

## MORPHOLOGICAL CHANGES IN THE TUBULOINTERSTITIAL COMPARTMENT IN PRIMARY GLOMERULOPATHIES\*

**Kostadinova-Kunovska S.,<sup>1</sup> Petrusevska G.,<sup>1</sup> Jovanovic R.,<sup>1</sup>  
Grcevska L.,<sup>2</sup> Bogdanovska M.,<sup>1</sup> Polenakovic M.<sup>2,3</sup>**

<sup>1</sup>*Institute of Pathology, Faculty of Medicine, Skopje, R. Macedonia*

<sup>2</sup>*Department of Nephrology, Clinical Centre, Skopje, R. Macedonia*

<sup>3</sup>*Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia*

**A b s t r a c t:** The renal interstitium structurally supports the functional renal units and is involved in almost all renal functions. The degree of renal disfunction strongly correlates to the changes in the tubulointerstitial compartment present in almost all types of glomerular diseases. A phenomenon arising in such an environment is epithelial-mesenchymal transition, i.e. a change of the cell's epithelial phenotype into a mesenchymal one.

Histochemical, immunohistochemical and morphometric analyses were made of 50 renal biopsies with primary glomerulopathies, as well as light-microscopy analyses of semi-thin sections embedded in epoxy resin. Double immunohistochemical stainings with pairs of epithelial and mesenchymal antibodies were also done. The results were analyzed and correlated with the clinical data of the renal function of the patients.

The immunohistochemical analyses of the atrophic tubular epithelial cells showed a loss of expression of Cytokeratin and E-cadherin, an enhanced expression of HLA-DR $\alpha$ , and a *de novo* expression of Vimentin and  $\alpha$ SMA as markers for epithelial-mesenchymal transition. The double immunohistochemical stainings with Cytokeratin/Vimentin and Cytokeratin/ $\alpha$ SMA showed a simultaneous expression of these antigens in atrophic tubular cells. Their proliferative index was mildly enhanced. Interstitial fibrosis was present in 98% of the analysed biopsies.

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The analyses show correlations among all the changes in the tubulointerstitial compartment as well as the concentration of creatinine in the serum as a parameter of renal function.

The study emphasizes the usefulness of the implementation of histomorphometrical and immunohistochemical techniques as well as ultrastructural and molecular analyses in the process of nephropathological diagnosis.

**Key words:** tubulointerstitium, fibrosis, epithelial-mesenchymal transition, morphometry, double immunohistochemistry, creatinine.

### *Introduction*

The tubulointerstitial renal compartment accounts for over 80% of the total renal volume [1] and comprises tubules and the interstitium.

The tubular segments which form the major portion of the nephron (proximal tubules, distal tubules, Henle's loop and collecting ducts) are arranged in a complex pattern and are structurally and functionally heterogeneous.

The interstitium is made of cellular elements (fibroblasts, macrophages and dendritic cells) and an extracellular matrix (ECM) (collagen I, III, VI, proteoglycans, fibronectin, laminin and interstitial liquid) [2]. The basal membranes made of collagen IV are also considered to be part of the interstitium as they form its boundaries. The interstitial volume, according to the stereological analyses, varies from 7–9% in the cortical compartment and 30–40% in the inner medulla [3].

The interstitium not only supports the functional renal units, but also takes part in a number of exchange processes, influences glomerular filtration, and produces a variety of local and systemic hormones, such as erythropoietin [4], adenosine, prostaglandins, etc.

Changes in the tubulointerstitial compartment have been found in almost all glomerular diseases (with or without the presence of glomerular inflammatory infiltrate), except in minimal change nephropathy, as well as in some systemic diseases with renal involvement [5, 6, 7].

Beginning in the late sixties, numerous studies in this field have emphasized the impact of tubulointerstitial changes on the renal function [8, 9, 10, 11]. The impairment of the glomerular filtration rate (GFR), defined with the serum creatinine level, creatinine clearance, PAH clearance and inulin clearance, was found to be strongly correlated to the degree of tubulointerstitial damage, defined with an interstitial inflammatory infiltrate, interstitial fibrosis and tubular atrophy. Histomorphometric studies showed that the histological degree of glomerular impairment corresponded to the intensity of the histological changes in the tubulointerstitial compartment [12, 13]. Other studies

showed a correlation between the extent of interstitial fibrosis and the GFR [9, 11, 12] and between the extent of tubular damage and clinical parameters [14, 15, 16, 17].

The pathogenesis of the tubulointerstitial changes in primary glomerulopathies is complex and can generally be divided into three consequent phases according to the localization and the dominant cell type that is involved in each of them [18]: phase one – glomerular damage and its spread in the tubulointerstitium; phase two – tubular cells produce a variety of mediators, thus contributing to the progression of the renal impairment, and phase three – activation of the fibroblasts resulting in interstitial fibrosis.

The induction of the tubulointerstitial damage in primary glomerulopathies is a result of *inflammation* passing through the hilar glomerular zone into the interstitium, *toxic effects* on the tubular epithelial cells (TECs) from the impaired permeability of the glomeruli, *immunological processes* of humoral type and *haemodynamic disorders* in the glomeruli [1, 5, 19, 20].

The damaged TECs are stimulated to produce various mediators and have the main role in the formation of the secondary inflammatory infiltrate in the interstitium, as well as in the process of fibrosis through stimulation of the resident fibroblasts and through the mechanism of epithelial-mesenchymal transition.

The phenomenon of epithelial-mesenchymal transition (EMT) is a process during which the mature epithelial cells lose their phenotype, acquiring a new, mesenchymal one [21].

The fibroblasts, both resident and newly formed through the process of EMT, deposit excessive amounts of ECM [22]. They are stimulated by the macrophages and the surrounding damaged TECs. This process results in the destruction of the renal interstitium, obliteration and atrophy of the tubules and peritubular capillaries and the formation of atubular glomeruli (missing the distal nephron segment). The final result is a consequent reduction of the GFR.

### *Material*

We analysed fifty renal biopsies in the period between 2000 and 2005, previously diagnosed as primary glomerulopathy with standard histological and immunofluorescent analyses. Only biopsies containing at least 5 glomeruli, cortical tubulointerstitium and arteriolar segments were included in the study. The clinical data were obtained from the clinical histories of the patients.

The control group consisted of 20 samples from kidneys nephrectomised due to renal carcinoma, from the same period, with a regular renal histomorphology.

### *Methods*

All the cases were stained histochemically and immunohistochemically and analysed on a light microscope.

The histochemical stainings included PAS, trichrom Masson and Silvermethenamine Jones.

Immunohistochemical staining was done with monoclonal antibodies against Cytokeratin 7 (Clone OV-TL 12/30), Vimentin (Clone V9),  $\alpha$ SMA (Clone 1A4), HLA-DR $\alpha$  (Clone TAL.1B5), E-cadherin (Clone NCH-38) and Ki-67 (Clone MIB1). The double staining method was applied, coupling Cytokeratin 7 with Vimentin and  $\alpha$ SMA.

Cytokeratin 7 is an antibody directed against the intermediate filaments of the epithelial cells from the glandular and transitional epithelium. We analysed the presence of a signal in the different tubular segments, as well as changes in the intensity of the staining in the atrophic tubules.

Vimentin is an antibody directed against the intermediate filaments of the mesenchymal cells and  $\alpha$ SMA (Smooth Muscle Actin) marking the actin cytoskeleton of the myocytes and myofibroblasts. We specifically sought for their presence in the atrophic tubular cells and performed a semiquantitative assessment of the presence of tubules that contain cells positive for Vimentin and  $\alpha$ SMA, the results of which were expressed as a percentage of the total analysed area.

HLA-DR $\alpha$  as a marker for antigen-presenting cells was looked for in atrophic tubules, and the intensity of its staining was interpreted in a semiquantitative manner as an absent signal (-), weak positivity (+), moderate signal (++) and strong signal (+++).

E-cadherin is a transmembrane adhesive molecule contributing to the intercellular adhesion between the epithelial cells and we were looking for changes in the intensity of its staining in the atrophic tubules in comparison to those with a regular morphology.

Ki-67 was used for the determination of the proliferative index, which was expressed as the number of cells with positive nuclei per 10 high power fields (HPF).

For morphometric analysis, we made colour extraction of the interstitial area on tissue sections stained with trichrom Masson, using the Lucia M–Nikon image analysing system, and expressed the results as a percentage of the total scanned area. Morphometry was also used to count the nuclei positive for Ki-67.

Furthermore, samples of the biopsies were embedded in epoxy resin, cut into semi-thin sections, stained with Toluidine blue and PAS Silvermethenamine and analysed on a light microscope.

Statistical analysis of the obtained data was made with commercial statistical software (StatSoft, 2001). The results were expressed as mean values, standard deviation, minimum and maximum. The correlation analyses were performed using the Spearman index of correlation (R). A p value < 0.05 was considered to be statistically significant.

### *Results*

The analysed group of biopsies consisted of 35 biopsies from men (70%) and 15 from women (30%), at a mean age of 41.8 (SD = 14.4; min = 15; max = 80).

The control group consisted of 13 men (65%) and 7 women (35%), at a mean age of 55.2 (SD = 14.41, min = 27, max = 76).

The histological analysis showed the presence of interstitial fibrosis and tubular atrophy in all of the analysed cases, unlike the cases from the control group.

The extent of interstitial fibrosis in the analysed group of biopsies varied between 8.6% and 32%, with a mean value of 18.75% (SD = 5.04), whereas the mean value of the interstitial fibrous tissue was 5.32% in the control group (min = 3.4; max = 7.3; SD = 1.03). The statistical analyses showed a significant difference in the presence of interstitial fibrosis in the analysed group in comparison to the control group (t-test:  $t = 11.84$ ;  $df = 68$ ;  $p < 0.01$ ).

The histological analyses showed tubular atrophy in the areas of interstitial fibrosis in all cases from the analysed group, unlike the control group. This featured simplification of the TECs, dilatation of the tubular lumina and reduced diameter of the tubular cross-sections. Furthermore, the analyses of the semi-thin sections showed vacuolization of the TECs' cytoplasm, loss of the brush border and intensive resorptive activity of the cells (Figure 1).

The immunohistochemical analyses showed that both the TECs from the analysed group and those from the control group were positive for Cytokeratin. Apart from that, in the analysed group the TECs of the atrophic tubules in the areas of fibrosis showed a loss of expression for Cytokeratin (Figure 2). These TECs also showed a less intensive and even absent signal for the adhesive molecule E-cadherin in comparison to those from the control group (Figure 3). On the other hand, the atrophic TECs showed a stronger positivity for HLA-DR $\alpha$  than the TECs from the control group (Figure 4). The intensity of the staining signal was assessed semiquantitatively as described above and the statistics showed that it was significantly stronger in comparison to the control group (t-test:  $t = 7.28$ ;  $df = 67$ ;  $p < 0.01$ ).

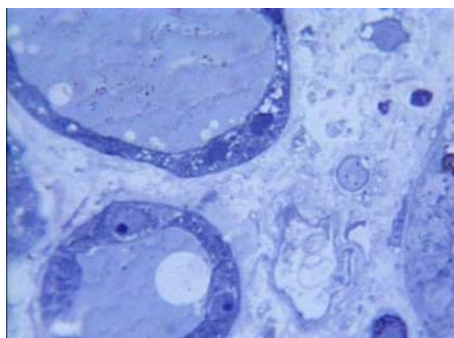


Figure 1 – Atrophic tubules, semi-thin section, Toluidine blue, (x1000);  
Slika 1 – Атрофични тубули, полутенок пресек, Toluidine blue, (x1000)

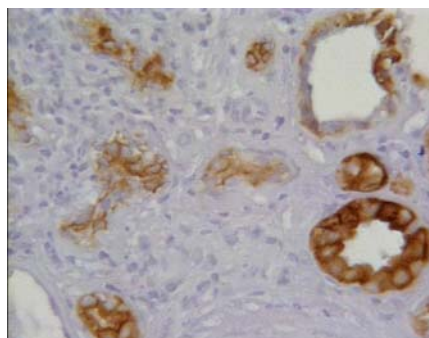


Figure 2 – loss of expression of Cytokeratin in atrophic tubules (x400);  
Slika 2 – Губење на експресија на цитокераин кај атрофични тубули (x400)

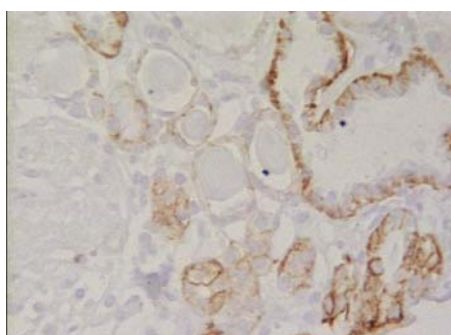


Figure 3 – Loss of expression of E-cadherin in atrophic tubules (x400);  
Slika 3 – Губење на експресија на E-cadherin кај атрофични тубули (x400)

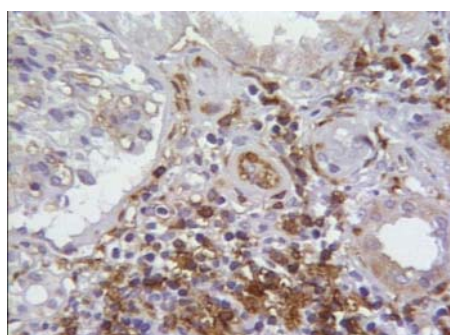


Figure 4 – Enhanced expression of HLA-DRα in atrophic tubules (x400);  
Slika 4 – Зголемена експресија на HLA-DRα кај атрофични тубули (x400)

The TECs in the areas of tubular atrophy and interstitial fibrosis also showed cytoplasmic positivity for the mesenchymal markers Vimentin (Figure 5) and  $\alpha$ SMA (Figure 6), which was not observed in the control group cases. These phenomena were better visualized with the double immunohistochemical staining method, using Cytokeratin as a first primary antibody and Vimentin (Figure 7) or  $\alpha$ SMA (Figure 8) as a second primary antibody. The semiquantitative assessment showed that atrophic tubules with cells positive for Vimentin and  $\alpha$ SMA were present in less than 10% and 5%, respectively, of the total analysed area. Nevertheless, the statistical analysis showed a significant difference in the presence of such cells in the analysed group in comparison to the control

group (Vimentin: t-test:  $t = 10.06$ ;  $df = 68$ ;  $p < 0.01$ ;  $\alpha$ SMA: t-test:  $t = 3.94$ ;  $df = 68$ ,  $p < 0.01$ ).

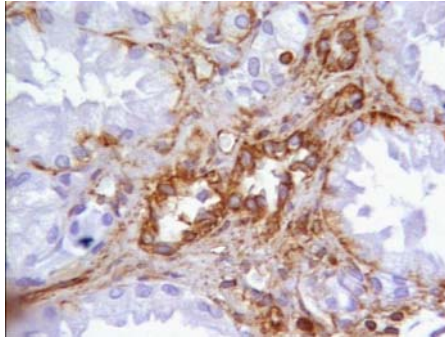


Figure 5 – Atrophic tubules positive for Vimentin (x400)

Slika 5 † Аїтрофични тїубули їозитїивни на вименїїин (x400)

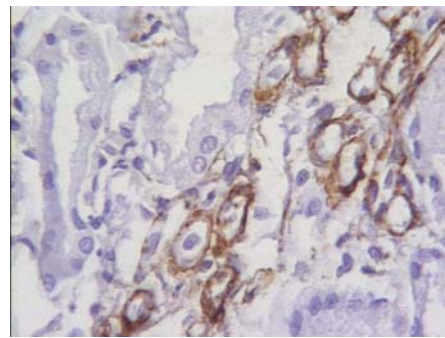


Figure 6 – Atrophic tubules positive for  $\alpha$ SMA (x400)

Slika 6 † Аїтрофични тїубули їозитїивни на  $\alpha$ SMA (x400)

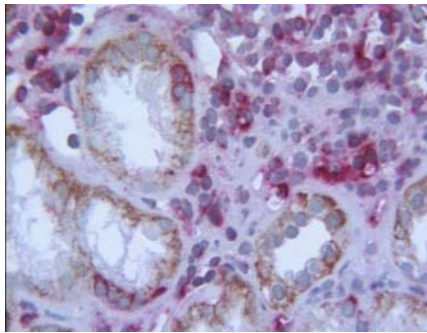


Figure 7 – Double immunohistochemical staining with Cytokeratin 7 (brown) and Vimentin (red) (x400);

Slika 7 † Двоїно їмунохїстїїохемїско боење со цїїїокератїїин 7 (кафено) и вименїїин (црвено), (x400)

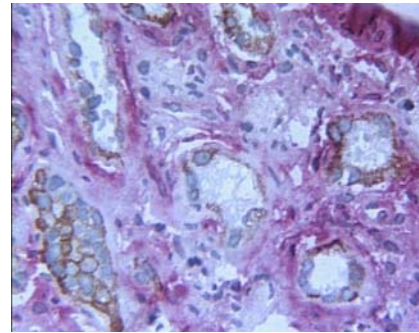
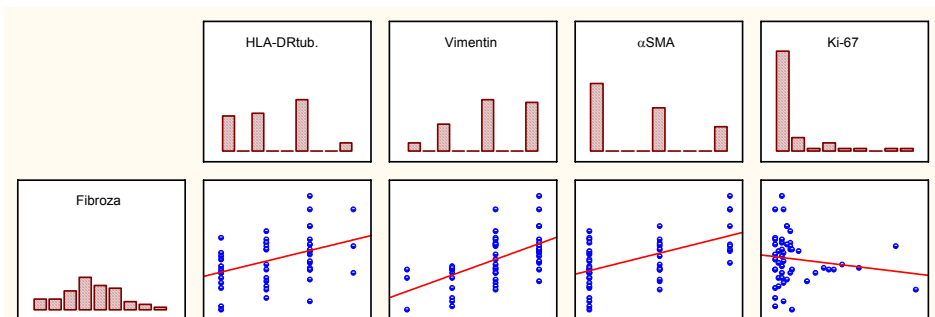


Figure 8 – Double immunohistochemical staining with Cytokeratin 7 (brown) and  $\alpha$ SMA (red) (x400)

Slika 8 † Двоїно їмунохїстїїохемїско боење со цїїїокератїїин 7 (кафено) и  $\alpha$ SMA (црвено), (x400)

The morphometric analysis of the tissue samples from the analysed group stained for Ki-67 showed positive nuclei in an average of 6.62 TECs/10 HPF (min = 0; max = 50; SD = 10.58). The control group cases contained 0.08 TECs/10HPF with nuclei positive for Ki-67 (min = 0; max = 3; SD = 0.83). The statistical analysis showed a significantly greater proliferative index of the TECs in the analysed group than in the control group (t-test:  $t = 2.45$ ;  $df = 68$ ;  $p < 0.05$ ).

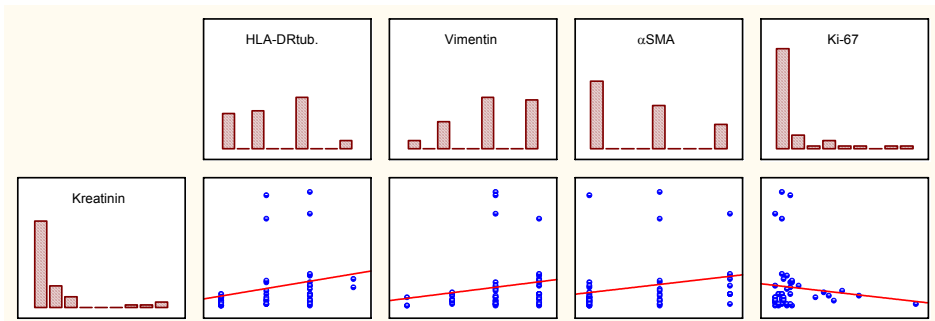
The above-stated parameters were correlated to each other, as well as to the clinical parameters. The extent of interstitial fibrosis correlated to almost all the markers of tubular cell injury: a significant positive correlation was found to the presence of atrophic tubules with TECs positive for Vimentin and  $\alpha$ SMA, as well as to the intensity of the staining of the TECs with HLA-Dr $\alpha$ , except with the proliferative index in the tubular cells (Graph 1).



*Graph 1 – Correlations between extent of interstitial fibrosis and morphological markers for tubular injury*

*Графикон 1 – Корелации меѓу засијаноста на фиброзиата и морфолошките параметри за тубуларно оштетување*

The analyses showed a statistically significant positive correlation between the serum creatinine level and the presence of atrophic TECs positive for Vimentin ( $R = 0.36$ ;  $p < 0.05$ ), for  $\alpha$ SMA ( $R = 0.45$ ;  $p < 0.01$ ) and the intensity of the staining of TECs with HLA-Dr $\alpha$  ( $R = 0.45$ ;  $p < 0.01$ ). There was no statistically significant correlation between the serum creatinine level and the proliferative index in the tubular renal segment (Graph 2).

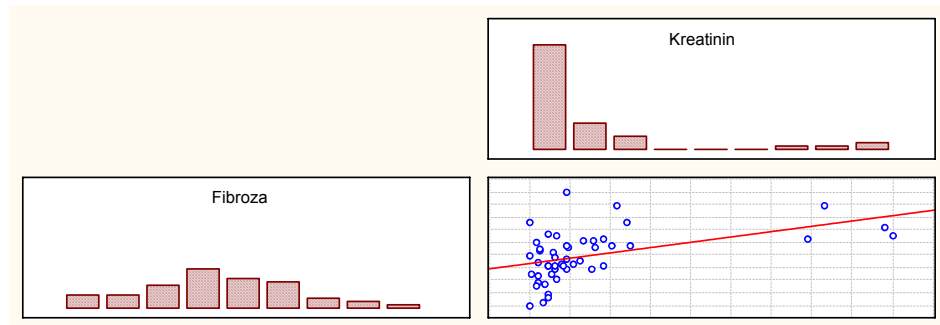


*Graph 2 – Correlations between serum creatinine level and morphological parameters for tubular injury*

*Графикон 2 – Корелација меѓу морфолошките параметри за тубуларно оштетување и концентрацијата на креатинин во серумот на пациентите*

The proteinuria was statistically significantly correlated only to the presence of cells positive for  $\alpha$ SMA ( $R = 0.39$ ;  $p < 0.01$ ).

Finally, there was a statistically significant correlation between the level of interstitial fibrosis and the serum creatinine level ( $R = 0,54$ ,  $p < 0,01$ ) (Graph 3).



Graph 3 – Correlation between extent of interstitial fibrosis and serum creatinine level  
Графикон 3 – Корелација међу застпаченошћом на фиброзију во интерстицијумој и концентратијом на креатинин во серумој на пациентијите

### Discussion

The results of our study suggest that in primary glomerulopathies the TECs have an active role in the development and progression of the disease. They convey the signal for the presence of antigens in the tubular lumina to the cells in the interstitium and they undergo epithelial-mesenchymal transition, thus becoming an executor of the synthesis of ECM.

According to numerous studies reviewed by Nath [1], the tubular epithelial cells have antigen-presenting properties, expressing HLA class II antigens, which are enhanced in cases of tubular damage. This is an important link in the promotion of secondary inflammatory infiltrate. The results from our study support these findings since all of the atrophic tubules in our analysed group, unlike the control group, had TECs positive for HLA-DR $\alpha$  with a different intensity of staining.

The TECs suffer phenotypic changes due to their permanent stimulation with numerous pro-inflammatory and pro-fibrotic mediators. The process of EMT is a multistep process. The first step is the entry of the TECs into the cell cycle, resulting in three possibilities: proliferation, apoptosis and EMT [23]. The proliferative index of the TECs, detected with nuclear positivity on Ki-67, in our study was low ( $< 10$  TECs/10 HPF), still being significantly higher than in the control group. However, both atrophic and vital TECs expressed Ki-67,

suggesting attempts of the TECs to repair the damage. Rastaldi *et al.* [14] also noted a higher proliferative index in the tubular segment in renal biopsies from patients with glomerulopathies.

Yang and Liu [24] identify four main consecutive events in the process of EMT: 1. loss of adhesive properties of the epithelial cells; 2. *de novo* expression of Vimentin and  $\alpha$ SMA in TECs; 3. disruption of the tubular basement membranes (TBM); 4. migration of the cells in the interstitium where they synthesize ECM.

Loss of expression of the adhesive molecule E-cadherin is an early event in EMT, allowing further dissociation in the structural integrity of the renal epithelia. This corresponds to our finding of loss of expression of E-cadherin in both the atrophic tubules and tubules with preserved morphology in areas of interstitial fibrosis.

The epithelial cell shifting into a mesenchymal phenotype is manifested with a change in the cellular morphology. In our study, we noticed that the atrophic tubules, besides dilatation of their lumina, also showed a spindle morphology of their epithelial cells. These cells were positive for Vimentin and  $\alpha$ SMA, unlike the TECs from the control group. Furthermore, all the tubules having cells positive for mesenchymal markers also showed expression for Cytokeratin, which was visualized by double staining. However, the intensity of the staining with Cytokeratin was markedly lower than in non-atrophic tubules and the tubules from the control group. Such properties make these cells potential candidates for EMT. These findings are consistent with those of Rastaldi *et al.* [14].

In our study the presence of atrophic tubules positive for  $\alpha$ SMA was significantly lower than the presence of atrophic tubules with cells positive for Vimentin. This is explained by the fact that Vimentin marks all cells with a mesenchymal cytoskeleton, while only myofibroblasts, a subpopulation of fibroblasts, are positive for  $\alpha$ SMA as a marker for their activation [25].

The final common pathway for many kidney lesions is renal fibrosis. The destructive fibrosis in the interstitial renal compartment initiates a vicious circle which progressively worsens the renal function [1, 22]. The results from our study, where the extent of interstitial fibrosis of more than 9% was found in 49 cases (98%) of the analysed group, are consistent with other studies [5, 6, 7]. Bearing in mind that interstitial fibrosis is also a component of an ageing kidney [1], our analyses showed that there was not any significant correlation between the age of the patients from the analysed group and the extent of interstitial fibrosis.

A few morphometric studies [9, 11, 12, 15] have suggested that the clinical parameters for renal function correlate better with the extent of the

interstitial fibrosis than with the degree of glomerular damage. These findings have generated the theory that once the glomeruli are damaged to a certain extent the progression of the renal disease becomes an irreversible process, depending on the status of the tubulointerstitial compartment.

The results of our study show the existence of a positive correlation between the extent of interstitial fibrosis and the clinical parameters for renal function. A strong positive correlation ( $R = 0.54$ ;  $p < 0.05$ ) was found with the serum creatinine level. Opposed to this, the proteinuria was not significantly correlated to the extent of interstitial fibrosis, which is inconsistent with the findings of other researchers [26, 27, 28, 29] and corresponds to the results of our previous study [30], as well as to the study of Rastaldi *et al.* [14]. A possible explanation for this is the fact that it is the quality and not the quantity of the proteins in the urine that contributes to the progression of the disease.

In conclusion, our study confirms the role of the tubular epithelial cells in the process of interstitial fibrosis and the progression of renal disease, independently of its etiology. An important event for the generation of myofibroblasts and excessive deposition of ECM is the epithelial-mesenchymal transition. These findings render the histomorphometric and immunohistochemical analyses of the changes in the tubulointerstitial compartment valuable tools for the assessment of the prognostic factors in primary glomerulopathies.

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## R e z i m e

**МОРФОЛОШКИ ПРОМЕНИ  
ВО ТУБУЛОИНТЕРСТИЦИЈАЛНИОТ КОМПАРТМАН  
КАЈ ПРИМАРНИ ГЛОМЕРУЛОПАТИИ\***

**Костадинова-Куновска С.,<sup>1</sup> Петрушевска Г.,<sup>1</sup> Јовановиќ Р.,<sup>1</sup>  
Грчевска Л.,<sup>2</sup> Богдановска М.,<sup>1</sup> Поленаковиќ М.<sup>2,3</sup>**

<sup>1</sup>Институт за патологija, Медицински факултет, Скопје, Р. Македонија

<sup>2</sup>Клиника за нефрологија, Клинички центар, Скопје, Р. Македонија

<sup>3</sup>Македонска академија на науките и уметностите, Скопје, Р. Македонија

Бубрежниот интерстициум обезбедува структурна поддршка на функционалните бубрежни единици, истовремено учествувајќи во речиси сите бубрежни функции. Степенот на бубрежна дисфункција силно позитивно корелира со промените во тубулоинтерстицијалниот компартман, кои се најдени скоро кај сите видови гломеруларни заболувања. Еден од феномените кои се случуваат во вакво опкружување е епително-мезенхималната транзиција, односно промена на фенотипот на епителните тубуларни клетки во мезенхимален.

Направени се хистохемиски, имунохистохемиски и морфометриски анализи на 50 бубрежни биопсии од примарни гломерулопатии, како и светлосно-микроскопски анализи на полутенки пресеци вкалупени во смола. Направени се и двојни имунохистохемиски боења со примена на парови од

\* Дел од проектот Тубулоинтерстицијални промени при нефропатиите – нефритите (клиничко-морфолошки иследувања) – бр. 07-80-3, финансиран од Македонската академија на науките и уметностите.

еден епителен и еден мезенхимален маркер. Добиените резултати беа статистички анализирани и корелирани со клиничките податоци за бубрежната функција на пациентите.

Имунохистохемиските анализи на атрофичните тубуларни епителни клетки покажаа губење на експресија на Cytokeratin и E-cadherin, зголемена експресија на HLA-DR $\alpha$ , како маркер за антиген-презентиращки клетки, како и *de novo* експресија на Vimentin и  $\alpha$ SMA, како маркери на епително-мезенхималната транзиција. Двојните имунохистохемиски боења со Cytokeratin/Vimentin и Cytokeratin/ $\alpha$ SMA покажаа истовремена експресија на овие антигени кај атрофичните тубуларни клетки. Покрај тоа, беше установен лесно зголемен пролиферативен индекс кај истите. Кај 98% од испитуваните биопсии беше установено постоење на интерстицијална фиброза.

Анализите покажаа меѓусебна зависност на сите опишани промени во тубулоинтерстицијалниот компартман, како и корелации со концентрацијата на креатинин во серумот, како битен параметар за процена на бубрежната функција.

Студијата укажува на користа од комбинираната примена на хистоморфометриска и имунохистохемиска техника, како и ултраструктурни и молекуларни анализи во процесот на нефропатолошка дијагностика.

**Клучни зборови:** тубулоинтерстициум, фиброза, епително-мезенхимална транзиција, морфометрија, двојна имунохистохемија, креатинин.

**Corresponding Author:**

**Kostadinova-Kunovska S.**  
**Department of Pathology**  
**Medical Faculty**  
**Ss Cyril and Methodius University**  
**1000 Skopje, Republic of Macedonia**  
**tel. +389 70 398 298**

**email: [skkunovska@yahoo.com](mailto:skkunovska@yahoo.com)**