Expression of tissue fibrosis genes in congenitally obstructed pyeloureteral junction and biomarkers of renal damage

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Citation: Kazlauskas V, Zilinskaite-Tamasauske R, Barasa P, et al. Expression of tissue fibrosis genes in congenitally obstructed pyeloureteral junction and biomarkers of renal damage. Cent European J Urol. 2024; doi: 10.5173/ceju.2023.218 [Epub ahead of print]

Article history
Submitted: Sep. 19, 2023
Accepted: Jan. 7, 2024
Published online: Feb. 25, 2024

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Introduction The aim of this study was to investigate the expression of fibrosis-related genes in obstructed ureteral tissue and determine its relationship with biomarkers of renal damage and preoperative renal scan findings.

Material and methods In all cases, bladder urine and blood samples were collected preoperatively. They were analysed for serum cystatin C, urinary albumin, urinary beta 2 microglobulin, and urinary neutrophil gelatinase-associated lipocalin concentrations, as well as their concentrations standardised by urine creatinine. Pyeloureteral junction obstruction tissue specimens were frozen in liquid nitrogen upon harvesting. RNA was extracted from the samples using TRIzol reagent. qPCR was performed, and the relative expressions of TGFβ1, MMP1, TIMP1, PAI1, CTGF, and VEGFA in stenotic ureteral tissue were calculated. Spearman's rank correlation test was used to calculate the correlation between the relative expression of investigated genes, urine, and blood biomarkers of renal damage and preoperative renal scan findings.

Results A total of 20 pyeloureteral junctions of 20 patients were harvested at the time of dismembered pyeloplasty. The median age of the patients at the time of the operation was 15.2 [9.07, 66.2] months. There was a significant negative correlation between urinary albumin concentration and relative TGFβ1 expression in pyeloureteral junction tissue (rho =-0.45, p = 0.047), as well as between uAlb and relative VEGFA expression (rho = -0.575, p = 0.008). No correlation with other urine biomarkers of renal damage or renal scan findings was found.

Conclusions Expression of fibrosis-related genes in the obstructive tissue of the pyeloureteral junction have no direct correlation with biomarkers of renal damage.

Key Words: pyeloureteral junction obstruction ‹› fibrosis-related genes ‹› biomarkers of renal damage ‹› stenotic ureteral tissue ‹› renal scan

INTRODUCTION

Despite a series of studies dedicated to explaining the mechanisms responsible for the development of congenital ureteral strictures, a comprehensive understanding of this pathology is still lacking. There is a growing body of evidence suggesting that urogenital pathology is closely associated with genetic determinants [1]. A large study of rare copy-number variations has recently been published. The study was conducted on 3000 CAKUT cases, 512 of which were obstructive uropathies, and the odds ratio for copy-number variations >250 kb was significant when compared to healthy controls. The study also identified several genetic susceptibility loci and genes, with TBX6 as a main genetic driver for CAKUT in subjects with chromosome 16p11.2 microdeletion syndrome [2].

Most studies investigating pathophysiological findings in pyeloureteral junction obstruction (PUJO)

tissue are comparative studies in which PUJO tissue is compared to ureteral tissue harvested upon nephrectomy due to renal tumour or other non-obstructive causes and autopsied [3–5]. In all cases, these control specimens are potentially affected by extrinsic or intrinsic factors such as chemotherapy, dysplasia, or tissue degradation before autopsy.

The use of a control group based on completely healthy human ureteral tissue is not feasible due to ethical restrictions. An explanation of the roles of tissue factors in the pathophysiology of the development of PUJO can be alternatively implemented by analysing potential relationships between the expression of tissue factors and obstruction signs, MAG-3 radionuclide renal scans, and biochemical obstruction markers in blood and urine.

The most common abnormal histological findings in ureteral stricture tissue are a reduction in the number of Cajal-like cells and increased collagen smooth muscle ratio [3, 6].

Scientific information related to the identification of pro-fibrotic gene expression in ureteral stricture tissue is scarce. Yang et al. observed that, according to the findings of immunohistochemistry, in situ hybridisation, and RT-qPCR, the expression of transforming growth factor β1 (TGFβ1) was upregulated and the expression of epidermal growth factor was downregulated in PUJO tissue when compared to the control group [7].

Koca et al. also described immunohistochemical analysis of PUJO, the findings of which were suggestive of increased staining intensity of TNFα in the control tissue mucosa, while TGFβ3 staining was more intensive in the same layer of ureteral stricture tissue [4]. Data considering the relationship between the expression of fibrosis-related factors and postoperative outcomes were identified in only 2 publications.

Seremetis et al. claimed that TGFβ expression was upregulated in PUJO tissue when compared to control ureteral tissue harvested at the time of ureteral reimplantation, due to reflux. High TGFβ expression correlated with good postoperative outcomes, good renal function, smooth muscle hypertrophy, and artificially induced obstruction in a murine model [5].

In a recent systematic analysis that encompassed data from 10 articles, 15 genes were found with altered expression profiles in ureteral stricture tissue. Genes that were upregulated included ET1, ACTA2, MCP-1, TGFB1, NFKB1, IL-6, HIF1A, S100A1, and SYP, and the expression of 6 genes was downregulated (ADM, NOS2, EGF, PDGFRA, UCHL1, and NGFR). The products of these genes are components of the HIF-1 signalling pathway, and they participate in the development of vasculature. Some of these factors also act as agonists of signalling pathways and particularly affect the Ras signalling system. There may also be a relationship of current factors with tissue hypoxia, formation of redundant fibrotic tissue, and inflammatory response [8].

Urine proteome analysis demonstrated elevated concentrations of 50–80 proteins in bladder urine when kidney obstruction was present, compared to controls [9–11]. As a result, a series of articles have been published to reveal the potential relationships between the concentrations of biomarkers in urine or blood and the degree of obstruction. The most frequent proteins utilised as potential markers of obstruction were urinary neutrophil gelatinase-associated lipocalin (uNGAL), monocyte chemotactic peptide-1, transforming growth factor β1, epidermal growth factor, and kidney injury molecule 1 [12]. In this study, we decided to analyse the most available, cost-effective, and ordinary biomarkers associated with renal obstruction (urinary albumin [uAlb], urinary β2 microglobulin [uβ2-M], and uNGAL concentrations and their concentrations normalised by creatinine [Cr] and blood serum cystatin C were measured) [13, 14, 15, 16]

We could not identify any article providing data with respect to the expression of connective tissue growth factor (CTGF), matrix metallopeptidase 1 (MMP1), or plasminogen activator inhibitor (PAI1) in ureteral stricture tissue in the MEDLINE database over the last 10 years. Additionally, publications revealing the relationship between the expression of fibrosis-related genes and renal-obstruction-associated biomarkers were absent. The aim of this study was to analyse the expression of the main known fibrosis-related factors (i.e., TGFβ1, tissue inhibitor of metalloproteinases 1 [TIMP1], vascular endothelial growth factor A [VEGFA], CTGF, PAI1, and MMP1) in congenital ureteral obstruction tissue and their relationships with perioperative ultrasound, renal scan findings, and obstruction biomarkers.

MATERIAL AND METHODS

From February 2019 until February 2021, all children with a diagnosis of PUJO were prospectively included in the study. The study group consisted of 20 patients (13 boys and 7 girls). All of them underwent open Hynes–Anderson pyeloureteroplasty due to PUJO. The median age of the patients at the time of the operation was 15.2 [9.07, 66.2] months. Both kidneys of each patient were evaluated by ultrasound scan preoperatively and postoperatively. Preoperative urine samples were collected using urine collection bags or urine collection containers. Venous blood was also obtained, and the blood se-

rum concentration of cystatin C was detected at the time of blood collection. All urine samples were frozen at -80°C. When the whole batch of urine samples was collected, they were analysed for uAlb, uβ2-M, and uNGAL concentrations, as well as their concentrations standardised by urine creatinine. For the biochemical analysis, we used an Abbot Architect analyser. The decision of whether to operate on the patient was based on our local protocol. The operation was performed when there was an increase in the antero-posterior diameter (APD) value by 20% on subsequent ultrasound scans, when the differential renal function (DRF) was <40% of the affected kidney or a decrease in DRF by >5% was observed on subsequent renal scans, or when pain or pyelonephritis occurred. None of the patients in our cohort at the time of inclusion and during the follow-up had any signs of urinary tract infection, and they had no history of fever or renal colic. All ultrasound scans were performed by the same evaluator. For the ultrasound scans, we used a LOGIQ V2 portable device. In total, 8 out of 20 patients were managed without a radionuclide renal scan because all of them had increasing dilatation during the follow-up, while 12 patients required RRS because they demonstrated a stable profile of hydronephrosis on subsequent ultrasound scans. Diagnosis of obstruction in all cases was confirmed by intraoperative findings.

RRS was performed using the Infinia2 dual detector visualising system. The Tc-99m MAG3 radionuclide F-20 protocol was applied when the evaluation of the obstructive curve was carried out 20 minutes after the radionuclide injection. DRF and tissue transit time (TTT) were the criteria evaluated in our study. A one-frame-per-second scan for the first 2 minutes and a one-frame-per-15-seconds scan for the remaining 40 minutes were used to assess TTT. TTT was assumed as the duration from the first sign of radionuclides in the renal parenchyma until the moment when radionuclides appeared in the renal collection system. TTT was evaluated by a single radiologist.

PUJO segments were excised according to routine procedures, and the extent of the procedure was not increased due to the study. PUJO tissue samples in all cases were frozen at -80°C in liquid nitrogen. When the whole batch of ureteral tissue samples was collected, they were analysed all at once.

Tissue RNA extraction and RT-qPCR were performed to detect the relative expression of fibrosis-related factors (i.e., TGFβ1, TIMP1, VEGFA, CTGF, PAI1, and MMP1). Relative expression of the genes was calculated using the GADPH and GPI genes as controls. The molecular study was conducted at Vilnius University Life Science Centre. RNA extraction from ureteral tissue was implemented by gradual defrosting of the frozen samples, after which the samples were centrifuged and TRIzol reagent (Thermo Fisher) was applied according to the manufacturer's protocol. Approximately 1 mL of TRIzol reagent was consumed for every 100 mg of tissue. After extraction, RNA was dissolved in nuclease-free water, and the RNA quantity and quality were evaluated using a NanoDrop device (Thermo Fisher).

Complementary DNA was synthesised using the Maxima H Minus First Strand cDNA synthesis kit (Thermo Fisher). Every sample had a reverse transcription (RT)-negative control. RT-qPCR was implemented using Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher) with a QuantStudio 3 quantitative PCR device. The geometric average of GADPH and GPI gene expression was used to calculate the relative expression of the genes of interest. Relative expression was calculated using the common base method [17].

Regarding the results of the aforementioned relative gene expression in ureteral stricture tissue, we calculated whether there was any correlation between the relative expression of the aforementioned genes and urinary obstruction biomarkers (i.e., uAlb, uNGAL, and uβ2-M, standardised and not standardised by urinary Cr) and blood markers of overall renal function (cystatin C). The same calculation was performed to verify whether there was any correlation between the relative expression of fibrosis-related factors and RRS findings such as DRF and TTT of the affected kidneys.

Spearman's rank correlation test was applied in all calculations. For the calculations, we used R commander, version 4.1.0. The level of significance was $\alpha = 0.05$.

RESULTS

A total of 20 patients with PUJO were included in this study. The median age of the patients at the time of the operation was 15.2 [9.0, 66.2] months, and the median duration of postoperative follow-up was 10.35 [4.26, 14.8] months. The baseline characteristics of every patient are provided in Table 1.

The relative expression of fibrosis-related factors in the pyeloureteral junction obstruction tissue samples is demonstrated in Figure 1.

There was a significant negative correlation between uAlb concentration and relative TGFβ1 expression in ureteral stricture tissue (rho = -0.45, p = 0.047), and between uAlb concentration and relative VEGFA expression in ureteral stricture tissue (rho = -0.575, p = 0.008) (n = 20; Figure 2, Table 2).

Moreover, the correlation of uAlb/Cr ratio with VEGFA (p = 0.095) and uNGAL alone, and of uNGAL/Cr

ratio with MMP1, was close to significant (p = 0.06 and p = 0.08, respectively) (Table 2).
Considering the correlation between fibrosis-related factors and cystatin C, there was a marginally significant correlation between cystatin C in blood serum and PAI1 in ureteral stricture tissue (rho = 0.478, p = 0.06), and a marginally significant negative correlation between cystatin C eGFR and PAI1 in ureteral stricture tissue (rho = -0.48, p = 0.06). We also identified a significant negative correlation between PAI1 and TTT on renal scans (rho = -0.63, p = 0.03) (Figure 3).

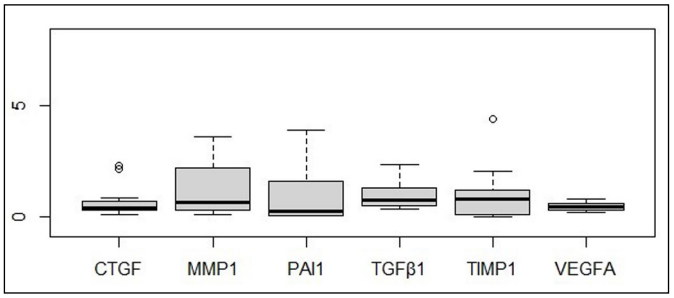

Figure 1. *Boxplots representing the relative expression of fibrosis-related factors in pyeloureteral junction obstruction samples. Outliers are identified by dots. Sample size n = 20.*

CTGF – connective tissue growth factor; MMP1 – matrix metallopeptidase 1; PAI1 – plasminogen activator inhibitor; TGFβ1 – transforming growth factor β1; TIMP1 – tissue inhibitor of metalloproteinases 1; VEGFA – vascular endothelial growth factor A

DISCUSSION

Unexpectedly, we did not find a positive correlation between biochemical markers of renal damage and fibrosis factors in ureteral tissue. Few previous studies were in favour of that finding. A study by Y. Yang et al. revealed that there was a particular upregulation of TGFβ1 RNA expression in ureteral stricture tissue when compared to normal controls [7]. Several other studies have described the potential involvement of other TGFβ variants in the development of congenital ureteral strictures. Koca et al. observed that immunohistochemical expression of TGFβ3 was upregulated in ureteral stricture tissue in com-

Table 1. *Baseline data for each patient included in the study*

Age (months)	Follow-up after operation (months)	Blood serum cystatin C (mg/l)	Cystatin C eGRF (ml/min per 1.73 m^2)	uAlb/Cr (mg/mmol)	Middle parenchymal thickness before operation (mm)	APD before operation (mm)
3.3	14.13	n/a	n/a	3.6*	10	27
3.3	14.17	1.89	39.08‡	34.78	6.5	37
6	23.03	1.09	65.24‡	2.29	7.6	26.5
6.9	5.73	n/a	n/a	2.88	8.1	33.6
8.7	15.3	1.08	65.81‡	2.67	8.8	28
9.2	31.87	n/a	n/a	5.06*	10.3	20
9.6	22.2	1.04	68.16‡	3.21	7.4	20
12.6	4.37	0.66	104.09	0.83	6.7	22
13.4	10.23	1.62	45.16‡	4.21*	5.5	20.8
14.3	23.47	1.97	37.6‡	17.74*	7.8	26
16.1	4.3	0.69	99.87	2.63	12.6	25.2
20.7	14.67	0.9	77.98	6.76*	10.3	16.6
23.4	1.8	0.92	76.4	7.47*	11.6	20
25.6	10.47	n/a	n/a	1.4	9.2	24.2
62	3.53	0.82	85.04	1	9.9	20
78.7	2.17	0.81	86.017	1.12	4	22
96.2	2.27	0.95	74.16‡	1.14	16.4	20
98.6	5.13	0.66	104.09	0.63	11	29
99.7	14.27	0.56	121.29	0.53	6.75	55.7
111.3	4.13	0.58	117.39	3.79*	11	21.5

APD – antero-posterior diameter; Cr – creatinine; n/a – value not available; uAlb – urinary albumin
Cystatin C clearance was calculated by the formula eGFR = 40.9 (1.8/cystatin C)$^{0.931}$ [26];
* Albuminuria was defined as uAbl/Cr ≥3.4 mg/mmol [27]; ‡eGFR <75 ml/min/1.73 m^2 was defined as abnormal [28]

Table 2. *Correlations between miscellaneous urine and blood obstruction biomarkers and the relative expression of fibrosis-related factors in ureteral stricture tissue (correlation coefficients and p-values below them in every field).*

Biomarker	CTGF (n = 20)	MMP1 (n = 12)	PAI1 (n = 20)	TGFβ1 (n = 20)	TIMP1 (n = 20)	VEGFA (n = 20)
uAlb (mg/l) (n = 20) M = 6.5 [4; 13]	0.156 p = NS	-0.39 p = NS	0.272 p = NS	-0.45 p = 0.047*	-0.295 p = NS	-0.575 p = 0.008*
uAlb/Cr (mg/mmol) (n = 20) M = 2.77 [1.13; 4.42]	0.174 p = NS	0.07 p = NS	0.302 p = NS	-0.224 p = NS	0.105 p = NS	-0.385 p = 0.095‡
uβ2-M (mg/l) (n = 19) M = 0.07 [0.04; 0.12]	0.045 p = NS	-0.492 p = NS	-0.03 p = NS	0.231 p = NS	0.297 p = NS	-0.24 p = NS
uβ2-M/Cr (mg/mmol) (n = 19) M= 0.03 [0.015; 0.064]	-0.044 p = NS	-0.027 p = NS	0.132 p = NS	0.342 p = NS	0.35 p = NS	-0.06 p = NS
uNGAL (ng/ml) (n = 19) M = 4.3 [1.9; 11.2]	0.00176 p = NS	0.6 p = 0.06‡	-0.36 p = NS	0.198 p = NS	0.276 p = NS	-0.148 p = NS
uNGAL/Cr (ng/µmol) (n = 19) M = 2.21 [0.649; 5.05]	0.023 p = NS	0.555 p = 0.08‡	-0.144 p = NS	0.29 p = NS	0.315 p = NS	-0.194 p = NS
Cystatin C in blood serum (mg/l) (n = 16) M = 0.91 [0.68; 1.08]	0.046 p = NS	0.33 p = NS	0.478 p = 0.06‡	0.146 p = NS	-0.04 p = NS	0.012 p = NS
Cystatin C eGFR (n = 16) M= 77.19 [65.67; 100.92]	-0.046 p = NS	0.333 p = NS	-0.48 p = 0.06‡	-0.146 p = NS	0.04 p = NS	-0.012 p = NS

Cr – creatinine; CTGF – connective tissue growth factor; DRF – differential renal function; M – medians and quartiles in square brackets; MMP1 – matrix metallopeptidase 1; NS – not significant; PAI1 – plasminogen activator inhibitor; TTT – tissue transit time; TGFβ1 – transforming growth factor β1; TIMP1 – tissue inhibitor of metalloproteinases 1; uβ2-M – urinary β2 microglobulin; uNGAL – urinary neutrophil gelatinase-associated lipocalin; VEGFA – vascular endothelial growth factor A
*Statistically significant correlation was assumed when α <0.05; ‡values approximating statistical significance when α (0.05, 0.1). Cystatin C clearance was calculated by the formula eGFR = 40.9 (1.8/cystatin C)$^{0.931}$ [26].

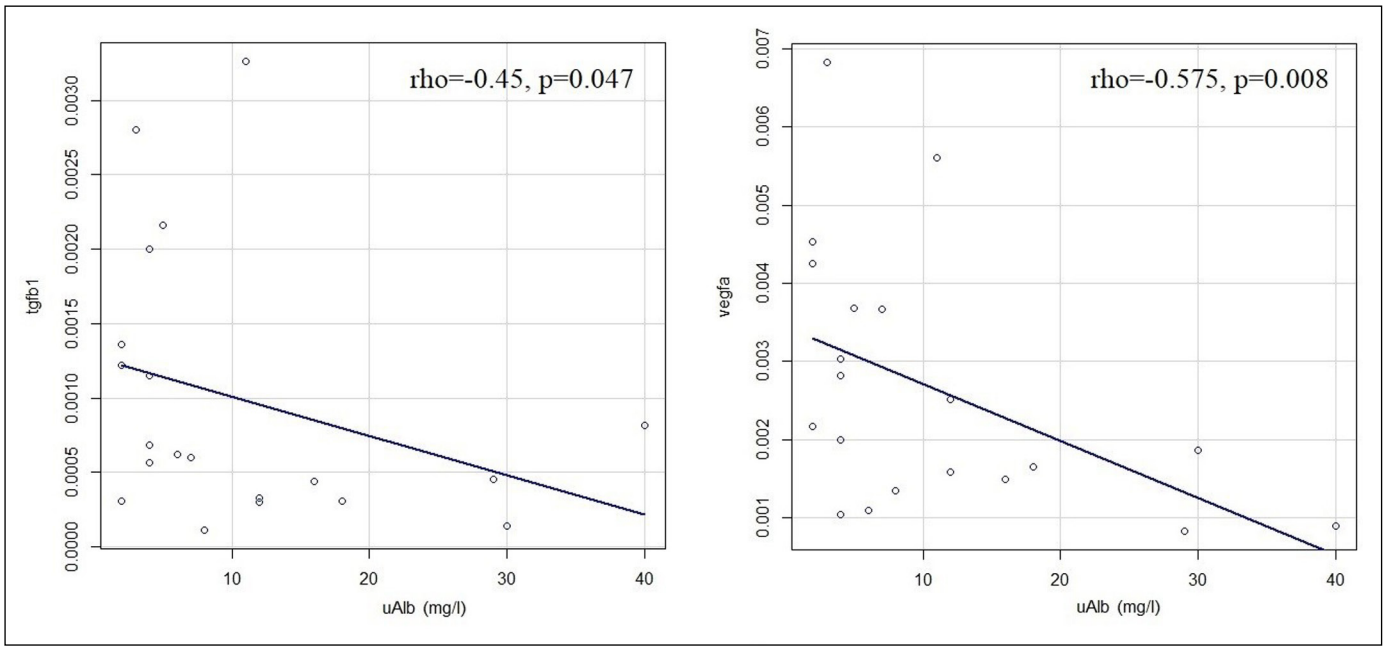

Figure 2. *Correlations between transforming growth factor β1 (TGFβ1), vascular endothelial growth factor A (VEGFA), and urinary albumin (uAlb) (n = 20). Blue curves were generated by the least-squares method.*
uAlb – urinary albumin; TGFβ1 – transforming growth factor β1; VEGFA – vascular endothelial growth factor A

Table 3. *The table representing the correlations between DRFa nd TTT, and fibrosis-related tissue factors in ureteral stricture tissue*

Renal scan	CTGF (n = 12)	MMP1 (n = 6)	PAI1 (n = 12)	TGFβ1 (n = 12)	TIMP1 (n = 12)	VEGFA (n = 12)
DRF of the affected side (n = 12)	-0.105 p = NS	0.036 p = NS	0.105 p = NS	-0.224 p = NS	0.042 p = NS	0.24 p = NS
TTT of the affected side (n = 12)	-0.151 p = NS	–	-0.629 p = 0.03*	0.45 p = NS	0.32 p = NS	0.33 p = NS

CTGF – connective tissue growth factor; DRF – differential renal function; MMP1 – matrix metallopeptidase 1; NS – not significant; PAI1 – plasminogen activator inhibitor; TTT – tissue transit time; TGFβ1 – transforming growth factor β1; TIMP1 – tissue inhibitor of metalloproteinases 1; VEGFA – vascular endothelial growth factor A
*statistically significant correlation was assumed when α <0.05. The calculation of correlation between TTT and Mmp1 correlation was not feasible, due to insufficient sample size to run Spearman's rank correlation test

parison to control tissue [4]. Experimental animal studies have also demonstrated that artificial unilateral ureteral obstruction upregulated TGFβ mRNA at the site of obstruction. This finding suggests that this mechanism is responsible for smooth muscle cell activation and increased collagen synthesis as a result of acute obstruction [5, 18].

Higher TGFβ1 expression can also be a feature of more extensive scarring, higher quantities of fibrotic tissue, and lower motility of the ureter at the site of obstruction, because TGFβ1 has been described as one of the central fibrosis-related factors in human tissue [19].

We could not find any publication in which the relationship between the expression of fibrosis-related factors in congenital ureteral tissue and biochemical obstruction markers was reviewed. In our data, we found a significant negative correlation between uAlb concentration and relative TGFβ1 expression in ureteral stricture tissue (rho = -0.45, p = 0.047). With regard to our previously published article, uAlb concentrations were significantly higher in the urine of children who underwent operations due to obstruction than in that of non-operated and control patients [20].

Altogether these findings support the hypothesis that congenital obstruction to urinary flow has different aetiology than experimentally induced or acute obstruction; both lead to renal damage detectable by established methods, as supported by correlation of biochemical markers and TTT in our previous study.

The significant negative correlation between uAlb concentration and VEGFA expression in PUJO tissue is of particular interest. We could not find any similar relationship described in the literature. Onions et al. published the results of their experimental study with mice, in which the activation of VEGFC expression reduced the concentration of albumin in renal glomeruli. According to the author's statement, VEGFA has the opposite effect on the permeability of glomeruli, and its upregulation increases the glomerular membrane's permeability to uAlb and, subsequently, increases its concentration in the urine. Our findings raise the question of whether

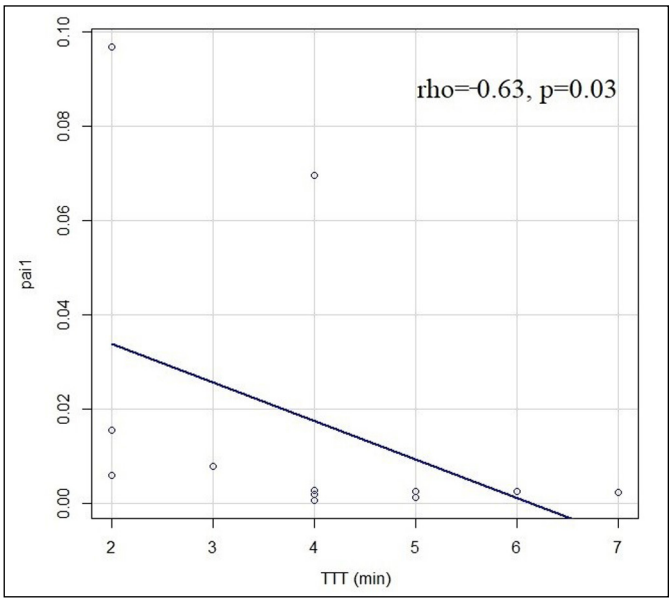

Figure 3. *Correlation between relative expression of plasminogen activator inhibitor; (PAI1) in ureteral stricture tissue and tissue transit time (TTT) scan (n = 12).*

PAI1 – plasminogen activator inhibitor; TTT – tissue transit time

significant correlation between uAlb and VEGFA is a sign of direct involvement of VEGFA in the pathophysiology of congenital ureteral stricture, or whether this correlation was present because VEGFA expression was organ-specific, and its expression in ureteral tissue may also be correlated with the expression of VEGFA in kidney tissue, potentially influencing the uAlb concentration. However, to prove this hypothesis, renal tissue biopsies would be necessary. Considering human ureteral strictures, we could find only one study whose aim was to identify immunohistochemical expression of VEGF in ureteral strictures and a control tissue. VEGF did not stain positively in the ureteral stricture or in the control tissue [21].

Considering the involvement of TIMP1 in the pathogenesis of congenital ureteral stricture, Reis et al. found that the expression of TIMP1 in PUJO tis-

sue was positively correlated with a degree of hydronephrosis at the time of surgery. The expression of TIMP1 was compared between SFU3 and SFU4 hydronephrosis grades [22].

MMP expression in congenital ureteral stricture tissue was described in one paper. According to Reis et al., MMP9 expression was downregulated and TIMP1 was upregulated in PUJO tissue when the degree of hydronephrosis was higher [22]. In the experimental murine model, MMP2 was described as a factor that was responsible for scarring when pressure on the renal parenchyma was present. MMP2-knockout mice demonstrated less extensive fibrosis in renal tissues following artificially induced obstruction [23]. In our data, we could not identify any significant correlation between MMP1 in ureteral tissue and urinary obstruction biomarkers. This result could have been influenced by the relatively low sample size. Due to the overall low levels of MMP1 gene expression, this estimation was available in only 12 samples, which was insufficient to run Spearman's rank correlation test.

The only study to reveal the significance of PAI1 in artificially induced urinary tract obstruction was conducted by Duymelinck et al., whose results showed that TIMP1 and PAI1 were the factors that participated in tubulointerstitial renal fibrosis [24]. Our calculations also revealed that the relative PAI1 expression in congenital ureteral stricture tissue was negatively correlated with TTT, which is recognised as an early sign of obstruction on RRS [25]. No correlation with differential renal function was found. None of the other investigated genes correlated with scintigraphic findings. The main drawback of our study is its relatively small sample size. The investigated parameters have small variation, so larger sample sizes would be necessary to elucidate differences. Nevertheless, such a dispersion speaks for independence of the investigated variables. Correlations between absolute urinary albumin and TGFβ1/VEGFA were significant; however, these values were not statistically significant when standardising for urinary creatinine, making the correlations borderline.

CONCLUSIONS

Expression of fibrosis-related genes in the obstructive tissue of PUJ have no direct correlation with biomarkers of renal damage.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This project received funding from the European Regional Development Fund (01.2.2-LMT-K-718-03-0087) under a grant agreement with the Research Council of Lithuania (LMTLT).

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