphology in the biopsies from AAV patients was classified by Berden's classification as either focal (≥50% of normal glomeruli), mixed (<50% of normal, <50% of crescentic and <50% of globally sclerotic glomeruli), crescentic (≥50% of glomeruli with cellular crescents) or sclerotic (≥50% of globally sclerotic glomeruli) [31].

Table 1. Baseline characteristics and baseline biopsy evaluation

Variables	Healthy	IgAN discovery	AAV discovery	IgAN validation	*n* (IgAN discovery/AAV discovery/IgAN validation)
n	10	49	47	85	(49/47/85)
Women, %	90	20	40	37	(49/47/85)
Age, years	41 (39–42)^{ns}	42 (33–56)	62 (55–69)****	43 (32–54)^{ns}	(49/47/85)
sCr, mg/dL	NA	1.6 (1.0–3.0)	3.6 (1.6–6.8)***	1.7 (1.1–2.4)^{ns}	(49/47/85)
eGFR, mL/min/1.73 m^2	NA	47 (20–95)	17 (8–42)****	45 (26–74)^{ns}	(49/47/85)
PU, g/day	NA	1.3 (0.8–3.6)	1.4 (0.6–2.0)^{ns}	2.0 (1.1–2.4)^{ns}	(49/47/82)
CRP, mg/L	NA	2.4 (1.0–4.4)	38.1 (5.4–104.8)****	NA	(44/47/NA)
Haemoglobin, g/L	NA	NA	101 (91–111)	NA	(NA/47/NA)
ANCA levels, IU/mL	NA	NA	77 (32–100)	NA	(NA/45/NA)
ANCA type, *n* (MPO/PR3)	NA	NA	(23/21)[a]	NA	(NA/47/NA)
CKD stages (1–5), %	NA	29, 12, 29, 10, 20	4, 9, 21, 26, 40	14, 19, 33, 26, 8	(49/47/85)
Level of fibrosis, %	NA	20 (10–30)	15 (14–30)^{ns}	30 (15–45)^{ns}	(45/42/83)
Sclerotic glomeruli, %	NA	NA	21 (8–43)	NA	(NA/44/NA)
Lung fibrosis (yes/no), yes %	NA	NA	17	NA	(NA/47/NA)
Banff score (ci0–ci3), %	NA	17, 31, 31, 21	9, 56, 19, 16	8, 37, 41, 14	(48/43/83)
T-score (T0–T2), %	NA	52, 27, 21	NA	NA	(48/NA/NA)
AAV classification scheme (focal, mixed, crescentic and sclerotic), %	NA	NA	27, 24, 22, 27	NA	(NA/45/NA)

Continuous variables are expressed as median or % (IQR) and categorical variables as % or *n*. The significant differences of continuous variables between groups were analysed with the Kruskal–Wallis test. NA, not available.

***P < 0.001.

****P < 0.0001 versus the IgAN patient group.

ns, not significant.

[a]Three of the 47 AAV patients were both MPO and PR3 positive and were not included in one of the ANCA type groups.

Assays

Serum levels and urinary excretion of ETP (S-ETP and U-ETP) were measured using a competitive ELISA, PRO-C6, according to manufacturer's instructions (Nordic Bioscience, Herlev, Denmark) [24, 32]. DKK-3 was measured in serum (S-DKK-3) and urine (U-DKK-3) with a Human DKK-3 ELISA Kit (Sigma Aldrich, St Louis, MO, USA) in the laboratories of the General University Hospital. Urine Cr levels were measured with the QuantiChrom^{TM} Creatinine kit (BioAssay Systems) and U-ETP as well as U-DKK-3 levels were normalized for urine Cr (U-ETP/Cr and U-DKK-3/Cr). Serum and urine samples for ETP and DKK-3 measurements were not available for all 49 IgAN (S-ETP $n = 49$, U-ETP $n = 47$, S-DKK-3, $n = 47$; U-DKK-3, $n = 39$) and 47 AAV patients (S-ETP $n = 47$, U-ETP, $n = 44$; S-DKK-3, $n = 45$; U-DKK-3, $n = 37$), or for the 85 IgAN patients included in the validation cohort (S-ETP, $n = 85$; U-ETP, $n = 61$).

Statistical analyses

For non-parametric statistical analysis, untransformed data were used and $\log_{10}$-transformed data were used for parametric statistical analysis. Spearman's rank correlation coefficient was used to estimate the correlation between variables. Multiple linear regression analysis was used to analyse the association of variables with fibrosis levels. The statistical differences between groups were analysed with the Mann–Whitney or the Kruskal–Wallis test. Receiver operating characteristic (ROC) curve analysis was used to evaluate discriminatory power of biomarkers for fibrosis scores and AAV histologic classification scheme. Comparison of C-statistics was used to evaluate the superiority of the different biomarkers to discriminate patients with a biopsy scored as ci3 according to the Banff score from patients with a biopsy scored as ci0–ci2. ROC curve analysis was also used to determine the ability of baseline ETP to discriminate between AAV patients with a decrease in CKD stage from patients with no change or an increase in CKD stage from baseline to the 3-year follow-up. Statistical analyses were performed using GraphPad Prism version 7.04 and MedCalc version 14.8.1. $P < 0.05$ were considered significant.

RESULTS

Baseline characteristics of the study cohort

The discovery cohort of this study included 49 patients with IgAN and 47 patients with AAV. The demographic and clinical data as well as the distribution of the IgAN and AAV patients in fibrosis classes according to the Banff score, the T-score (IgAN only) and the glomerular morphology (AAV only) are summarized in Table 1. Ten healthy volunteers were included as well as a validation cohort of 85 patients with IgAN. The validation cohort was not significantly different from the IgAN patients in the discovery cohort (Table 1).

Associations of ETP and DKK-3 with baseline clinical parameters

Both S-ETP and U-ETP/Cr measured in IgAN and AAV patients correlated with sCr, PU and inversely correlated with eGFR (Table 2). S-ETP and U-ETP/Cr were highly correlated ($r = 0.72$, $P < 0.001$). In AAV patients, ETP levels correlated with CRP and inversely correlated with haemoglobin, whereas there was no correlation with ANCA levels (Table 2). S-ETP and U-ETP/Cr levels were significantly higher in patients with

Table 2. Spearman's rank correlation coefficients of S-ETP, U-ETP/Cr and fibrosis levels with clinical parameters

Variables	IgAN and AAV discovery	IgAN discovery			AAV discovery			IgAN validation		
	Level of fibrosis	S-ETP	U-ETP/Cr	Level of fibrosis	S-ETP	U-ETP/Cr	Level of fibrosis	S-ETP	U-ETP/Cr	Level of fibrosis
Age	0.21*	0.37**	0.10	0.31*	0.27	0.35*	0.20	0.24*	0.38**	−0.03
sCr	0.53****	0.86****	0.62****	0.75****	0.81****	0.69****	0.37*	0.67****	0.54****	0.57****
eGFR	−0.53****	−0.86****	−0.66****	−0.76****	−0.82****	−0.72****	−0.44**	−0.71****	−0.59****	−0.53****
PU	0.22*	0.26	0.17	0.19	0.43**	0.32*	0.24	0.10	0.25	0.12
CRP	0.00	0.17	0.13	−0.03	0.41**	0.35*	0.04	NA	NA	NA
Haemoglobin	NA	NA	NA	NA	−0.55****	−0.54***	0.04	NA	NA	NA
ANCA levels	NA	NA	NA	NA	0.11	0.049	−0.04	NA	NA	NA
Level of fibrosis	–	0.70****	0.48**	–	0.42**	0.40*	–	0.64****	0.39**	–
Sclerotic glomeruli	NA	NA	NA	NA	0.36*	0.47**	0.69****	NA	NA	NA
S-DKK-3	0.34**	0.52***	0.15	0.35*	0.26	0.25	0.36*	NA	NA	NA
U-DKK-3/Cr	0.39**	0.62****	0.77****	0.65****	0.72****	0.87****	0.30	NA	NA	NA

Statistical significance.
*P < 0.05.
**P < 0.01.
***P < 0.001.
****P < 0.0001.
NA, not available.

CKD stages 3–5 and CKD stages 4 and 5 compared with CKD stage 1, respectively (Figure 1A). Whereas levels of S-DKK-3 were only increased in CKD stage 5, levels of U-DKK-3/Cr increased gradually from CKD stages 3–5 (Figure 1B).

Association of ETP with kidney fibrosis

S-ETP and U-ETP/Cr levels correlated significantly with the extent of fibrosis in the kidney (Figure 2). In a multiple regression analysis, S-ETP [$\log_{10}$(S-ETP) $r_{partial} = 0.32$, P = 0.003; $\log_{10}$(sCr) $r_{partial} = 0.10$, P = 0.35], but not U-ETP/Cr [$\log_{10}$(U-ETP/Cr) $r_{partial} = 0.07$, P = 0.52; $\log_{10}$(sCr) $r_{partial} = 0.34$, P = 0.002], was independently associated with fibrosis levels when adjusting for sCr.

S-ETP and U-ETP/Cr increased with increasing interstitial fibrosis and tubular atrophy [evaluated using both the Banff score (Figure 1C) and the T-score according to the MEST-C classification (Figure 1E)]. S-ETP [area under the curve (AUC) = 0.799, P < 0.001] and U-ETP/Cr (AUC = 0.759, P = 0.002) were able to discriminate patients with advanced interstitial fibrosis (ci3 compared with ci0–ci2) (Table 3).

S-ETP [AUC = 0.861 (95% CI 0.730–0.943), P < 0.001] and U-ETP/Cr [AUC = 0.839 (95% CI 0.701–0.931), P < 0.001] were able to discriminate patients with advanced interstitial fibrosis and tubular atrophy (T2 compared with T0–T1).

Association of DKK-3 with kidney fibrosis

Levels S-DKK-3 and U-DKK-3/Cr correlated significantly with the extent of fibrosis in patients with IgAN ($r = 0.35$, P < 0.05 and $r = 0.65$, P < 0.0001, respectively; Table 2) but did not correlate with sCr ($r = 0.05$, P = 0.76 and $r = -0.02$, P = 0.88, respectively). In patients with AAV, U-DKK-3/Cr correlated with sCr ($r = 0.72$, P < 0.001), but there was only a borderline significant correlation with the extent of fibrosis ($r = 0.30$, P = 0.07; Table 2), and there was a correlation between S-DKK-3 and the extent of fibrosis ($r = 0.36$, P < 0.05; Table 2), but not with sCr ($r = 0.27$, P = 0.07).

In IgAN and AAV patients, both S-DKK-3 and U-DKK-3/Cr levels were increased in biopsies with the highest ci3 compared with ci0–ci2 (P < 0.05 and P < 0.001, respectively; Figure 1D). However, levels of S-DKK-3 were not significantly elevated in IgAN patients with kidney biopsy scored as T1 or T2 compared with T0 (T-score; Figure 1F). In addition, U-DKK-3/Cr (AUC = 0.755, P < 0.001; Table 3) but not S-DKK-3 (AUC = 0.605, P = 0.15; Table 3) was able to discriminate biopsies with advanced interstitial fibrosis (ci3).

Comparison of biomarker association with fibrosis levels

Based on multiple regression analyses including S-ETP, U-ETP/Cr, S-DKK-3 and U-DKK-3/Cr (all $\log_{10}$-transformed), only S-ETP was independently associated with the level of fibrosis [Model 1; $\log_{10}$(S-ETP) $r_{partial} = 0.47$, P < 0.001; Table 4]. To assess whether the investigated biomarkers added to sCr, sCr was added to the previous model. Only S-ETP [$\log_{10}$(S-ETP) $r_{partial} = 0.38$, P < 0.01], and not sCr [$\log_{10}$(sCr) $r_{partial} = 0.04$, P = 0.72], was retained in the model and correlated significantly with the level of fibrosis (Model 2; Table 4).

We investigated the univariate association of all variables available with the extent of fibrosis in the combined IgAN and AAV discovery cohort (Table 2), sCr, PU, S-ETP, U-ETP/Cr, S-DKK-3 and U-DKK-3/Cr (all $\log_{10}$-transformed) had a significant univariate association with the extent of fibrosis. All these variables were included in a multiple regression analysis with sequential forward selection (Model 4). Only S-ETP was retained in the final model (Model 4; $\log_{10}$ $r_{partial} = 0.65$, P < 0.0001; Table 4).

Comparison of biomarker discrimination ability for advanced fibrosis

S-ETP and U-ETP/Cr had superior discriminatory power compared with S-DKK-3 and U-DKK-3/Cr, respectively

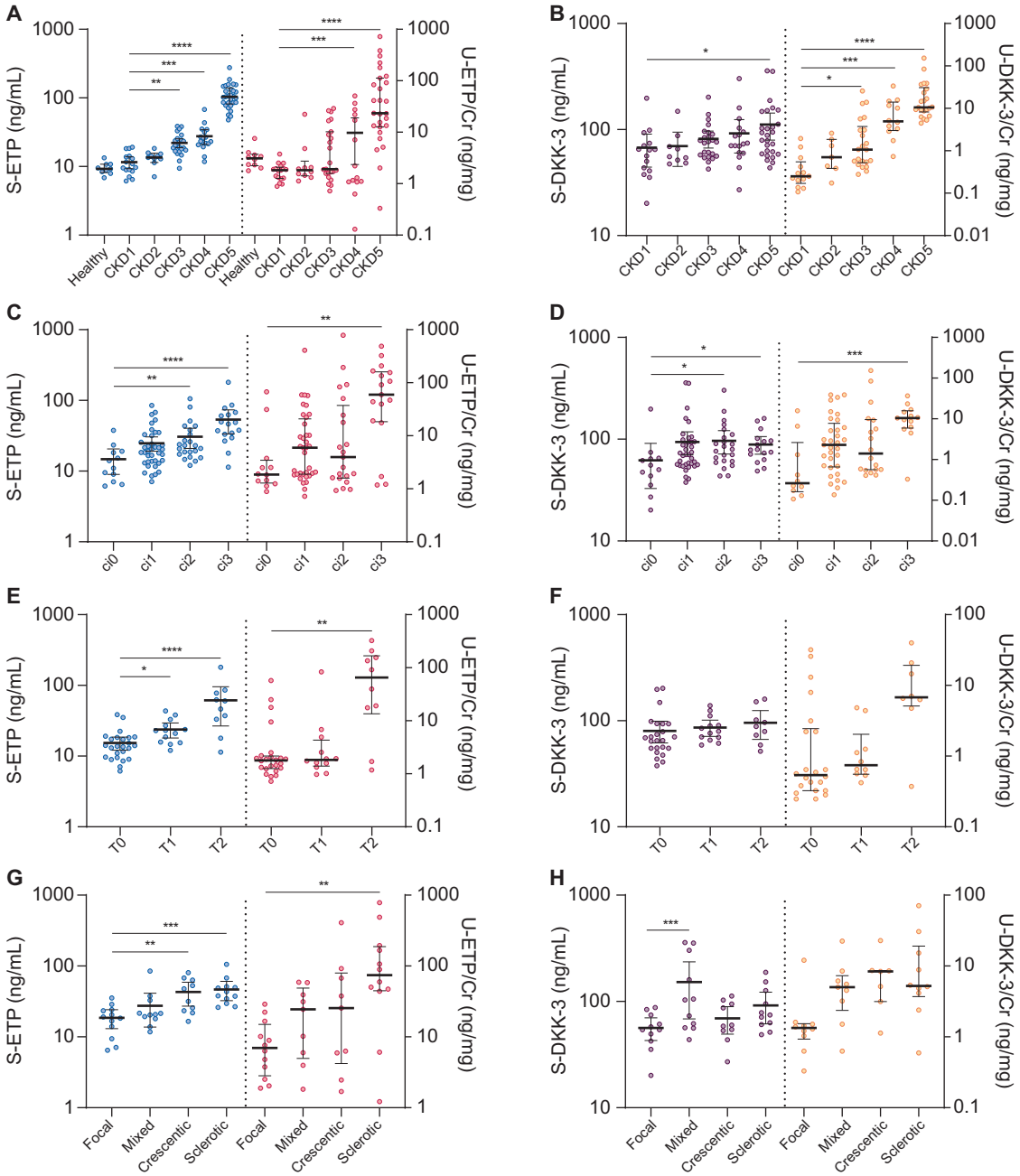

FIGURE 1: ETP and DKK-3 levels according to CKD stages, interstitial fibrosis and tubular atrophy, and glomerular morphology classes. Serum and urinary levels of ETP (A) and DKK-3 (B) in IgAN and AAV patients according to CKD stages (CKD1–5). Serum and urinary levels of ETP (C) and DKK-3 (D) in IgAN and AAV patients according to Banff score (ci0–ci3). Serum and urinary levels of ETP (E) and DKK-3 (F) in IgAN patients according to T-score (T0–T2). Serum and urinary levels of ETP (G) and DKK-3 (H) in AAV patients divided into different glomerular morphology classes (focal, mixed, crescentic or sclerotic). Data are presented on a $\log_{10}$ scale as median with IQR and statistical differences were assessed by Kruskal–Wallis test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

(comparison of ROC curves, $P = 0.03$ and $P = 0.05$), but were not significantly different from sCr (AUC $= 0.795$, $P < 0.001$; comparison of ROC curves, $P = 0.90$ and $P = 0.39$) (Table 3).

Validation of the association of ETP with fibrosis levels and its discrimination ability for advanced fibrosis

The validation cohort of 85 patients with IgAN confirmed the association of S-ETP and U-ETP/Cr with the level of kidney fibrosis (Figure 3B and C, Table 2) and the discrimination ability for advanced fibrosis (Table 3). In a multiple regression analysis, both S-ETP [$\log_{10}$(S-ETP) $r_{partial} = 0.35$, $P < 0.01$; $\log_{10}$(sCr) $r_{partial} = 0.32$, $P < 0.01$] and U-ETP/Cr [$\log_{10}$(U-ETP/Cr) $r_{partial} = 0.29$, $P = 0.03$; $\log_{10}$(sCr) $r_{partial} = 0.29$, $P = 0.02$] were independently associated with fibrosis levels when adjusting for sCr, whereas only S-ETP and not U-ETP/Cr was independently associated with fibrosis levels in the discovery cohort.

Morphology evaluation of AAV biopsies

In the AAV population, renal biopsies were classified according to the histologic classification scheme for glomerular damage as focal, mixed, crescentic or sclerotic [33] with the focal phenotype being associated to the best and the sclerotic phenotype being associated to the worst prognosis. The percentage of sclerotic glomeruli evaluated in the AAV biopsies correlated significantly with S-ETP, U-ETP/Cr and the level of fibrosis (Table 2). S-ETP levels were higher in AAV patients with biopsies classified as both crescentic and sclerotic compared with patients with biopsies classified as focal ($P < 0.01$ and $P < 0.001$, respectively; Figure 1G). U-ETP/Cr levels were significantly elevated in patients with the sclerotic phenotype ($P < 0.01$; Figure 1G). S-DKK-3 levels were significantly elevated in patients with the mixed compared with the focal phenotype ($P < 0.001$; Figure 1H). Based on ROC curve analysis, both S-ETP and U-ETP/Cr could discriminate patients with biopsies classified as focal from the other classifications [AUC for S-ETP = 0.816 (95% CI 0.672–0.915), $P < 0.001$; AUC for U-ETP/Cr = 0.758 (95% CI 0.602–0.877), $P < 0.001$].

ANCA types

There was no difference in S-ETP {median MPO: 23.36 [interquartile range (IQR) = 18.01–37.78] ng/mL, PR3: 25.52 (IQR = 18.73–49.27) ng/mL, P = 0.83} and U-ETP/Cr [median MPO: 16.24 (IQR = 5.98–54.49) ng/mg, PR3: 24.44 (IQR = 3.76–59.78) ng/mg, P = 0.83] levels between AAV patients with different ANCA types. The level of fibrosis was not significantly different in the AAV patients with different ANCA types (MPO versus PR3, P = 0.10).

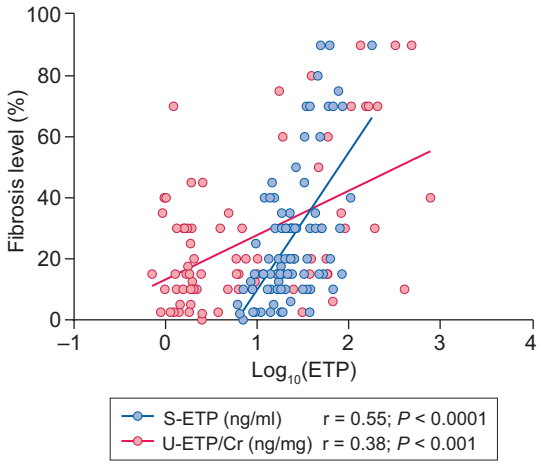

FIGURE 2: Spearman's rank correlations of serum and urinary levels of ETP with fibrosis levels in the combined IgAN and AAV discovery cohort. ETP levels are presented on a $\log_{10}$ scale.

Pulmonary fibrotic changes

We investigated whether the elevated ETP levels in the patients with AAV were influenced by pulmonary involvement with signs of fibrotic changes. Neither S-ETP nor U-ETP/Cr were influenced by pulmonary involvement [S-ETP median with pulmonary fibrotic changes: 24.70 (IQR = 21.69–34.17) ng/mL, without pulmonary fibrotic changes: 25.64 (IQR = 19.21–40.83) ng/mL, P = 0.79; U-ETP/Cr median with pulmonary fibrotic changes median: 8.26 (IQR = 6.07–47.90) ng/mg, without pulmonary fibrotic changes median: 24.94 (IQR = 4.59–58.70) ng/mg, P = 0.78].

Change in kidney function from baseline to follow-up

A 1-year clinical follow-up was available for all IgAN and AAV patients. S-ETP and U-ETP/Cr baseline levels were not significantly different between patients that regressed, remained stable and progressed in CKD stage (IgAN S-ETP: P = 0.85; IgAN U-ETP/Cr: P = 0.50; AAV S-ETP: P = 0.10; AAV U-ETP/Cr: P = 0.09).

A 3-year-long clinical follow-up was available for 31 AAV patients. These patients were primarily treated with corticosteroids combined with other immunosuppressive drugs. Sixteen patients regressed, 11 patients remained stable and 4 patients progressed in CKD stage. Patients who experienced a decrease in CKD stage (regression) had significantly higher S-ETP levels at baseline than patients who increased in CKD stage (progression) ($P < 0.05$; Figure 4A). In fact, baseline levels of S-ETP could discriminate the AAV patients with a decrease in CKD stage from patients with no change or an increase in CKD stage from baseline to the 3-year follow-up [AUC = 0.807 (95% CI 0.615–0.931), $P < 0.001$].

DISCUSSION

Reliable biomarkers for non-invasive assessment of kidney fibrosis burden and progression in patients with kidney diseases are lacking. The ECM holds great promise as a source of new markers for the detection of early alterations leading to disease progression [34]. The main finding of this work is that the matrikine ETP, measured by the PRO-C6 assay, correlated with burden of fibrosis in the kidneys of IgAN patients and AAV

Table 3. ROC curve analysis to determinate discriminatory power of biomarkers for advanced fibrosis

Cohort	Biomarker	AUC	95% CI	P-value	Sensitivity	Specificity	Criterion
IgAN and AAV discovery	sCr	0.795	0.698–0.872	<0.0001	87.5	69.3	>3.0
	S-ETP	0.799	0.702–0.876	<0.0001	87.5	73.3	>26.0
	U-ETP/Cr	0.759	0.655–0.845	0.002	81.3	72.9	>16.7
	S-DKK-3	0.605	0.496–0.708	0.15	73.3	56.2	>73.1
	U-DKK-3/Cr	0.755	0.642–0.848	<0.001	91.7	66.1	>3.8
IgAN validation	sCr	0.834	0.738–0.906	0.0001	75.0	87.7	>2.6
	S-ETP	0.822	0.725–0.897	<0.0001	83.3	68.5	>15.4
	U-ETP/Cr	0.847	0.732–0.926	<0.0001	100	61.8	>2.4

ROC criteria: Banff score ci3 (advanced interstitial fibrosis) versus ci0–ci2 (low to moderate interstitial fibrosis).

Table 4. Multiple regression analysis to determine the association of biomarkers with the extent of fibrosis (%)

Cohort	Model	Method	$r_{partial}$					
			Log_{10}(S-ETP)	Log_{10}(U-ETP/Cr)	Log_{10}(S-DKK-3)	Log_{10}(U-DKK-3/Cr)	Log_{10}(sCr)	Log_{10}(PU)
IgAN and AAV discovery	1	Enter	0.47**	0.08	0.02	−0.09	–	–
	2	Enter	0.38*	0.07	0.03	−0.10	0.04	–
	3	Enter	0.37*	0.07	0.02	−0.10	0.05	0.03
	4	Forward	0.65***	NR	NR	NR	NR	NR

Statistical significance.
*P < 0.01.
**P < 0.001.
***P < 0.0001.
Method: Enter (enter all variables in the model in one single step), Forward (enter significant variables sequentially).
NR, not retained in the model.

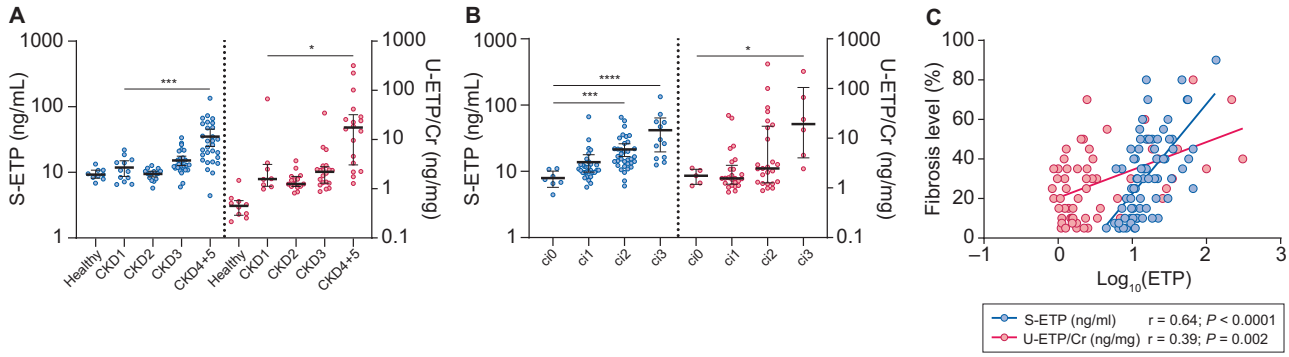

FIGURE 3: ETP levels in the validation cohort according to CKD stages (A), Banff scores (B) and Spearman's rank correlations with fibrosis levels (C). (A and B) Data are presented on a log_{10} scale as median with IQR and statistical differences were assessed by Kruskal–Wallis test; *P < 0.05, ***P < 0.001, ****P < 0.0001. (C) ETP levels are presented on a log_{10} scale.

patients. Based on multiple regression analysis including S-ETP and sCr, only S-ETP and not sCr was independently associated with the degree of fibrosis. Moreover, high baseline S-ETP levels were able to identify AAV patients that regressed in CKD stage within a follow-up period of 3 years. In addition, ETP was superior to the known fibrosis biomarker DKK-3 in reflecting fibrosis burden.

It is well-established that not only are baseline eGFR and PU risk factors of CKD progression, but the degree of glomerulosclerosis, interstitial fibrosis and tubular atrophy are also important predictors of long-term kidney outcome in patients with CKD [35, 36]. As tubulointerstitial fibrosis remains a powerful predictor of future disease progression [13, 14], biomarkers reflecting altered turnover of key components of fibrosis hold great promise as a surrogate to monitoring changes in the tissue. Such changes may reflect the destruction of the renal parenchyma. Seminal studies have shown that collagens are not merely structural entities but can release fragments with paracrine and endocrine properties, termed matrikines [15, 16]. COL VI is expressed at low levels in healthy adult kidneys [18] and is markedly upregulated in patients with renal fibrosis [19, 20]. When COL VI is produced by fibroblasts, ETP, a fragment with important signalling properties, is released from the α3 chain [21, 37]. The PRO-C6 assay detects the released fragment, and thus reflects both COL VI formation and levels of ETP. ETP was previously identified as a novel prognostic biomarker of kidney-related outcome in different populations of CKD patients [19, 24, 25]. ETP was highly present in human kidneys with fibrotic foci and inflammatory cell infiltration, in areas co-localizing with markedly increased total COL VI staining. Even though COL VI was present in histologically normal kidneys, no ETP staining was observed [19].

Here, we explored for the first time whether levels of ETP in serum and urine samples taken on the same day of renal biopsy reflect the fibrosis burden in patients with IgAN and in patients with AAV. The increased levels of ETP were related to an increased level of fibrosis in the biopsies, regardless of the used fibrosis score. When investigating which variables best described the extent of histologically confirmed fibrosis, S-ETP was the only variable retained in the final model. By measuring ETP, a molecule that is directly involved in the structural changes that takes place during development of renal fibrosis, earlier diagnosis and detection of disease progression might be possible. This is especially important as surrogate markers of kidney function such as sCr reflect delayed changes due to the concept of 'renal reserve'; during progressive destruction of nephrons, hyperfiltration and compensatory hypertrophy of the remaining healthy nephrons can maintain a normal GFR even though pathological structural changes are already in place [38, 39]. S-ETP and U-ETP/Cr levels were also able to discriminate between AAV patients whose glomerular injury in biopsies were classified as focal, associated with a good renal outcome, and

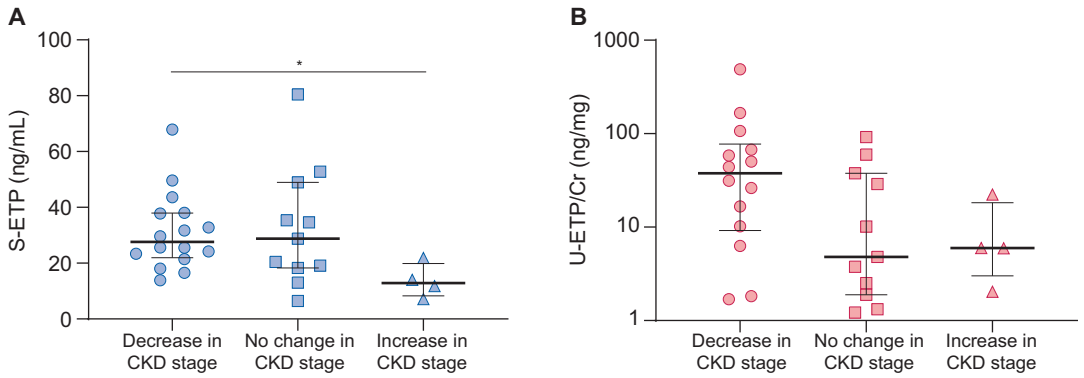

FIGURE 4: Baseline ETP levels in patients with AAV divided into groups based on changes in CKD stage from baseline to the 3-year follow-up. Serum (A) and urinary (B) ETP levels. Data are presented on a linear (A) and $\log_{10}$ scale (B) as median with IQR and statistical differences were assessed by Kruskal–Wallis test; *$P < 0.05$.

sclerotic, associated with the poorest prognosis (advancement to ESKD and death within 1 year after diagnosis) [33]. ETP levels may in part reflect increased deposition of COL VI in the glomerulus, which is in line with previous studies showing that COL VI replaces COL IV in the glomerular basement membrane in diabetic glomeruli [40].

In alignment with previous findings [26], we showed that U-DKK-3/Cr correlated to the extent of interstitial fibrosis in the biopsies from IgAN patients. However, ETP appeared to have a superior association to fibrosis burden than did DKK-3.

ETP has previously been explored as a marker of lung diseases and high S-ETP levels were associated with an increased risk of mortality in chronic obstructive pulmonary disease [41]. Our data indicate that the elevated ETP levels were not influenced by pulmonary involvement with signs of fibrotic changes. In this study, it was not possible to quantify the degree of lung fibrosis in the AAV patients; therefore, it is not possible to draw conclusions on the relationship of the marker with lung fibrosis, as the nature of pulmonary involvement in these patients may not be related to fibrosis.

During the 3-year follow-up, AAV patients who had an improved kidney function also had a higher baseline level of S-ETP compared with patients with stable or decline in kidney function. Since all AAV patients received treatment after diagnosis, this might indicate that high levels of S-ETP at baseline identified patients with a more active disease, who are more prone to respond to immunosuppressive treatment. This would also be in line with the effects of ETP, which is a matrikine promoting a proinflammatory environment that drives fibrosis [37]. There was no difference between the groups at the 1-year follow-up, either in the IgAN or in the AAV population. However, as a 1-year follow-up time is short, especially for IgAN patients who have a slowly progressing disease, this could be expected.

The association of ETP with the level of fibrosis in the kidney was verified in the validation cohort of patients with IgAN, confirming the robustness of ETP, measured in both serum and urine, as a marker of histologically confirmed fibrosis.

In conclusion, both S-ETP and U-ETP/Cr levels reflect the burden of histologically confirmed fibrosis in the kidneys from patients with IgAN and patients with AAV. The high correlation between S-ETP and U-ETP/Cr indicates that changes in the biomarker levels in serum of patients in this study are not a result of impaired kidney function, but rather a reflection of an actual increased COL VI production, which accurately reflects the fibrosis burden in the kidney. The results presented in this study emphasize the potential of ETP as a non-invasive biomarker of interstitial fibrosis and tubular atrophy that might supplement kidney biopsies and may aid in the evaluation of anti-fibrotic compounds.

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AUTHORS' CONTRIBUTIONS

V.T., Z.H., F.G. and N.S. designed the study. N.S. performed the statistical analysis, made the figures and drafted the paper. All authors acquired the data, contributed to the interpretation of findings, revision of the paper and approval of the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

F.G., D.G.K.R. and M.A.K. are full-time employees at Nordic Bioscience. N.S. is full-time employee at Nordic Bioscience and the University of Copenhagen. Nordic Bioscience is a privately owned, small- to medium-sized enterprise partly focused on the development of biomarkers. None of the authors received fees, bonuses or other benefits for the work described in this article. F.G. and M.A.K. hold stock in Nordic Bioscience. The patent for the PRO-C6 ELISA used in this work is owned by Nordic Bioscience. The funder provided support in the form of salaries for authors N.S., F.G., D.G.K.R. and M.A.K., but did not have any additional role in

the study design, data collection and analysis, decision to publish or preparation of the manuscript. All other authors report no competing financial interests relevant to this article. The results presented in this article have not been published previously in whole or part, except in abstract format.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

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