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Implication of epithelial and mesenchymal interrelation in the evaluation of chronic kidney disease
Clinical, histological and immunohistochemical study

ABSTRACT

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CONTENT

I. STATE OF KNOWLEDGE

I.1. Introduction

In many epidemiological studies is highlighted the importance of diagnosis of renal impairment in early stages of chronic kidney disease, the etiology diagnosis being difficult based only on clinical criteria [41-43].

Thus, various studies support that renal biopsy is essential to obtain a conclusive diagnosis regarding the etiology of renal impairment, clinical suspicion being confirmed by renal biopsy in only 51% of patients [44].

However, it is noted that, in patients with advanced chronic kidney disease in renal biopsy procedure has a higher risk of complications and histopathological diagnosis results in a modification based therapy [45].

Multiple studies have demonstrated the presence of non-reversible healing mechanisms hold a key pathogenic factor in the development of chronic kidney disease regardless of initial histological lesions [92]. Thus, chronic injury to the parenchyma, similar to well differentiated tissue structures replacement with fibrosis in the healing process, are specific pathogenetic mechanisms in the development of chronic parenchymal insufficiency in different tissues such as liver, kidneys and lungs [93].

The purpose of this study was to demonstrate the relationship between epithelial phenotype and mezemchymal expression in renal interstitial fibrosis development process, as described, as the expression of new immunohistochemical markers predictive for renal function and in development and interaction between the expression of these markers and treatment with vitamin D or vitamin D substitutes.

I.2. Epidemiology of chronic kidney disease

The term chronic kidney disease was introduced in 2002 by the working group "The Kidney Disease Outcome Quality Initiative" (K / DOQI) and is defined as decreased glomerular filtration rate (GFR) in 60ml/min/1.73 sqm. body surface , persisting more than 3 months, with / without renal damage or renal impairment as the presence of more than 3 months, demonstrated by pathological changes or the presence of injury markers such as albuminuria / proteinuria, urinary sediment and kidney changes detectable pathological imaging [6].

In recent years the term of chronic renal failure was replaced with chronic kidney disease because it highlights the existence of chronic renal disease in patients with normal GFR (> 90 ml/min/1.73mp- BCR stage I) or slightly reduced from 60 to 90 ml/min/1.73mp or stage II chronic kidney disease and because is including renal transplant patients [6].

Chronic kidhey disease (CKD) is a major public health problem worldwide because of higher prevalence of 18.3% in Australia (AUSDIAB) [7] and 11% in the U.S. (NHANES III) [8]. In Western Europe the prevalence of CKD stages 3-5 is about 10% of the adult population [6]. In 2004, there were 1,783,000 people of patients in renal replacement therapies whith 1,371,000 (77%) patients on dialysis and 412,000 (23%) patients with transplanted kidney.

In Romania there is an increase in the number of dialysis patients (5447 in 2003) and roughly double in 2009 and increasing age of initiation of renal function sustitution therapy.

Multiple studies support that renal transplantation improves life expectancy by about 3-15 years compared with renal replacement therapy by dialysis but consistent with the presence of various features for donors and recipients such as age at transplantation [5] .

Although renal transplantation is the best choice for patients with chronic kidney disease due to improved quality of life compared to dialysis, there is a problem identified as the global demand for kidney transplants far exceeds the actual possibilities to achieve it [10].

Regarding the survival rate at 1 year for kidney transplant was as follows: 93.7% probability of survival at 1 year after transplantation and cadaver donor transplantation for life supravițuire rate is 97, 6% (Annual Report of USRDS data 2003). 5-year survival rate presented in the same report for the afost kidney transplant: 80.6% probability of survival at 5 years after transplantation from cadaver and 90.4% probability for a living donor.

In Romania from 1980 until June 1997 were performed 45 kidney transplants, and from June 1997 to December 2001 were performed 255 kidney transplants in which the donor graft 39 brain dead. In the Annual Report of 2011 of the Romanian Renal Registry has been reported that although growth in the number of patients with renal graft function is higher than the European average, the percentage of renal transplantation among renal replacement therapy methods is still small, so hemodialysis remains the primary method of renal replacement therapy in Romania (88%) [11].

I.3. Histopathological characteristics in chronic kidney disease evolution

In the kidney, the interstitial fibrosis was considered a common mechanism of progression of chronic kidney disease, which at some point becomes independent of the original cause kidney damage and is linked to a pathogenic imbalance between submission and degradation of extracellular matrix components [13]. This process is stimulated by a variety of cytokines and growth factors.

Growth factors belonging to the TGF-β family are the most intensively studied molecules derived from macrophages that are related to renal fibrosis [14].

Macrophages, tubular epithelial cells, and myofibroblasts are able to synthesize TGF-β in different stages of renal fibrotic lesions. Thus, the observation that suppression of expression of macrophages in the chronic lesion progression to fibrosis suggests that these cells are among the main producers of TGF-β [15].

Once activated this factor, TGF-β, signals through SMAD proteins transmembrane receptor that activates transcription of genes that will regulate important processes as those coding for collagen synthesis. In the kidney, TGF-β derived from macrophages may promote fibrosis by activating paracrine expression of extracellular matrix and myofibroblasts synthesis and also promote tubular epithelial cells trasdiferentiatign in myofibroblasts [16].

Thus, initiation of epithelial- mesenchymal transdifferentiation is an evolving process evoluating to decline in renal function and renal replacement therapy initiation. In addition, macrophages can synthesize and secrete collagen and TGF-β can also turn off, macrophages and induce expression of the macrophage inflammatory phenotype characterized by the production of collagen type VI [17].

In a recent study, it was observed that a single hematopoietic cell was able to differentiate into mesangial cells in an experimental model of hematopoietic cell transplantation, thus these

observations demonstrates that these cells derived from bone marrow can rebuild mesangial and interstitial cells [18].

In a recent study has observed that GFP + bone marrow cells migrate to the glomeruli and interstitium thus contributes to normal cellular bone turnover [19].

Although the majority of regenerated tubular epithelial cells derived from intrarenal source of asmenea, bone marrow-derived cells can contribute to replacing tubular epithelial cells through a process of cell fusion [17].

Thus, hematopoietic cell responsible for cell fusion process is not fully known, although there is evidence that macrophages can be reponsabilă of this process in the liver [18]. Thus, despite adequate stocks of cells and mechanisms for repair of damaged, in most cases, the kidneys or liver fibroblasts are used in the development process of fibrosis [16]. For example, if glomerular epithelial cells are the only non regenerating line of cells, endothelial cells, mesangial or tubular cells have high proliferation index providing adequate structural elements in the healing processes.

One factor that seems to be acting in tubular epithelial cells is represented by angiotensin II, which acts as a growth factor thereby stimulating local, cellular hypertrophy and production of collagen type IV and the main growth factors involved in this process is the TGF β1 [13]. Thus, this mechanism could explain the decrease in TGF-β1 in urine samples after administration of corticosteroids or inhibitors of angiotensin converting enzyme (ACE).

However, combination therapy with ACE inhibitors and angiotensin II receptor blockers, significantly enhances this phenomenon. The main factors that trigger TGF-β1 secretion are angiotensin II and proteinuria. Thus, in patients with diabetic nephropathy and proteinuria high levels of TGF-β1 in the urine compared to patients without proteinuria [20].

The most important mechanisms involved in chronic injury at tubulo-interstitial level in nephropathies evolving with proteinuria are: direct injury to tubular epithelial cells by consumtion of large amounts of protein through phagocytosis and accumulated in excess, tubular cells ischemia is a phenomenon triggered by multiple chemokines and growth factors that reach tubular cells, new antigens expression of adhesion molecules and proinflammatory features tubular cells after attaching to cell surface proteins as well as proteinuria and hypoxia [22].

TGFβ-1 by SMAD pathway is considered as the main mediator in regulating mechanisms of epithelial to mesenchymal transition (EMT), together with the factors likely inducers of hypoxia. Fibroblasts cells are pivotal effectors of fibrogenesis, initially these cells were identified by light microscopy and electron microscopy based on the characteristics

mofologice but more recently are identified by fibroblast markers. In kidney, Bowman identified two distinct cell populations in the cortex and the medulla internal morphological and functional changes in the future in accordance with stage of disease [22].

Given the complexity of cell lines, fibroblasts renal origin remains controversial, currently the most common theory prioritizes local membership interstitial cells but other authors claim that migrated leukocytes derived to local fibroblasts [13]. In recent studies performed in cultured cells and in experimental models of nephropathy has been proposed that tubular epithelial cells through EMT become collagen-producing cells. According to this hypothesis epithelial cells should undergo several stages such as proliferation and phenotypic changes to eventually synthesize extracellular matrix proteins.

Presence of EMT in renal fibrosis was first demonstrated by Strutz et al. [23], using fibroblast-specific protein (Fsp1) as a marker, these authors showed that tubular epithelial cells could express Fsp1, calcium binding proteins associated with the cytoskeleton, which is normally expressed in the fibroblasts.

In obstructive nephropathy induced by unilateral ureteral obstruction, multiple cells showed co-expression of both α-SMA and tubular markers, indicating that they are in a transitional stage between epithelium and mezenchyme [25]. These tubular epithelial cells lost their specific epithelial markers and gained mezemchymal features such as α-SMA and vimentin, resulting in production of interstitial matrix components such as fibronectin and collagen type I. According to studies EMT was also observed in tissue obtained by renal biopsy [2].

Recent studies on biopsy specimens provide new arguments in support of EMT that seems to play a role in progressive renal fibrosis, tubular epithelial cells that undergo phenotypic changes, as demonstrated by the expression of α-SMA and cytokeratines loss [24]. Because EMT often occurs in areas with severe structural tubular damage, a concern that has been raised over the years is that the α-SMA positive tubular cells may represent an interstitial infiltration of miofibroblaste.

In conclusion, renal tubulo-interstitial fibrosis characterized by depositing excess tissue in the renal parenchyma is an extensive process that finally results in progressive renal function impairement, independent of the primary renal disease.

Hepatocyte growth factor (HGF) and BMF-7 is the TEM molecule inhibitors for which experimental and clinical puteapreveni at interstitial fibrosis process. Recent studies indicate that administration of these molecules prevents peritoneal fibrosis in experimental models [23].

II. PERSONAL CONTRIBUTION

II.1. CLINICAL STUDY

Clinico-statistical study objectives were represented by: description of demographic characteristics for the study group and determining population distribution by sex, age and risk factors in chronic kidney disease, evaluation of the onset and confounding pathologies in chronic kidney disease onset, clinical form of the disease, investigating the factors involved in the development of renal pathology.

II.1.1. Matherials and methods

The clinical study was conducted in Nefrology Hospital "Carol Davila", and represents a retrospective study on a group of 87 patients who underwent renal biopsy procedures from 2008 to 2011 and followed up for a period of 21 ± 11 months or until renal function decline and initiate dialysis or death.

The protocol was approved by the Ethics Committee of the Hospital "Carol Davila" and was conducted in accordance with the ethical principles of the Helsinki Convention.

Information on clinical and laboratory parameters were extracted from electronic databases and medical records of each patient. Collection and reporting of these data was approved by the ethics committee of the institution and informed consent for the use of confidential data was obtained from each patient.

Exclusion criteria were represented by: lack of clinical and laboratory data at the time of biopsy and follow-up, lack of tissue in the renal biopsy fragments viable to perform classical histology procedures.

Renal function was assessed by serum creatinine levels measured at frequent intervals and by estimating glomerular filtration rate (GFR) by MDRD formula [26].

Basal medical history and clinical parameters were recorded at the time of renal biopsy and biochemical and clinical reassessment was performed at intervals determined for each patient according to the recommendations of "Kidney Disease Outcomes Quality Initiative" for assessing chronic kidney disease [27].

Induction immunosuppressive therapy for primary glomerulonephritis was the cortisol associated with cyclophosphamide. Immunosuppression in some primary glomerulonephritis consisted of the combination of prednisone and mycophenolate mofetil.

Renal biopsies were performed under ultrasound guidance by puncture with a needle mounted in an automatic spring, the main indications for biopsy were: isolated proteinuria, decreased renal function assessed by serum creatinine increase over 25% from previous values, the association between decreased kidney function and proteinuria.

Biological samples were obtained from the Department of Nephrology each patient fasted for 12 hours and all biochemical analyzes were performed in the same laboratory.

Systemic hypertension was defined as blood pressure values above the limit of consecutive repeated 140/90mmHg or use of at least one anti-hypertensive drug.

Regarding statistical analysis, continuous variables were expressed as mean values ± SD. Differences between groups were determined by Student t test, Mann-Whitney test and ANOVA, where indicated. Linear regression and logistic regression were used to perform single and multi-variable analysis. In all analyzes statistical significance was established for P values <0.05. Statistical analysis was performed using SPSS software version 9.

II.1.2. Results

In the clinical study were evaluated 87 patients who underwent renal biopsy procedures between April 2008 - September 2011 and followed up for 21 ± 11 months or until the decline of renal function and initiation of dialysis.

During the study period a total of 8 (9%) patients had renal function decline with renal sustitution therapy initiation.

Regarding chronic kidney disease stages it was observed a constant ratio between the incidence of glomerular filtration rate between 70 and 90ml/min/1.73m ², in the cases in which there has been observed the loss of renal function it was observed an increased incidence of cases with a glomerular filtration rate less than 60 ml/min/1.73m at renal biopsy moment.

For the group of patients who had renal function decline, with the initiation of renal replacement therapy, the average time to the loss of renal function was 15 ± 10 months.

Study on gender distribution of the studied population showed male predominance in both groups, so there is a percentage of 61.3% of male patients in the group of patients in whom there was no decline in renal function. In the stratification of patients in terms of age there was no difference between the two groups, the average age of the study group was 44.7 ± 11 years.

Following distribution by age, a maximum incidence of patients with eGFR> 90ml/min/1.73m ² was observed in the group aged 30-40 years and also can be observed the

continuing decline in the incidence of case with GFR within 60-90 ml/min/1.73m ² with renal biopsy indication, in age groups over the 50 years.

As the incidence of eGFR decline in the age groups, there was observed an increasing trend in the frequency of cases in which there was observed a decreased kidney function in the age group between 40-49 years and the age group 50-59 years.

Clinical and laboratory criteria that represent the indication to perform renal biopsy in the group with normal glomerular function, with eGFR calculated by the MDRD formula> 90ml/min/1.73m ², co-existence of proteinuria/24 h> 1g was in 87% of cases the main reason to perform renal biopsy in association with the presence of micro or macrohematuriei.

Isolated proteinuria with less than 1g/24h, did not influence the decision to perform renal puncture under normal renal function, even with modified values of serum proteins.

Regarding the presence of cardiovascular risk factors was found that the group who had renal function decline presented in 62.5% of cases high blood pressure values and in 34% of cases were associated with more than 3 anti- hypertensive medication.

It was observed in this study the presence of inflammatory syndrome in 75% of cases where there has been observed the decline in renal function and serum levels of C-reactive protein at the time of renal biopsy related with glomerular filtration rate (eGFR) during follow-up ($r = -0.21$, $p = 0.021$).

To determine the impact of several factors on renal function decline and initiation of renal replacement therapy we applied logistic regression statistical model.

The strongest predictor of initiation of substitutive renal therapy, in this model, was serum creatinine at the time of renal biopsy. Thus, any unit decrease in glomerular filtration rate increases during follow-up of 1.46 times the risk of starting renal replacement therapy by dialysis.

Short time tracking is also a predictor of starting dialysis with a probability rate of 1.16, this means that each month tracking further reduce risk of 1.16 times.

Also, the association of several cardiovascular risk factors relate to decreased glomerular filtration rate, as was observed that in 75% of cases where there has been obseved the decline in renal function has been emphasized the biological inflammatory syndrome during follow-up and in 62.5% of cases initiated renal replacement therapy were evolving metabolic acidosis which was corrected treated during follow-up.

II.1.3. Discussions

In the clinical-statistical study it was investigated a group with different etiology of renal impairment and was observed that performing renal biopsy puncture at a greater glomerular filtration rate was associated with improved survival, respectively, fewer cases had initiated renal replacement therapy.

Preserved renal function at the time of renal biopsy puncture was an important predictive factor for chronic kidney disease progression. Thus, immunosuppressive therapy or modification of this therapy after renal biopsy puncture resulted in improved renal function in most cases of glomerulonephritis. The response to treatment is comparable to existing data in other studies in groups of subjects with pathological diagnosis of glomerulonephritis in different stages [28-31].

This study did not compare individual immunosuppressive therapy in groups of patients with different etiology of glomerular damage, these subgroups were too small for such an analysis.

In terms of population distribution by gender was observed in this study, an increased incidence of males in certain age groups. This observation is supported by a number of studies that emphasizes higher life expectancy for women [48].

Thus, Haas et al. reported an almost equal number of women and men aged over 80 years who underwent renal biopsy procedures for the investigation of acute kidney injury (AKI) [49].

In our study, there was a similar incidence of cases diagnosed with membranous nephropathy and primary focal segmental glomerulosclerosis in the age group over 50 years. In the age groups below 50 years it was seen a high incidence of cases diagnosed with diabetic nephrosclerosis and glomerulopathy with minimal damage.

An important aspect described in this study is represented by underlining the importance of cardiovascular risk factors in the decline of renal function.

Thus, biological inflammatory syndrome with elevated CRP levels and hypertension were risk factors associated with decline in glomerular filtration rate ($p = 0.001$ and $p = 0.032$).

In analyzing the factors involved in the development of renal pathology an important aspect was the predictive power of serum creatinine at the renal biopsy moment ($p = 0.48$), and decreased glomerular filtration rate during follow-up was a predictive factor for the initiation of renal replacement therapy .

II.2. HISTOLOGICAL STUDY

Histological study objectives were represented by: histopathological description of the main features in the study group, highlighting the presence of inflammatory features at interstitial and tubular expression and describing interstitial fibrosis score and periglomerular fibrosis in the development of renal pathology.

II.2.1. Matherials and methods

Histological study was performed on a total of 87 pieces of renal biopsy obtained from patients who underwent renal biopsy procedures between April 2008 - September 2011 and followed for 21 ± 11 months or until the initiation of dialysis. There were selected only cases analyzed by microscopy in Nepropathology Laboratory of the Hospital of Nephrology "Carol Davila", Bucharest.

Microscopic aspects were studied in the tubulo-interstitial and glomerular area and the degree of inflammation and fibrosis. There were selected renal biopsies that had sufficient tissue for the diagnosis of all three techniques: optical microscopy, electronic miroscopie direct imunonofluorescenţă.

For the study histology were used to quantify the histological changes at least three sectiones in the usual histological stains for each biopsy, a section with hematoxylin-eosin staining, two sections with Goldner-Szeckeli trichrome staining, all sections had more than 5 glomeruli. In all biopsies for histological diagnosis there were reviewed by two independent pathologists before enrollment. Biopsies were evaluated and classified according to the diagnostic criteria [69].

Regarding the interstitial evaluation there was used a semiquantitative score evaluation by visual rating conducted by two independent pathologists through a scoring system from 0 to 3, as measured percentage interstitial inflammatory infiltrate and interstitial fibrosis percentage of total cortical area, there were excluded glomerular and tubular areas. Thus, score 0 = no inflammatory infiltrates or interstitial fibrosis; score 1 = presence of changes in <25% of cortical area assessed, score 2 = 25-50% presence of changes in cortical area assessed, score 3 = presence of changes in> 50% of the cortical area assessed.

Interobserver reproductivity was assessed by Spearman's correlation test that varied between 0.82 and 0.96 and the Student t test for paired data revealed no significant difference between observers.

The morphometric study was aimed to study histological semiquantitative measurement of the density of collagen fibers. Tubulo-interstitial area images in trichrome Goldner-Szeckeli staining were previously processed in Adobe Photoshop before the morphometric study was conducted in order to remove the overestimation of fibrosis by exclusion of tubular basement membrane.

Regarding statistical analysis, continuous variables were expressed as mean values ± SD. Differences between groups were determined by Student t test, Mann-Whitney test and ANOVA, where indicated. Also, Kaplan-Meier analysis was used to assess survival, statistical significance was established for P values <0.05. Statistical analysis was performed using SPSS software version 9.

II.2.2. Results

The most common histopathological diagnoses encountered in the study group were represented by membranous nephropathy (17.2%), minimal change glomerulopathy (16%) and focal segmental glomerulosclerosis (11.2%) and in the group that was observed the decline in renal function the most common forms of pathologic diagnosis was chronic tubulo-interstitial nephropathy, membranoproliferative glomerulonephritis and renal amyloidosis.

In 13% of cases diagnosed with membranous nephropathy was observed glomerular capillary walls with weak electron-dense deposits epimembranare and common areas of resorbtion, glomerular basement membranes on electron microscopy they have characteristics of stage IV membranous nephropathy .

Of the 8 patients who had renal function decline, respectively initiated renal replacement therapy, 5 biopsies (62.5%) had renal function decline in more than 12 months after the renal biopsy puncture. In these biopsies it was observed the overall inflammation score greater than 0 in 80% of cases and a score of inflammation in interstitial fibrosis area greater than 0 in 20% of cases.

In the cases that had a total inflammation score greater than 0 forms were the predominant histopathological lesions nephropathies with active lesions: membranoproliferative glomerulonephritis, vascular nephropathy.

In renal biopsies in which inflammation score <1 was observed a lower percentage of progression to initiation of renal replacement therapy during follow-up, thus, only in one case was initiated the renal replacement therapy in the follow-up period of the 28 cases of biopsy

who had a total score of inflammation equal to 0 and 2 cases of 50 biopsies who had a score of inflammation in interstitial fibrosis area equal to 0.

In 64% of cases that showed vascular congestion this was associated with the presence of total inflammation score greater than 1 ($r = 0.29$, $p = 0.031$). It was observed a characteristic vascular congestion associated with the presence of plasma cell inflammatory lymphocytes in the interstitial area.

A characteristic noted was the association of periglomerular fibrosis in early stages of glomerulosclerosis characterized by capillaries with open lumen compared with the presence of periglomerular fibrosis with well-defined aspect of stratified collagen deposition surrounding Bowmann capsular basement membrane in a severe glomerulosclerosis scoring.

Average score of tubulointerstitial fibrosis in the interstitial area was 42.3 ± 11.1% for renal biopsies with interstitial fibrosis confirmed by visual assessment.

In renal biopsies with interstitial fibrosis semiquantitative score 0 the average fibrosis assessed by computer analysis was 9.1 ± 7.4%. The interstitial fibrosis was observed prominently as a deposition of fibrous bands at the interstitial and peritubular level and in some cases this is associated with an inflammatory infiltrate with plasmo-lymfocitary cells.

Thus, there was observed the presence of interstitial fibrosis score higher in the group of patients that recorded the decline in renal function during the follow-up ($r = 0.61$, $p = 0.03$). Also, interstitial fibrosis score was associated with expression of periglomerular fibrosis ($r = 0.21$, $p = 0.001$).

II.2.3. Discussions

In the study group was observed that the most common histological types were represented by membranous nephropathy, minimal change glomerulopathy and focal segmental glomerulosclerosis. These data are consistent with the literature, which emphasizes the high frequency of primary glomerulonephritis and the high prevalence of histopathological forms of membranoproliferatve glomerulonephritis, focal segmental glomerulosclerosis and minimal change glomerulopathy [74].

Recent data indicate that lymphoid neogenesis can play an important role in maintaining immune responses and thus, promote chronic inflammation [77].

Thus, tissues housing a chronic inflammatory process express a high density of effectors cells such as T lymphocytes, macrophages, dendritic cells, B lymphocytes which can be

organized as secondary lymphoid follicles that can be structured in lymphatic nodes and lymphatic T cells areas [78,79].

The impact of inflammation on renal function reflects association with progressive disease, presenting histopathological markers of inflammation expressed both in areas with interstitial fibrosis and no fibrosis areas, supporting the assertion that renal dysfunction is not primary caused by the independent inflammatory process [80]. Thus, severe injury to the nephron causes an inflammatory reaction allowing the nephron and stromal remodeling as an attempt to healing tissue.

It was observed the presence of tubular atrophy, interstitial fibrosis in severe degree in the group that recorded the decline in glomerular filtration rate over a period of time greater than one year from the moment of renal biopsy. Thus, in these biopsies the association of histopathological markers of inflammation in areas with or without fibrosis, interstitial fibrosis, inflammatory reaction is caused due to loss of nephron, this explains why inflammation markers correlates with the decline of renal function [88].

Various studies as the study by Remuzzi et al. revealed that single sections correctly estimated advanced glomerulosclerosis but overestimated by about 30% the percentage of normal glomeruli [89]. Thus, in this study there are limitations imposed by histopathological evaluation of a small number of sections in the correct classification of individual glomerulosclerosis score for each section.

Regarding periglomerular fibrosis in our study was observed the association between expression of periglomerular fibrosis and glomerulosclerosis score. These results are consistent with data presented in various studies that emphasizes the importance of assessing periglomerular fibrosis in routine histopathological analysis of renal biopsies [90].

Multiple studies have demonstrated the presence of non- reversible healing mechanisms as a key pathogenic factor in the development of chronic kidney disease regardless of initial histological lesions [92]. Thus, chronic injury to the parenchyma, similar to well differentiated tissue structures replacement by fibrosis in the healing process, are specific pathogenetic mechanisms in the development of chronic renal parenchymal insufficiency of tissues such as liver, kidneys and lungs [93].

II.3. IMMUNOHISTOCHEMICAL STUDY

Immunohistochemical study objectives were represented by: epithelial phenotype characteristics observing the histopathological forms of chronic lesions in the renal biopsy, the description of the immunohistochemical expression of epithelial and mezemchymal markers in tubulo-interstitial, evaluation of the importance of tubulo-interstitial markers assesment in the evaluation of chronic kidney disease.

II.3.1. Matherials and methods

The immunohistochemical study was conducted on a total of 36 renal biopsies selected following exclusion criteria, from the initial population of 87 pieces of renal biopsy, obtained from patients who underwent renal biopsy procedures between April 2008 - September 2011 and followed for a period of 17 ± 11 months or until the initiation of renal replacement therapy or death.

An important exclusion criterion in the study was the absence of immunohistochemical biopsy material, which was used in many cases almost entirely for diagnostic histopathology. Another exclusion criterion was the histopathological forms of acute kidney injury or acute proliferative lesions such as proliferative diabetic nephropathy, acute interstitial nephritis, acute tubular necrosis, vasculitis or thrombotic microangiopathy.

To highlight the immunohistochemical method used is two-staged tissue antigens based on a polymer network visualization system (DAKO EnVision).

Similar to the histological the immunohistochemically stained slides were then investigated using a Nikon Eclipse microscope (Nikon, Apidrag, Romania) equipped with a 5MP CCD objectives and an AxioCam MRc5 digital vidocamera.

Quantitative analysis was performed using imaging morphometric analysis dedicated software Image Pro Plus (MediaCybernetics) [68]. Areas of interest were photographed with the same lighting and contrast settings and the images were stored in TIF format in a database. Immunohistochemical study was conducted on a database of more than 200 microscopic images captured with 20x and 40x objectives.

Antibodies used in the immunohistochemical study are presented below:

Tabel 1. Antibodies used in the immunohistochemical study

Antibody	Marker	Dilution	Antigen retrieveal	Source
α- SMA	Myofibroblasts	1:50	Citrat buffer, pH6	Dako
CD68	Macrophages/monocites	1:50	Citrat buffer, pH6	Dako
Cytokeratine (MNF116)	Cytokeratines with intermediate molecular weight	1:50	Citrat buffer, pH6	Dako
E-cadherin	Epithelial adesion molecule	1:500	Citrat buffer, pH 6	Epitomics
Vimentin	Mezemchymal marker	1:150	Citrat buffer, pH6	Invitrogen
Ki-67	Proliferation marker	1:50	EDTA, pH 9	Dako

The morphometric study aimed to measure the intensity and percentage of area occupied by the signal, the number of items of interest identified and the distance between the elements of interest.

Has only been studied aspects tubulo-interstitial area were excluded from morphometric analysis of glomerular areas or structures in the vicinity of large vessels.

The expression of immunohistochemical markers was expressed as a percentage of cortical tubulointerstitial area intestiţială, busy signal, exception made CD-68 expression being represented by the number of positive cells / interstitial area analyzed.

Similar histological study was used a semiquantitative scores to quantify tubulointerstitial lesions in the interstitial area represented by diagnostic scores ranging between 0 and 3, level chronic glomerular lesions, vascular, tubular or interstitial [90] .

Regarding statistical analysis, continuous variables were expressed as mean values ± SD. Differences between groups were determined by Student t test, Mann-Whitney test and ANOVA, where indicated. Also, Kaplan-Meier analysis was used to assess survival in the study group, the primary endpoint being the serum cretininei increase by more than 25% from baseline at the time of renal biopsy (T0), observed during the tracking. Statistical analysis was performed using SPSS software 9.

II.3.2. Results

It has been observed the male predominance in the group of patients included in the immunohistochemical study, similar to the original population. In this group was not observed any case where there has been encountered the decline in glomerular filtration rate with the initiation of renal replacement therapy during follow-up.

Also it was observed during follow-up in 63.8% of cases serum creatinine increase during follow-up with more than 25% compared to the value recorded at the renal biopsy moment. Thus, it was observed in the study group the classification according to the stage of chronic kidney disease, the presence of 25% of cases in stage III chronic kidney disease, 38.8% of stage II cases of chronic kidney disease and 41.6% in stage I chronic kidney disease at the time of renal biopsy.

The main forms of histopathological diagnosis were represented by focal segmental glomerulosclerosis in 27.7% of cases, followed by membranous nephropathy in 22.2% of cases and diabetic nephropathy in 16.6% of cases.

In the main histopathological features in the population for immunohistochemical study, there was a percentage of glomerulosclerosis expression by 14 ± 4.2%, in this study group no relationship was observed between the expression score of glomerulosclerosis and renal function parameters evolution.

It was also observed the expression of interstitial fibrosis score with an average of 31.2 ± 16.1%. Another aspect observed was the expression of interstitial fibrosis of 11.1 ± 3.5% in the expression of inflammatory infiltrate with a score> 1, compared with the expression of interstitial fibrosis of 23.1 ± 8.2% in the absence of inflammatory infiltrate ($p = 0.03$).

Morphometric expression of interstitial fibrosis was associated with development of renal function parameters. Thus, in cases in which there has been observed the increase in serum creatinine during follow-up, showed a higher expression of interstitial fibrosis with a mean of 54 ± 11.7% compared with the group that was not observed the increase in serum creatinine during follow-up ($p = 0.01$).

In this study, cytokeratines expression was assessed as the percentage expression observed in tubular sections for each biopsy, cytokeratines and E-cadherin expression was not observed in interstitial area, also, no expression was observed at the glomerular epithelial area.

An important aspect observed in this study was the decreased expression of cytokeratines expression in biopsies with interstitial fibrosis semiquantitative score greater than 1.

Thus, cytokeratines expression was observed with an average of 31.8 ± 5.9% of tubular sections. It has also been observed in this study a decrease in the expression of cytokeratines in 34% of renal biopsies who had interstitial fibrosis score greater than 1 ($r = -0.61$, $p = 0.03$).

In the study group were observed association of low expression of tubular cytokeratines epithelium expression with predominant expression of myofibroblasts ($r = -0.32$, $p = 0.02$) and vimentin ($r = -0.25$, $p = 0.002$) in the tubulo-interstitial area.

Similar to expression of cytokeratines it was evaluated the E-cadherin expression which is a transmembrane adhesion molecule important in defining the tubular epithelial phenotype characteristics, this marker was not observed in interstitial or glomerular area.

The E-cadherin expression was observed in the tubular epithelium with an average of 37.1 ± 11% of tubular sections. Also observed in the group who had increased serum creatinine during follow-up E-cadherin expression was with an average of 24.5 ± 11.5%, lower than the expression of E-cadherin in the group that showed no increase in serum creatinine during follow-up ($p = 0.02$).

Vimentin expression in the tubulo-interstitial area was associated with the interstitial fibrosis and also it was observed the immunohistochemical presence of vimentin in tubular epithelial cells.

In the evolution of renal function parameters was observed in the group that did not show tubular epithelial expression of vimentin association with a glomerular filtration rate calculated by the MDRD formula with an average of 43.8 ± 14.5 mil/min/1.73m ^{2} higher than group showing vimentin expression in tubular epithelium ($p = 0.002$).

In this study, it was not observed the myofibroblasts expression in tubular epithelial cells but mainly at interstitial area and also was observed at the glomerular area. Interstitial expression of myofibroblasts are mainly in the areas of interstitial fibrosis or in the adjacent areas with vimentin expression.

There was no relationship between immunohistochemical expression of interstitial myofibroblasts and other parameters of renal function evaluation such as proteinuria/24h or glomerular filtration rate at renal biopsy moment.

Also in this study it was observed the relationship between myofibroblasts expression and serum creatinine variation during follow-up. Thus, there is a negative association between immunohistochemical expression of α-SMA and changes in serum creatinine during follow-up ($r = -0.23$, $p = 0.01$).

Imnunohistochemical expression of macrophages was not observed in the glomerular capillaries and tubular epithelial cells. Immunoexpression of interstitial CD-68 was between 0.4 and 11.2 positive cells / field.

In the group of patients who presented increased serum creatinine during follow-up, there was the expression of a larger number of CD-68 positive cells with a mean of 5.5 ± 3.2 positive cells compared with the group did not had increased creatinine during follow-up ($p = 0.04$).

An important aspect observed in this study was the association of a immunoexpression of CD-68 interstitial positive cells higher in the patients who were not treated with vitamin D analogues or substitutes with an average of 6.4 ± 2.1 cells / field compared to the group receiving vitamin D therapy ($p = 0.02$).

There was not observed the presence of proliferation markers in tubular epithelial cells at interstitial level.

In the multivariate analysis found no relationship between expression of myofibroblasts and vimentin and evaluated clinical parameters such as age, male gender and the presence of several cardiovascular risk factors.

However, analyzing the vimentin expression in renal function predictivity evolution, only the immunohistochemical markers demonstrated a diagnostic specificity and sensitivity statistically significant even at low levels of expression, in the prediction of serum creatinine increase during the follow-up (AUC 0.680, $p = 0.0026$).

II.3.3. Discussions

Tubular expression of vimentin was observed to be important for prognosis of renal function progression when its expression is associated with interstitial infiltration of myofibroblasts.

In this study, vimentin expression was associated with the degree of renal dysfunction at the biopsy moment, but also is a strong predictor of the development of renal function parameters during follow-up.

In addition, the presence of tubular vimentin expression showed an association with inflammatory infiltrate, indicating that the inflammatory events and those related with EMT

are closely related and possibly represent different stages in the same sequence pathogenic outcome in the progressive deterioration of renal function [95].

According to these results, a previous study reported an association between changes in phenotype tubular interstitial area, the degree of interstitial fibrosis and renal function parameters expression, respectively, serum creatinine [96].

However, it should be noted that, although it has been observed in various studies, the association between EMT and chronic tubulo-interstitial fibrosis and chronic deterioration of renal function have not yet found evidence to support a causal role of EMT in decline in glomerular filtration rate.

Also, interstitial expression of myofibroblasts are demonstrated as a predictor of development of renal function in renal biopsy protocol performed in less than 12 months post-transplant biopsies [94].

It is important to note that the association between vimentin expression and miofibroblaştilor on the one hand, and the evolution of renal function on the other hand, was independent of serum creatinine or renal biopsy proteinurei/24h at the time. Based on these results, it is clear that a wider use of these markers may represent an important element in the development of renal prognosis.

Another important aspect described in the immunohistochemical study was represented of interstitial macrophages immunoexpression evaluation, this expression is associated with progression of chronic lesions in the renal biopsy.

Multiple studies have described glomerular or interstitial nephropathies as characterized by the accumulation of macrophages in interstitial area, this process can become chronic and further stimulate the production of growth factors similar to the healing process [99,100].

The association between tubular expression of vimentin and myofibroblasts infiltration in the immunohistochemical study has not been demonstrated to be a process of epithelial to mesenchymal transition (EMT), respectively, in the analysis of histological sections was not observed the tubular basement membrane discontinuation.

Although in this study was not investigated the interstitial myofibroblasts origin, the association of myofibroblasts infiltration and macrophages in the areas of early fibrosis calls for possible dual origin of these cells or fibroblasts at local or systemic origin of the cells derived from bone marrow

These observations are consistent with the study by Yu et al. denying the presence of a epithelial to mesenchymal transdifferentiation process at tubulo-interstitial renal in the fibrosis development process [102].

IV.CONCLUSIONS

1. In terms of population distribution by gender was observed both in the clinical and statistical study as well as for immunohistochemical study, an increased incidence of males in certain age groups. After the age of 60 years was observed predominance of female patients in renal biopsy indication.
2. In the clinical study, conducted in a statistical group of patients with different etiology of renal impairment was observed that performing renal biopsy at a glomerular filtration rate greater was associated with improved survival or fewer cases in which was observed the initiation of renal replacement therapy.
3. In patients with diabetes and proteinuria/24h> 3g revealed a small number of cases with indication to perform renal biopsy, included in the study versus those with altered renal function, eGFR calculated by the MDRD formula <90ml/min/1.73m ^{2} and nephrotic proteinuria.
4. In analyzing the factors involved in the development of renal pathology an important aspect was the predictive power of serum creatinine when renal biospiei and decreased glomerular filtration rate during follow-up was a predictive factor for the initiation of renal replacement therapy.
5. The clinical-statistical study also described the importance of treatment with vitamin D, both at the biopsy moment and during follow-up. A negative association was observed between biological inflammatory syndrome and vitamin D treatment, but no improvement was observed in the development of chronic kidney disease in the vitamin D treated group.
6. The impact of inflammation on renal function reflects association with progressive disease, histopathological showing inflammation markers expressed both in areas with interstitial fibrosis and no fibrosis areas, is supporting the observation that the decline of renal function is due not dependently of the inflammatory process.
7. Interstitial fibrosis coefficient determined by morphometric study represent a predictive factor for decline in renal function.

8. Regarding periglomerular fibrosis it has been observed the association between expression of periglomerular fibrosis and glomerulosclerosis.

9. Also, immunohistochemical study confirmed the importance of assessing interstitial inflammatory infiltration in the early phase of the development of interstitial fibrosis process.

10. Another important aspect that was described in the immunohistochemical study was represented of the interstitial macrophages immunoexpression evaluation, this expression is associated with progression of chronic lesions in the renal biopsy.

11. Tubular expression of vimentin was observed to be important for prognosis of progression for renal function when its expression is associated with interstitial infiltration of myofibroblasts.

12. In this study, vimentin expression was correlated with the degree of renal dysfunction at the biopsy moment, but also is a strong predictor of the development of renal function during follow-up.

13. In the immunohistochemical study, α-SMA expression was not found in tubular epithelial cells, but only in the interstitial compartment in areas with a higher degree of tubulo-interstitial fibrosis, collagen deposition and tubular epithelial cells expressing vimentin.

14. It is important to note that the association between vimentin expression and myofibroblasts on the one hand, and the evolution of renal function on the other hand, was independent of serum creatinine or proteinuria/24h.

15. An important aspect observed in the immunohistochemical study was the association between interstitial macrophage infiltration and treatment with vitamin D or vitamin D analogs

16. An original aspect described in this study is the association between the expression of histological epithelial and mesenchymal markers describing the relationship in the development of tubulo-interstitial fibrosis in the evaluation of renal function.

17. Immunohistochemical expression of epithelial and mesenchymal markers in the tubulo-interstitial area, are important prognostic factors in the evolution of renal function.

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