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Towards clinical translation of urinary vitronectin for non-invasive detection and monitoring of renal fibrosis in kidney transplant patients

Marta Clos-Sansalvador[1,2†], Omar Taco[1†], Paula Rodríguez-Martínez[3], Sergio G. Garcia[1,2], Miriam Font-Morón[1], Jordi Bover[1], Anna Vila-Santandreu[1], Marcella Franquesa[1], Javier Juega[1] and Francesc E. Borràs[1,4*]

Abstract

Background Interstitial fibrosis and tubular atrophy (IFTA) is a critical factor in the prognosis of kidney health. Currently, IFTA quantitation in kidney biopsy samples is crucial for diagnosis and assessing disease severity, but the available non-invasive biomarkers are not satisfactory. Proteomic studies identified urinary vitronectin (VTN) as a potential biomarker for kidney fibrosis. As mass spectrometry techniques are not practical for use in clinical settings, we tested whether evaluation of urinary VTN levels through enzyme-linked immunosorbent assay (ELISA) can help monitor fibrotic changes in kidney transplant recipients and prove the clinical viability of the assay.

Methods A total of 58 kidney transplant (KTx) patients who underwent renal biopsy were included in the study. Patients were categorized into two groups referred as no fibrosis (0%) or with fibrosis (≥ 5%) based on their histological findings. In a subsequent/follow-up analysis, the time elapsed from transplantation was also considered. The urinary levels of VTN were measured using ELISA.

Results VTN ($p = 0.0180$) and VTN normalized by urinary creatinine levels ($p = 0.0037$), were significantly increased in patients with fibrotic grafts. When focusing on patients with long-term grafts (> 3 years from transplantation, $n = 36$), VTN exhibited superior potential in identifying fibrotic grafts compared to albuminuria (VTN $p = 0.0040$ vs. albuminuria $p = 0.0132$). Importantly, in this group, while albuminuria correctly identified 71% of fibrotic patients, the combination of VTN plus albuminuria correctly classified 89% of fibrotic grafts detected by renal biopsy.

Conclusions VTN has emerged as a valid indicator of renal fibrosis. Of interest, urinary levels of VTN in combination with conventional clinical parameters (such as albuminuria) significantly improved the non-invasive detection of renal fibrosis in kidney transplant patients.

Keywords (3–10): Biomarker, Kidney fibrosis, kidney transplant, Nephrology, Extracellular matrix, Liquid biopsy, ELISA

†Marta Clos-Sansalvador and Omar Taco contributed equally to this work.

*Correspondence:
Francesc E. Borràs
feborras@igtp.cat

Full list of author information is available at the end of the article

Background

Kidney fibrosis is widely recognized as the common final pathway and a key factor in the progression of various kidney diseases to end-stage renal failure [1–3] Interstitial fibrosis and tubular atrophy (IF/TA) are consistently identified as major contributors to kidney allograft failure and present a significant challenge to long-term allograft survival in kidney transplantation [4, 5]. This progression occurs regardless of the underlying disease, making fibrosis an important therapeutic target [6].

Fibrosis is a complex pathological process characterized by excessive accumulation of extracellular matrix (ECM) in the renal parenchyma, which leads to a progressive loss of kidney function [7]. Underlying pathophysiological mechanisms are diverse, most of them involving chronic injury to renal cells, particularly tubular epithelial cells, that trigger a maladaptive repair response. This may lead to activation and proliferation of myofibroblasts, persistent inflammation and activation of immune inflammatory cells promoting fibrogenic signals [8]. Also, imbalance between matrix metalloproteinases and their inhibitors favours ECM accumulation [9], and microvascular rarefaction leads to chronic hypoxia, further promoting fibrosis [10]. These interconnected mechanisms, among others, create a self-perpetuating cycle of injury, inflammation, and fibrosis that progressively replaces functional renal tissue with scar tissue, ultimately leading to end-stage renal disease [11, 12].

Assessment of kidney fibrosis is still based on kidney biopsy. This technique allows direct visualization of the tissue sample, provide specific diagnoses, quantify fibrosis, and offer detailed cellular information. However, despite recent advances in automation, biopsies are still predominantly based on subjective interpretations from nephropathologists and are invasive, posing risks, patient discomfort, economic burdens, and limitations in representing the entire organ's status [13]. Thus, efforts are underway to develop objective, non-invasive, and quantitative diagnostic methods [14] while enhancing our understanding of molecular mechanisms to identify potential targeted therapies [3].

In this sense, non-invasive biomarkers for assessing IFTA in kidney transplantation have evolved significantly over the past few decades. From unspecific determinations such as serum creatinine, albuminuria and estimated glomerular filtration rate (eGFR), attention shifted to urinary biomarkers, such as transforming growth factor-β (TGF-β) [15], monocyte chemoattractant protein-1 (MCP-1) [16], matrix metalloproteinase-2 (MMP-2) [17] and α1-microglobulin (A1M) [18] in urine among others. Also, advances in omic technologies allowed the study of complex panels of urinary proteins. In this sense, notable developments include the CKD273 test, which uses a panel of urinary peptides [19, 20] in a clinical setting. Thus, urinary proteomic studies successfully identified several biomarkers aiming to complement or replace invasive kidney biopsies, especially when the latter are contraindicated [21]. Yet, proteomic techniques are hindered by their cost and time requirements, impeding their easy translation to clinical practice [22]. Therefore, there is a pressing need for translating identified biomarkers to facilitate the monitoring of transplanted kidneys in a clinically applicable manner. In this scenario, a previous proteomic study by our group identified vitronectin (VTN) as a potential biomarker for kidney fibrosis [23]. In that study, urine VTN showed differential expression in patients with chronic IFTA lesions, specifically in those with ci and ct mean scores greater than 2 according to Banff criteria.

VTN is a multifunctional glycoprotein that accumulates in the renal interstitium during fibrogenesis [24] and has been identified as one of the most upregulated extracellular matrix proteins in fibrotic kidney tissue scaffolds [25]. While previous studies suggested that VTN may not be essential for fibrogenesis [24], it has been established that VTN interacts with several key proteins involved in fibrosis, including plasminogen activator inhibitor-1 (PAI-1) [26] and integrin receptors among others [27]. Moreover, VTN is primarily expressed and secreted by activated macrophages in chronic kidney disease models and has been recently related to contribute to the fibrotic environment [25]. All these data suggest VTN plays a significant role in the complex process of kidney fibrosis, potentially serving as both a diagnostic marker and a therapeutic target.

Considering the translational limitations of the proteomic analyses, and the potential of VTN to monitor renal fibrosis in a non-invasive manner, we aimed to assess whether urine VTN levels could reflect the findings determined by the gold standard (renal biopsy) using a clinically established technique such ELISA (Enzyme-Linked Immunosorbent Assay). This widely used technique is cost-efficient, and offers higher throughput and easier sample preparation among other advantages [28].

To this end, we conducted a pilot pre-clinical evaluation of urinary VTN levels in kidney transplanted patients [23]. Notably, our cross-sectional study confirmed statistically significant differences between the two examined groups (biopsy-proven non-fibrotic vs. fibrotic patients), highlighting the potential of using a clinically applicable VTN test as a promising approach for non-invasively detecting and monitoring renal fibrosis.

Methods

Patients and study design

This was a single-center, cross-sectional study in which kidney transplanted patients underwent renal biopsy at the Hospital Universitari Germans Trias i Pujol

(HUGTiP, Badalona, Spain) between July 2019 and January 2022. The protocol was conducted in accordance with the Declaration of Helsinki and the recommendations of the Guideline for Good Clinical Practice from the "Comitè d'Ètica de la investigació clínica de HUGTiP", who also approved the protocol (PI-20-083). This protocol did not alter at any time the standard procedure of patient's medical care. To protect the identity of the participants, an independent arbitrary code was used for sample identification.

Adult kidney transplant recipients from a living or cadaveric donor able to provide written informed consent were evaluated for eligibility and enrolled based on predetermined inclusion criteria. These criteria included patients who had undergone renal transplantation and were referred for biopsy based on clinical grounds (indication biopsy), regardless of whether they presented acute or chronic allograft dysfunction. Also included patients with stable allograft function and donor-specific antibody (DSA) positivity (surveillance biopsy). Lastly, patients undergoing monitoring treatment response or investigation of unexplained symptoms. Notably, none of our recruited patients had been treated with Sodium-Glucose Cotransporter-2 (SGLT2) inhibitors, Endothelin Receptor Antagonists, or Mineralocorticoid Receptor Antagonists for at least 6 months prior to their biopsy. The main exclusion criteria were acute kidney injury (AKI) or urinary tract infection (UTI) at presentation, age < 20 years, diagnosis of Hepatocellular Carcinoma (HCC), HCV infection and unwillingness to participate in the study.

In addition, 14 healthy volunteers were included in some analyses as our control group. These were selected based on a normal estimated glomerular filtration rate (eGFR) (>90 mL/min/1.73m2) because of ethical considerations related to performing invasive procedures (renal biopsy) on subjects who may not need them. None of these controls had a history of cardiovascular disease, diabetes, UTI or urinary system disease, and their good health status was confirmed through laboratory assessments.

Patient data collection

Our analyses included the following clinical variables: age, sex, diabetes mellitus, hypertension, cardiovascular disease, estimated glomerular filtration rate (GFR), spot urinary protein-to-creatinine ratio (UPCR), serum creatinine, albuminuria, treatment with ACEi, time after transplantation, body mass index (BMI), and immunosuppression regimen. GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation [29].

Assessment of renal scarring

Allograft kidney biopsies ($n=58$) were blindly evaluated by nephropathologists (P.R and L.H) according to the Banff 1997 criteria and their subsequent updates [30]. Specifically, 2-micron thickness histological sections from formalin-fixed paraffin-embedded blocks were made and stained for haematoxylin-eosin, PAS, Jones methenamine silver, and Masson's trichrome. BenchMark Special Stains System™ (Roche) was used and protocols provided by the manufacturer were followed. The number of glomeruli in each biopsy was over ten. Quantitative evaluation was assessed using visual methods and scoring according to Banff cut-offs for interstitial fibrosis (ci) [ci0: ≤ 5%, ci1: 6–25%, ci2: 26–50%, ci3: > 50%] and tubular atrophy (ct). As high grades of fibrosis > 50% were poorly detected ($n=3$), the patients were classified into the absence of interstitial fibrosis (non-fibrotic; ci/ct < 1; 0% fibrosis) or with the presence of interstitial fibrosis (fibrotic; ci/ct≥1; ≥ 5% fibrosis; mean grade of fibrosis detected 20%).

To better capture the relationship between the percentage of renal fibrosis and other variables in our analysis, we used the percentage of interstitial fibrosis as a continuous variable for the subsequent analyses. This was a methodological choice aimed at optimizing the analysis of continuous data and ensuring the accuracy and reliability to assess the impact of different factors on the progression of renal fibrosis and capture the nuances and variations present; this conversion allows for a more precise analysis of the relationship between the percentage of fibrosis and other continuous variables, such as biomarker levels.

Urine sample collection

All urine samples from kidney transplant (KTx) patients were collected up to 8 h before renal biopsy was performed. Samples were maintained at 4ºC before further processing and aliquoting as recommended [31] and for a maximum of 6 h. Approximately, 25 mL of urine from each patient was collected, centrifuged at 600 xg for 15 min to eliminate cells and cell debris, and stored immediately at -80ºC.

Creatinine normalization

As water reabsorption in the kidneys affects urinary solute concentrations, urinary biomarker levels are frequently reported as a ratio to urinary creatinine (U.Creat) to provide a standardized measure for comparison across samples and individuals. This approach often enhances the accuracy of biomarker measurements and their correlation with disease states or kidney function, and it facilitates the practical use of spot urine samples. Consequently, this practice is widely accepted in both clinical and research settings.

Enzyme-linked immunosorbent assay (ELISA)

All urine samples from KTx patients and healthy controls were analyzed using a commercial Vitronectin (VTN) ELISA kit (Cloud-Clone Corporation, USA), following the manufacturer's recommendations. One milliliter of each urine sample was thawed overnight at 4ºC and concentrated as reported before [23]. The urine concentrate was finally adjusted to 100 µL with the kit's dilution buffer and analyzed using the ELISA kit.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (9.0 version). Normal distribution of data was checked by a Shapiro-Wilk test and then the appropriate statistical test for each case was chosen. A Mann-Whitney test (non-parametric) was used to compare two groups of samples. Multiple group comparisons were conducted using the Kruskall-Wallis with Dunn's multiple comparison test (non-parametric). The correlation of multiple variables was checked using Spearman's correlation test. Receiver operating characteristic (ROC) curve analyses were performed to calculate the area under the curve and the sensitivity and specificity of each parameter. Finally, Venn diagrams were employed to visually represent the separated or overlapped potential in the identification of fibrotic patients by the two non-invasive biomarkers compared (albuminuria and VTN).

Results

Clinical parameters and patient stratification

Fifty-eight urine samples were collected from kidney transplant (KTx) patients and based on histopathological findings, were classified into two groups, namely "absence of fibrosis" (0%, non-fibrotic) and "presence of fibrosis" (≥5%, fibrotic) as described in materials and methods. Table 1 summarizes the relevant clinical parameters, biological variables, and immunosuppressive treatment of these patients.

The comparison of these parameters and variables revealed significant differences between the two groups in the vitronectin (VTN, $p=0.0180$) and VTN levels corrected by urinary creatinine (VTN/U.Creat, $p=0.0037$) in the fibrotic group, and in the number of patients treated with ACEi/ARBs ($p=0.0279$). No significant differences were observed regarding age, sex, cardiovascular disease, diabetes mellitus, body mass index, immunosuppression regimens, serum creatinine, estimated glomerular filtration rate, proteinuria, albuminuria, and "years from transplantation".

Correlation of clinical parameters and VTN with fibrosis

To elucidate the association between fibrosis and the monitored parameters, we focused on statistically significant parameters extracted from Table 1, along with other clinical indicators that exhibited a closer relationship to

Table 1 Clinical parameters of the patients

All patients ($n=58$)	Fibrosis 0% ($n=14$)			Fibrosis ≥ 5% ($n=44$)			*p*-value	Sig
Age (mean ± SD)	53.57	±	15.46	58.57	±	13.62	0.2725 [M]	ns
Sex (n (male), %)	10		71%	26		59%	0.533 [M]	ns
DM (n, %)	5		36%	16		36%	> 0.9999 [M]	ns
CVD (n, %)	5		36%	16		36%	> 0.9999 [M]	ns
HTA (n, %)	13		93%	44		100%	0.2414 [M]	ns
BMI (mean ± SD)	26.04	±	4.788	24.93	±	5.75	0.4687 [M]	ns
ACEi/ARBs	4		28%	29		66%	0.0279 [M]	*
Immunosuppression								
PDN + CsA (n, %)	0		0%	1		2.5%	> 0.9999 [M]	ns
PDN + CsA + MPS (n, %)	0		0%	1		2.5%	> 0.9999 [M]	ns
PDN + TAC (n, %)	1		7%	0		0%	0.2414 [M]	ns
PDN + TAC + MPS (n, %)	12		86%	36		81%	0.6713 [M]	ns
PDN + TAC + EVE (n, %)	1		7%	6		14%	> 0.9999 [M]	ns
S.Creat (mean ± SD)	2.49	±	1.26	2.60	±	1.35	0.7841 [M]	ns
eGFR (mean ± SD)	32.21	±	15.60	30.70	±	17.02	0.7429 [M]	ns
Proteinuria (mean ± SD)	742	±	824.40	1322	±	1630	0.1594 [M]	ns
Albuminuria (mean ± SD)	359.70	±	645	725.20	±	1070	0.096 [M]	ns
VTN (mean ± SD)	31.37	±	33.30	55.38	±	36.45	0.0180 [M]	*
VTN/U.Creat (mean ± SD)	0.46	±	0.52	1.15	±	0.86	0.0037 [M]	**
Years from Tx (mean ± SD)	4.214	±	1.968	5.068	±	3.121	0.5636 [M]	ns

[M]Mann-Whitney test

DM, diabetes mellitus; CVD, cardiovascular disease; HTA, hypertension; BMI, body mass index; ACEi/ARBs, angiotensin-converting- enzyme inhibitor/angiotensin receptor blocker; PDN, prednisone; CsA, cyclosporine A; MPS, mycophenolate sodium; TAC, tacrolimus; EVE, everolimus; S.Creat, serum creatinine; eGFR, estimated glomerular filtration rate; VTN, Vitronectin; U.Creat, urinary creatinine; Sig, significance; ns, non-significant (p-value > 0,05); *p-value < 0.05 and **p-value < 0.01

fibrosis, namely proteinuria ($p=0.1594$), albuminuria ($p=0.096$), and the variable "years from transplantation," which has been previously established as correlated with allograft fibrosis [32]. Subsequently, a Spearman's correlation test and the discrimination capacity of these variables between the non-fibrotic and fibrotic groups were assessed.

Global analysis ($n=58$) revealed no significant correlation between proteinuria and albuminuria levels and the presence of fibrosis in the biopsies. Notably, a weak yet significant correlation with VTN ($r=0.28$, $p=0.036$) and a stronger and significant correlation with "years from transplantation" ($r=0.43$, $p=0.0008$) were observed (Fig. 1A). Further examination of mean differences between the non-fibrotic and fibrotic groups (Fig. 1B-E) demonstrated statistically significant differences only in VTN ($p=0.0180$) (Fig. 1D) and normalized VTN levels ($p=0.0037$) (Fig. 1E).

Interestingly, the receiver operating characteristic (ROC) curves for VTN and VTN/U.Creat, showed a significant discriminatory potential for the patients with and without fibrosis, with an area under the curve (AUC) values of 0.7094 (Fig. 1H) and 0.7541 (Fig. 1I), respectively. Conversely, "years from transplantation" (Fig. 1F) and albuminuria (Fig. 1G) displayed no significant discriminatory capacity between the two patient groups.

Analysis of VTN levels in non-fibrotic and fibrotic patients segregated by "years from transplantation"

Although the parameter "years from transplantation" did not exhibit discriminatory potential in distinguishing the absence or presence of fibrosis, its strong correlation with the percentage of fibrosis prompted us to delve deeper into its impact on VTN as the unique variable that demonstrated such discriminatory capability. For this, we stratified patients based on "years from transplantation" and the absence or presence of fibrosis, and subsequently, we examined the values of VTN levels across these distinct groups.

Our analysis revealed that in recently transplanted patients (<1 year) and those extending up to the third-year post-transplantation (1–3 years), all individuals exhibited elevated levels of VTN regardless of the observed fibrosis in renal biopsies. From the third year onwards (>3 years), non-fibrotic patients displayed noticeably reduced VTN levels (Fig. 2), whereas in the fibrotic group, VTN levels remained high. This dichotomous pattern in VTN levels prompted us to categorize patients based on their "years from transplantation" as either "recent" transplanted (≤3 years) or "long-term" transplanted (>3 years).

The "recent" transplanted group, exhibited few discernible differences, except age which was notably higher in the fibrotic group than in the non-fibrotic group. However, none of the other biological variables, clinical parameters, or biomarkers demonstrated the ability to distinguish between the two groups (Supplementary Table 1). The analysis of the "long-term" group is described in the next section.

Clinical parameters of the "long-term" transplanted patients

In the analysis of "long-term" transplanted patients, we included 36 individuals from the overall group. Table 2 succinctly outlines the clinical parameters, biological variables, and immunosuppressive treatments for this subset of patients.

Within this time-specific patient group, notable distinctions were detected between non-fibrotic and fibrotic patients. Specifically, significant differences were observed in VTN ($p=0.0040$), VTN/U.Creat ($p=0.0037$), proteinuria ($p=0.0363$), and albuminuria ($p=0.0132$). No significant differences were identified in any other parameter, biological variable, or immunosuppression treatment within this context (Table 2).

Correlation of clinical parameters and VTN with fibrosis: "long-term" transplanted patients' analysis

Spearman's correlation test applied to the parameters deemed statistically significant in Table 2, alongside the "years from transplantation" variable in the long-term transplanted patients, revealed an absence of significant correlation with the percentage of fibrosis for any of the analyzed variables. Notably, VTN still emerged as the parameter with the highest correlation value ($r=0.29$, $p=0.0849$) (Fig. 3A).

However, the potential significance of these variables became evident when comparing the means of non-fibrotic and fibrotic patients, as all parameters (except "years from transplantation", Fig. 3B) exhibited significant differences between both groups. Of particular interest, the means of non-fibrotic and fibrotic patients demonstrated statistically greater discrimination in VTN (Fig. 3D) and normalized VTN (Fig. 3E) levels compared with albuminuria levels (Fig. 3C).

Moreover, the receiver operating characteristic (ROC) curves for these three variables underscored their discrimination capacity for fibrotic patients, with VTN/U.Creat exhibiting the highest efficacy (AUC=0.8438, $p=0.0034$, Fig. 3I), followed by VTN (AUC=0.8259, $p=0.0055$, Fig. 3H), and lastly, albuminuria (AUC=0.7857, $p=0.0149$, Fig. 3G).

Given that VTN, normalized VTN, and albuminuria demonstrated the capacity to differentiate between non-fibrotic and fibrotic patient groups, our subsequent analysis aimed to identify which patients with fibrosis were specifically detected by each parameter or their combinations. To achieve this, we established thresholds for

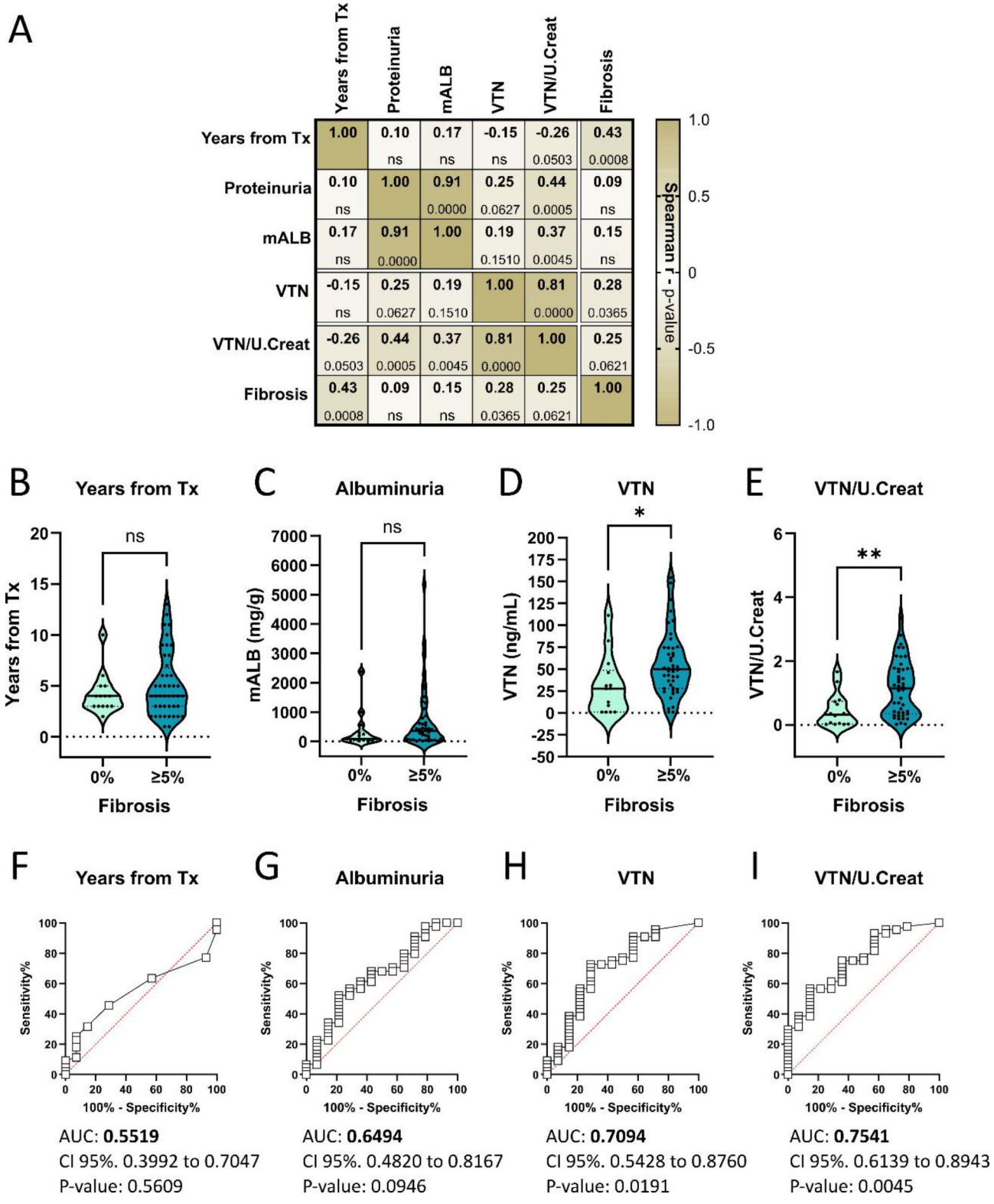

Fig. 1 Analysis of all KTx patients. (**A**) Spearman's correlation coefficient matrix. Spearman r values are indicated in bold and *p*-values of the correlation are indicated at the bottom if *p*-value < 0.2. *P*-values > 0.2 are indicated as ns. (**B**) Violin plots showing the descriptive statistics and statistical differences according to the parameter "years from transplantation", (**C**) albuminuria levels, (**D**) VTN levels obtained by ELISA and (**E**) VTN levels normalized by urinary creatinine, stratified regarding the percentage of fibrosis observed in the kidney biopsies. Statistical differences are indicated for $*p < 0.05$ and $**p < 0.01$ by a Mann-Whitney test. (**F**) ROC curve based on "years from transplantation", (**G**) albuminuria (**H**) VTN, and (**I**) VTN normalized by urinary creatinine, as a unique biomarker to differentiate the two groups of patients (no fibrosis vs. fibrosis). VTN, Vitronectin; U.Creat, urinary creatinine; mALB, albuminuria

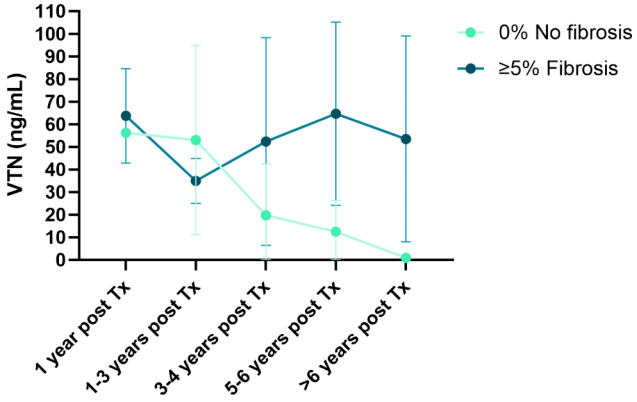

Fig. 2 VTN levels of all the patients stratified by their years from transplantation and fibrosis percentage. Data are shown as mean ± SD in all groups

each variable based on their ROC curves (VTN > 12.80 ng/mL; VTN/U.Creat > 0.13; Albuminuria > 95.65 mg/g), designating values above these thresholds as positive. Venn diagrams delineated the number and percentage of fibrotic patients identified by each parameter individually (VTN, VTN/U.Creat, and albuminuria), or in combination (VTN + albuminuria, and VTN/U.Creat + albuminuria) (Fig. 4A and B). In addition, because albuminuria is a widely used clinical parameter, we repeated the analyses using an established clinical threshold (>300 mg/g) (Fig. 4C and D) [33].

The results showed a consistent pattern, regardless of the albuminuria threshold. Most patients with fibrosis were identified by both VTN (or normalized levels) and albuminuria. However, an important proportion (17–25%) of patients were identified solely by VTN or VTN/U.Creat, and a minimal subset (0-3.6%) by albuminuria alone (Fig. 4).

To confirm that the VTN threshold chosen using the ROC curve was not biased into a sensitivity overestimation due to our specific group of non-fibrotic patients, we also analyzed 14 healthy controls with confirmed healthy eGFR (>90 mL/min/1.73m^2).

The mean ± SD VTN levels obtained from this analysis were 8.70 ± 10.6 ng/mL and the median was 5.16 ng/mL (Fig. 5A **and panel)**, which was much lower than the threshold used for the previous approach (12.80 ng/mL). Furthermore, no statistically significant differences were observed between healthy controls and non-fibrotic KTx patients (Fig. 5A). The discrimination potential of VTN between healthy controls and fibrotic patients was also analyzed (Fig. 5C), leading to an AUC of 0.89, specificity of 82.14%, and sensitivity of 85.71% (Fig. 5D). The same results were observed for normalized VTN levels (Fig. 5B).

Table 2 Clinical parameters of the "long-term" (more than 3 years) kidney transplant patients

> 3 years Tx patients (*n* = 36)	**Fibrosis 0% (*n* = 8)**			**Fibrosis ≥ 5% (*n* = 28)**			***p*-value**	**sig**
Age (mean ± SD)	58.63	±	12.41	56.36	±	13.86	0.8009 [M]	ns
Sex (n (male), %)	5		63%	17		61%	> 0.9999 [M]	ns
DM (n, %)	3		38%	11		39%	> 0.9999 [M]	ns
CVD (n, %)	3		38%	10		36%	> 0.9999 [M]	ns
HTA (n, %)	8		100%	28		100%	> 0.9999 [M]	ns
BMI (mean ± SD)	28.7	±	4.477	24.95	±	6.794	0.0575 [M]	ns
ACEi/ARBs	2		25%	18		64%	0.1034 [M]	ns
Immunosuppression								
PDN + CsA (n, %)	0		0%	1		4%	> 0.9999 [M]	ns
PDN + CsA + MPS (n, %)	0		0%	1		4%	> 0.9999 [M]	ns
PDN + TAC (n, %)	1		13%	0		0%	0.2222 [M]	ns
PDN + TAC + MPS (n, %)	6		75%	24		86%	0.5963 [M]	ns
PDN + TAC + EVE (n, %)	1		13%	2		7%	> 0.9999 [M]	ns
S.Creat (mean ± SD)	2.368	±	0.6803	2.277	±	0.7484	0.7865 [M]	ns
eGFR (mean ± SD)	27.75	±	9.067	32.79	±	17.18	0.5941 [M]	ns
Proteinuria (mean ± SD)	534.6	±	683.6	1562	±	1901	0.0363 [M]	*
Albuminuria (mean ± SD)	190.5	±	339.6	929.3	±	1262	0.0132 [M]	*
VTN (mean ± SD)	14.71	±	17.81	56.79	±	42.80	0.0040 [M]	**
VTN/U.Creat (mean ± SD)	0.18	±	0.23	0.99	±	0.80	0.0037 [M]	**
Years from Tx (mean ± SD)	5.25	±	2.053	6.679	±	2.803	0.1789 [M]	ns

[M]Mann-Whitney test

DM, diabetes mellitus; CVD, cardiovascular disease; HTA, hypertension; BMI, body mass index; ACEi/ARBs, angiotensin-converting enzyme inhibitor / angiotensin receptor blocker; PDN, prednisone; CsA, cyclosporine A; MPS, mycophenolate sodium; TAC, tacrolimus; EVE, everolimus; S.Creat, serum creatinine; eGFR, estimated glomerular filtration rate; VTN, Vitronectin; U.Creat, urinary creatinine; Sig, significance; ns, non-significant (*p*-value > 0,05); **p*-value < 0.05 and ***p*-value < 0.01

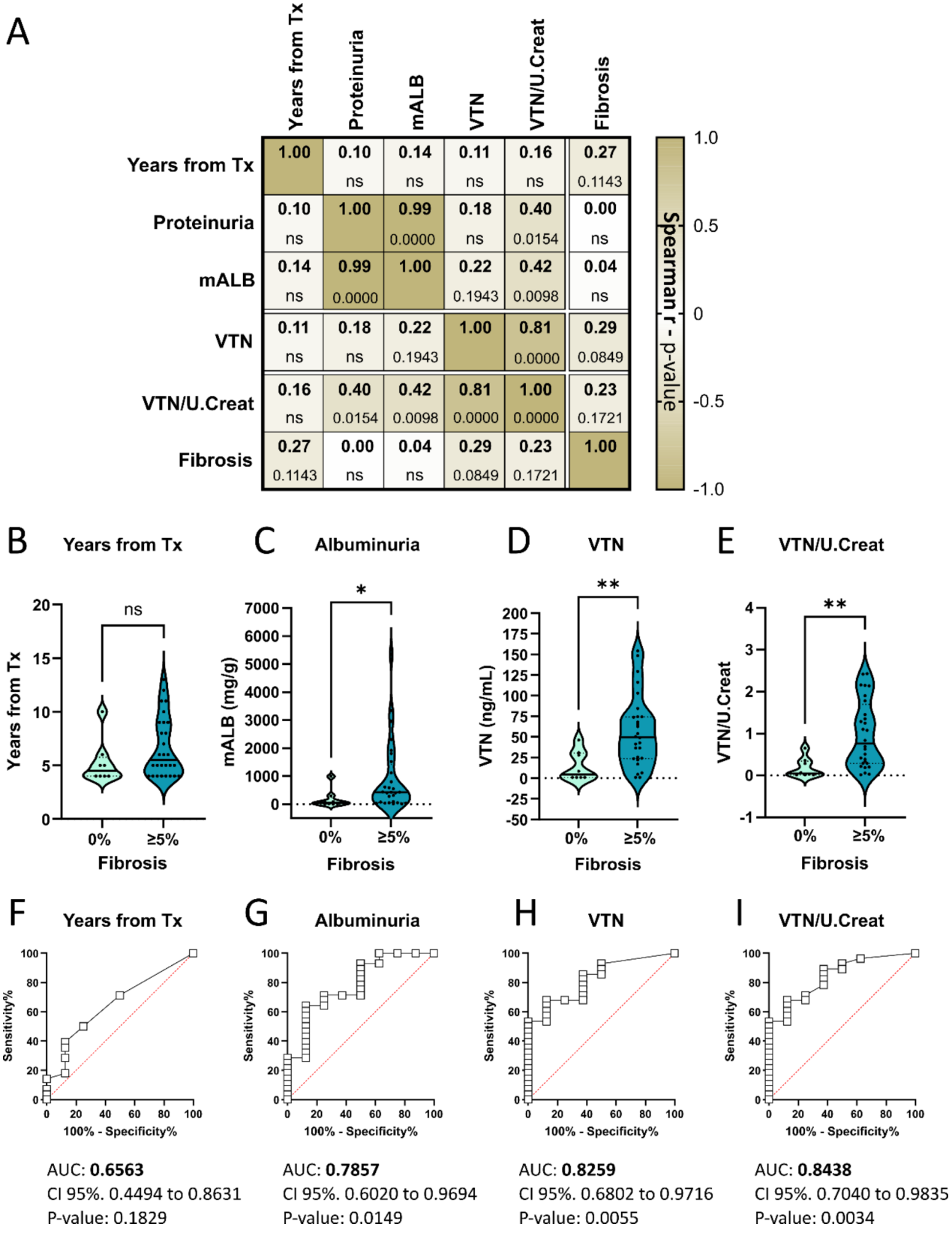

Fig. 3 (See legend on next page.)

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Fig. 3 Analysis of the "long-term" KTx patients. (**A**) Spearman's correlation coefficient matrix. Spearman r values are indicated in bold and *p*-values of the correlation are indicated at the bottom if *p*-value < 0.2. *P*-values > 0.2 are indicated as ns. (**B**) Violin plots showing the descriptive statistics and statistical differences according to the parameter "years from transplantation", (**C**) albuminuria levels, (**D**) VTN levels obtained by ELISA and (**E**) VTN levels normalized by urinary creatinine, stratified regarding the percentage of fibrosis observed in the kidney biopsies. The statistical differences are indicated for $*p < 0.05$ and $**p < 0.01$ by a Mann-Whitney test. (**F**) ROC curve based on "years from transplantation", (**G**) albuminuria (**H**) VTN, and (**I**) VTN normalized by urinary creatinine, as a unique biomarker to differentiate the two groups of patients (no fibrosis vs. fibrosis). VTN, Vitronectin; U.Creat, urinary creatinine; mALB, albuminuria

Discussion

In this study, we evaluated vitronectin (VTN) as a non-invasive biomarker for detecting and monitoring renal fibrosis in long-term kidney transplant (KTx) patients using an ELISA approach.

In the context of kidney transplantation, fibrosis is a leading cause of allograft loss, particularly in the later stage post-transplantation [34]. The need for early diagnosis and continuous monitoring of fibrosis is critical to surpass the limitations of renal biopsy and prevent the development of chronic allograft nephropathy. But common clinical parameters, such as serum creatinine, eGFR, and proteinuria, are inaccurate in detecting tubulointerstitial scarring at early stages. The sensitivity and specificity limitations/concerns associated with serum creatinine [35] and the glomerular-centric nature of albuminuria [36] highlight the necessity for additional biomarkers.

Biomarkers in renal diseases provide several advantages, including (i) a non-invasive method to assess kidney fibrosis, avoiding the risks and discomfort associated with biopsy procedures, (ii) a more comprehensive assessment of the whole kidney, overcoming the sampling error limitations of biopsies (iii) a more frequent monitoring of disease progression and treatment response, through repeated measurements from blood or urine samples, and (iv) the potential detection of early stages of fibrosis before kidney functional decline is detected [37]. Some recognized biomarkers for kidney fibrosis include transforming growth factor β (TGF-β) [15], monocyte chemoattractant protein-1 (MCP-1) [16], matrix metalloproteinase-2 (MMP-2) [17], kidney injury molecule-1 (KIM-1) [38], neutrophil gelatinase-associated lipocalin (NGAL) [39], and urinary vitronectin (VTN). Additionally, markers of tubule dysfunction like α1-microglobulin (α1M) [40] and uromodulin (UMOD) [41] have shown promise. While these biomarkers show potential, it is important to note that kidney biopsy still remains the gold standard for assessing renal fibrosis due to its ability to directly visualize and quantify fibrotic changes [42]. Thus, a direct comparison to renal biopsy results is required for evaluating the performance of the proposed biomarker. In this sense, our study included a direct comparison of the urinary VTN levels with the results of the timely coincident kidney biopsy.

Analyzing a set of KTX patients ($n=58$), our study revealed a significant correlation between VTN and fibrosis, outperforming conventional clinical parameters (Fig. 1). Notably, when analyzing the long-term KTx group, VTN exhibited superior potential in identifying fibrosis compared with albuminuria (Fig. 3). In this specific group, albuminuria was detected in approximately 71% of fibrotic patients, whereas VTN was identified in 86%, and the combination of both correctly classified 89% of fibrotic grafts detected by renal biopsy (Fig. 4). This enhanced efficacy may be attributed to the diverse origins of urinary VTN. Both VTN and albumin share a similar molecular weight. Therefore, both proteins can leak from the bloodstream when glomerular damage appears. However, VTN can also be produced in situ in the kidney [43]. Thus, fibrotic patients with tubulointerstitial scarring but no glomerular damage (no protein leakage) cannot be identified by albuminuria but could be detected by urinary VTN. Importantly, the normalization of VTN by urinary creatinine to reduce the potential bias of urine concentration due to uncontrolled factors did not modify the results (Figs. 3 and 4).

As a potential new biomarker, we established our pilot thresholds based on the values shown by ROC curves; based on this, some of the non-fibrotic patients showed overlapping VTN levels with the fibrotic group. A possible explanation for this observation could be a misclassification of some patients based on biopsy results. Of note, sampling bias during renal biopsy due to a heterogeneous distribution of the histopathological lesions or analysis errors in their further interpretation might be present in up to 20% of cases [44, 45]. Importantly, the reduced levels of urinary VTN found in healthy controls increased the potential of VTN to discriminate fibrosis (AUC=0.89) (Fig. 5).

An intriguing observation in our study was the lack of statistical differences in VTN levels between the non-fibrotic and fibrotic patients in the "recent" transplanted group (**Suppl. Table 1**). Our results suggest that high levels of VTN during the first-year post-transplantation are not related to the degree of fibrosis (Fig. 2). This observation could be explained by the pleiotropic participation of VTN in different pathways, such as complement regulation [46], fibrosis [47], and adhesion molecules [48]. Thus, in the complex scenario after a renal transplantation procedure, where an active injury-repair response occurs, including surgical recovery, immunosuppressive treatment, and tissue remodeling, among others, it is probable that VTN could contribute to these complex dynamics, leading to temporarily increased levels. When

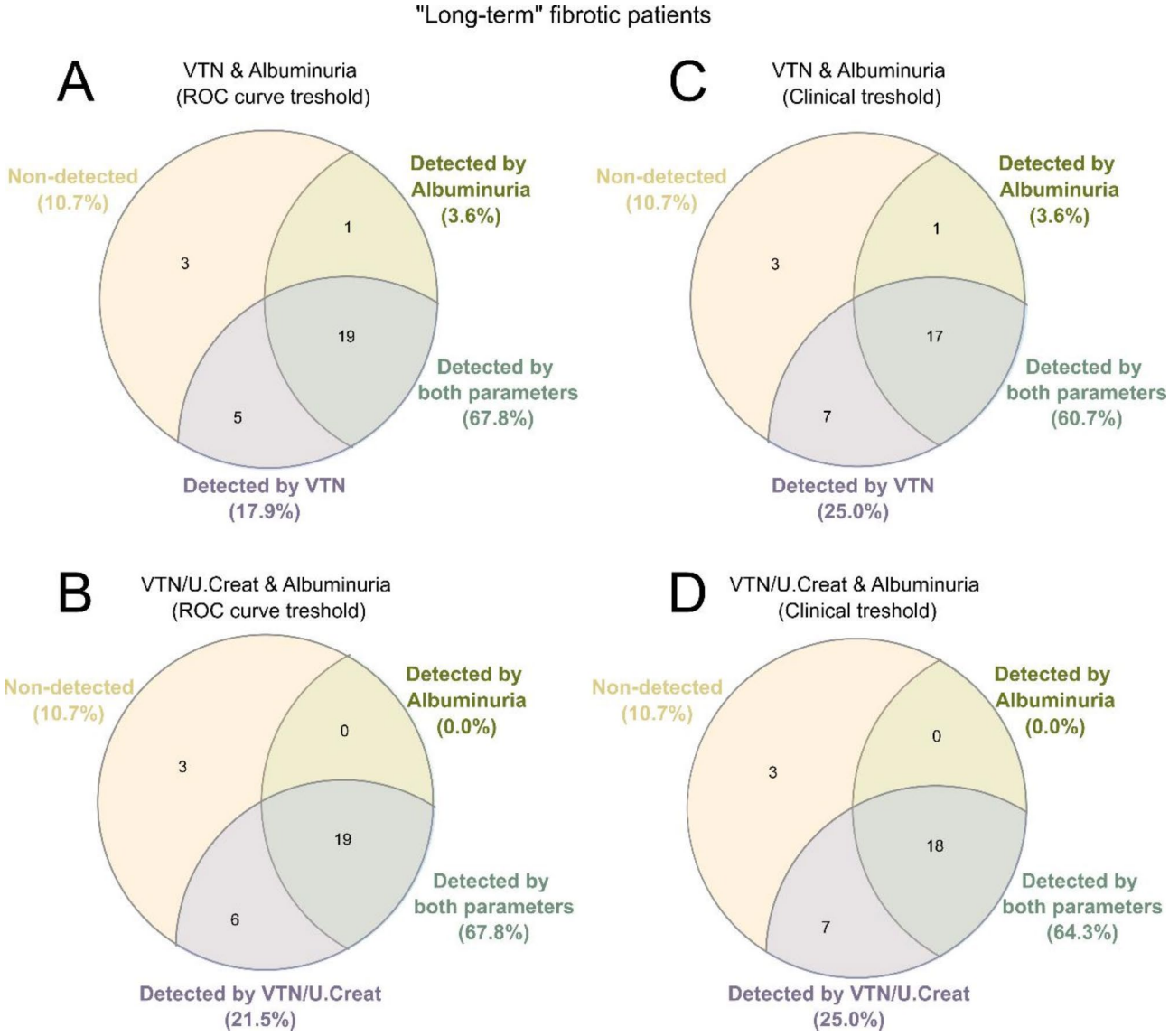
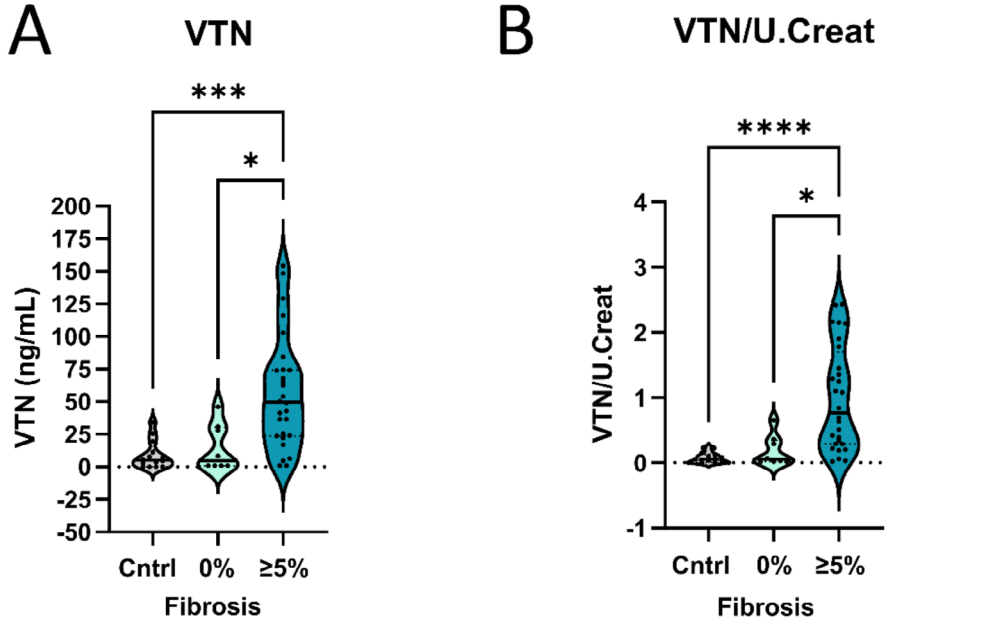


Fig. 4 Analysis of the "long-term" KTx patients. Venn diagrams showing the number and percentage of patients with fibrosis that would be detected by VTN or VTN/U.Creat levels, albuminuria levels or both, without a biopsy. (**A and B**) VTN, VTN/U.Creat, and albuminuria threshold levels have been stablished based on the ROC curves of these parameters (VTN considered positive > 12.80 ng/mL; VTN/U.Creat considered positive > 0.13; albuminuria considered positive > 95.65 mg/g). (**C and D**) The clinical albuminuria threshold (> 300 mg/g) was considered for the analysis. VTN, vitronectin; U.Creat, urinary creatinine

these processes return to homeostatic values, VTN levels seem to slowly decrease in patients without fibrosis at longer time points (>3 years) while remaining high in fibrotic patients (Fig. 2). This hypothesis needs to be confirmed in future studies.

In fact, the lack of a longitudinal follow-up of KTx patients is a limitation of this study. It has restricted the capacity to determine the exact time point after transplantation at which VTN would be more useful as an add-on non-invasive biomarker. Additional limitations are the low number of patients without fibrosis in the "long-term" group ($n=8$), the rather low number of patients with high fibrosis grades (>50%) (3/58), and the inclusion of patients based only on clinically indicated biopsies.

Other urinary biomarkers, have been investigated in recent years for their potential to detect kidney fibrosis. Compared to VTN, most of these biomarkers have been evaluated in specific chronic kidney disease (CKD) populations rather than in a broader group of kidney transplant (KTx) recipients. Moreover, not all studies have provided an area under the curve (AUC) value, thus limiting direct comparison of their diagnostic performance [49]. Yet, among those studies reporting AUC values, urinary CTGF has been identified as a biomarker for renal fibrosis in KTx patients, with an AUC of 0.63 at the time



	Cntrl	0%	≥5%
Minimum	0	1	1
25% Percentile	0	1	23.72
Median	5.16	4.77	49.73
75% Percentile	13.26	30.22	74.37
Maximum	34.29	46.18	154.2
Range	34.29	45.18	153.2
95% CI of median			
Actual confidence level	98,71%	99,22%	96,43%
Lower confidence limit	0	1	25.62
Upper confidence limit	19.75	46.18	73.88
Mean	8.704	14.71	56.79
Std. Deviation	10.6	17.81	42.8
Std. Error of Mean	2.832	6.296	8.089
Lower 95% CI of mean	2.586	-0.1834	40.19
Upper 95% CI of mean	14.82	29.59	73.39

	Cntrl	0%	≥5%
Minimum	0	0.02	0.02
25% Percentile	0	0.02	0.29
Median	0.0537	0.05	0.765
75% Percentile	0.1251	0.3425	1.698
Maximum	0.2347	0.65	2.43
Range	0.2347	0.63	2.41
95% CI of median			
Actual confidence level	98,71%	99,22%	96,43%
Lower confidence limit	0	0.02	0.34
Upper confidence limit	0.1507	0.65	1.35
Mean	0.07601	0.1825	0.9864
Std. Deviation	0.0797	0.232	0.7998
Std. Error of Mean	0.0213	0.08202	0.1511
Lower 95% CI of mean	0.03	-0.01145	0.6763
Upper 95% CI of mean	0.122	0.3765	1.297

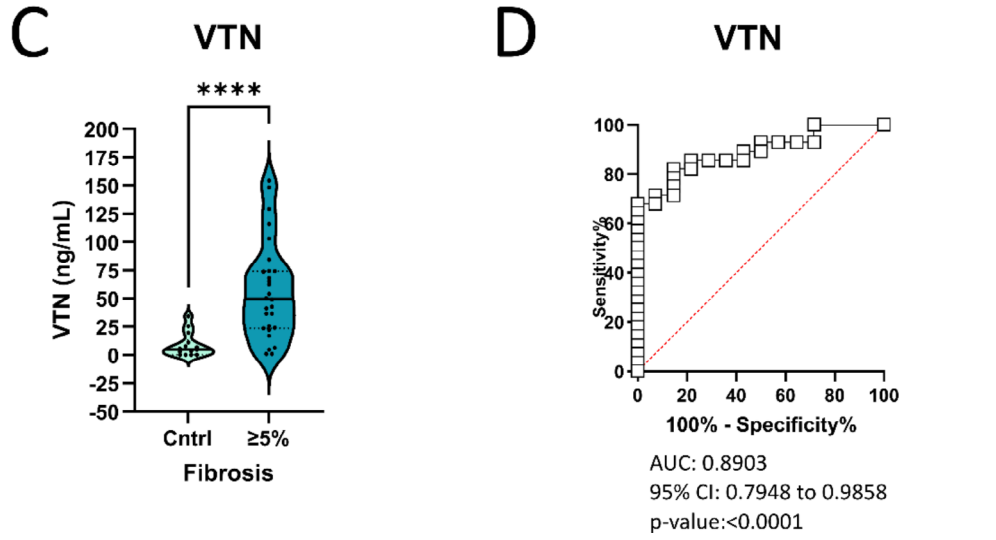


Fig. 5 (**A**) VTN levels of healthy controls and "long-term" KTx patients stratified by percentage of fibrosis, with descriptive statistics at the bottom. (**B**) VTN levels normalized by urinary creatinine of healthy controls and "long-term" KTx patients stratified by percentage of fibrosis, with their descriptive statistics at the bottom. Statistical differences are indicated for $*p < 0.05$, $***p < 0.001$ and $****p < 0.0001$ by a Kruskal Wallis test with Dunn's post-hoc analysis. (**C**) VTN levels obtained by ELISA stratified regarding the percentage of fibrosis observed in the kidney biopsies. Statistical differences are indicated for $****p < 0.0001$ by a Mann-Whitney test. (**D**) ROC curve based VTN as a unique biomarker to differentiate the two groups of patients (healthy controls vs. fibrotic patients). Cntrl, Healthy controls; VTN, Vitronectin; U.Creat, urinary creatinine

of renal biopsy [50]. In lupus nephritis, urinary MCP-1, Hepcidin, L-FABP [16], and TGF-β [15] have been associated with renal fibrosis, displaying AUC values of 0.66, 0.48, 0.60, and 0.90, respectively. Of these biomarkers, only urinary TGF-β exhibits a slightly better AUC than VTN. Nevertheless, in this particular case, patient populations differed from KTx recipients, limiting a broader comparability of these findings.

Early detection and management of fibrosis could significantly reduce the risk of graft loss by preserving and extending graft function. This may be partially tackled through fine tuning of immunosuppressive therapy. Yet, as several anti-fibrotic drugs are on clinical trials [51], new lines of treatment will be available for these patients. Thus, VTN detection may help to early identify those patients susceptible to receiving those treatments, and to non-invasively monitor their response.

Conclusions

VTN has emerged as a valid indicator of renal fibrosis, providing a potential non-invasive biomarker for clinical use. Of interest, the combination of this new biomarker with conventional clinical parameters (such as albuminuria) significantly improves the detection of renal fibrosis in kidney transplant patients. A longitudinal study in a new cohort of patients will help to fully assess the potential of VTN as early diagnostic and prognostic biomarker for renal fibrosis, and its implication in renal function and graft survival.

Abbreviations

ACEi/ARBs	angiotensin-converting enzyme inhibitor / angiotensin receptor blocker
AUC	Area under de curve
BMI	Body mass index
CsA	Cyclosporine A
CVD	Cardiovascular disease
DM	Diabetes Mellitus
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EVE	Everolimus
HTA	hypertension
IFTA	Interstitial fibrosis and tubular atrophy
KTx	Kidney transplant
mALB	albuminuria
MPS	Mycophenolate sodium
PDN	prednisone
ROC	Receiver operating characteristics
S.Creat	Serum creatinine
TAC	Tacrolimus
U.Creat	Urinary creatinine
VTN	Vitronectin

Supplementary Information

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Supplementary Table 1. Clinical parameters of the recent (less than 3 years) kidney transplant patients.

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Author contributions

Conceptualization, Francesc E. Borràs; *Data curation*, Marta Clos-Sansalvador and Omar Taco; *Formal analysis*, Marta Clos-Sansalvador and Omar Taco; *Funding acquisition*, Francesc E. Borràs and Javier Juega; *Methodology*, Marta Clos-Sansalvador, Omar Taco, Paula Rodríguez-Martínez, Sergio G. Garcia, Miriam Font-Morón and Javier Juega; *Supervision*, Javier Juega and Francesc E. Borràs; *Writing – original draft*, Marta Clos-Sansalvador, Omar Taco and Francesc E. Borràs; *Writing – review & editing*, Marta Clos-Sansalvador, Omar Taco, Paula Rodríguez-Martínez, Sergio G. Garcia, Miriam Font-Morón, Jordi Bover, Anna Vila-Santandreu, Marcella Franquesa, Javier Juega and Francesc E. Borràs.

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Declarations

Competing interests

F.E.B. holds a related patent with the International Application Number PCT/EP2020/087290. The other authors declare no conflicts of interest.

Author details

[1]REMAR-IGTP Group, Germans Trias i Pujol Research Institute (IGTP) & , Nephrology Department, University Hospital Germans Trias i Pujol (HUGTiP) , Can Ruti Campus, Badalona (Barcelona), Catalonia, Spain
[2]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain
[3]Department of Pathology, University Hospital Germans Trias i Pujol, Badalona (Barcelona), Spain
[4]Department of Cell Biology, Physiology and Immunology, Universitat de Barcelona (UB), Barcelona, Spain

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