Morphological changes in membranoproliferative glomerulonephritis – a quantitative approach

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Abstract
Our study aimed to investigate the quantitative profile of the renal corpuscle components in membranoproliferative glomerulonephritis (MPGN). We have analyzed digital color images corresponding to relevant microscopic fields from renal biopsies (10 cases type I MPGN and 10 cases type II MPGN). A computerized morphometric algorithm was designed and applied in both red-green-blue (RGB) and hue-lightness-saturation (HLS) color spaces, allowing the automated measurement of areas for the following morphological characteristics of the renal corpuscles (RCs): glomerulus, Bowman space, cells, mesangial matrix and glomerular basement membranes, and capillaries. Student’s t-test comparatively applied between the numerical data obtained for the measured morphological characteristics, for each individual color space, showed significant differences between type I MPGN and type II MPGN for Bowman space area (p=0.008) and for mesangial matrix and glomerular basement membranes area – exclusively in RGB color space (p=0.013). We have also demonstrated larger RCs and glomerular size in type II MPGN, comparative to those in type I MPGN. Consequently, we assume that the morphometrical characterization of RCs histological components could be used as an additional criterion not only in the diagnosis of MPGNs, but also in the stratification of evolution and prognosis of patients diagnosed with type I and II MNGN, respectively.

Keywords: membranoproliferative glomerulonephritis, renal corpuscle, histological components, digital images, computerized morphometry.

Introduction
In spite of progresses made by biomedical sciences in the last decades, the triggers that initiate the pathogenic mechanism of the most glomerular diseases are still unknown [1]. Strictly related to membranoproliferative glomerulonephritis (MPGN), two new findings contributed to a better understanding of its pathogenicity, specifically that of crucial role of the hepatitis C virus and the comprehensive view on the biologic influence of complement system dysregulation [1], and underpinned a novel classification of this clinicomorphological glomerular entity [2]. The treatment decision-making is dependent on the knowledge of the glomerular disease pathobiology [1], reflected by the lesions spectrum identified in kidney biopsy as well as of correlated clinical and biological data. Although kidney biopsy is considered the gold standard of renal diseases diagnosis [3], the results are mainly qualitative interpretations with low reproducibility due to sampling, measurement, and interpretation errors. Considering these circumstances, the establishment of therapeutic guidelines is a very difficult and frustrating process for the nephrologists, since it is time and resource consuming.

Morphometry techniques offer the quantitative support for the qualitative interpretation of kidney biopsies, by the introduction of quantifiable features (descriptors) and therefore aim to transform a qualitative (subjective) investigation into a quantitative (more objective) one [4–6]. Beyond diagnosis accuracy, morphometry has opened new possibilities of important characteristics’ description related to early pathogenic events and disease progression. Although nearly 50 years have passed from the first published data, the method is still not very popular among renal pathologist, and the number of publications addressing this sensitive topic is smaller as compared to other specific nephrology research themes. However, we cannot take into account the contribution of morphometrical characterization (by classical, manual methods) of normal renal corpuscles (RCs), in light and electron microscopy [7–9]. Moreover, the following studies based on the implementation of the modern methods supported by assisted-image analysis and computerized morphometry [10] created the possibilities for the development of this research direction, with multiple clinicomorphological valences, already initiated by classical, manual morphology techniques.

Consequently, computerized morphometry became a preferential option [11, 12] in demonstration of the direct relationship between morphological substrate of glomerular lesions, evolution, and prognosis. Concrete proofs towards this research direction have been provided by several research papers in mainstream publications which approach by the means of quantitative analysis diagnosis, evolution and/or treatment response in variable types of glomerulonephritis, such as: minimal change disease [13–16], mem-
branchous [17–21], with IgA deposits [22, 23], membranoproliferative [15, 16, 24, 25], extracapillary [26], and with thin membrane/thin membrane syndrome associated to benign familial hematuria [27–32]. A special attention has been paid to the morphological investigation in focal and segmental glomerulosclerosis, both in children [13–16, 33] and adults [34–37], and also in lupus nephritis [38] and renal transplant pathology [39–43]. Quantitative changes of RCs have been reported in numerous experimental studies [44–46]. Another research category is oriented toward interstitial changes associated to glomerulonephritis [47–51].

Within this context, the aim of our study has been a computerized morphometrical evaluation of MPGN specific histological features. We have focused on numerical information that is able to characterize the differences between type I and II MPGN particular lesions, which subsequently might be used as outcome predictors. To increase the possibility for automatic identification of these differences, our morphometrical approach operates in two color spaces [52], namely RGB (red-green-blue) and HLS (hue-lightness-saturation), strategy expecting that at least one of the two spaces could reveal the differences.

Materials and Methods
Specimens and digital images

The study group included 20 renal biopsies diagnosed by microscopy and immunofluorescence as primary type I MPGN (10 cases) and type II MPGN – dense deposit disease (10 cases). The analyzed renal biopsy specimens had a medium count of 18 RCs.

The digital color images corresponding to the relevant microscopic lesions on biotic fragments stained with light green trichrome staining were acquired by image-microscopic lesions on bioptic fragments stained with trichrome staining were acquired by image-microscopic lesions on bioptic fragments stained with RGB and HLS space, respectively.

The computerized quantitative analysis has been based on a MPGN dedicated algorithm (detailed in Table 1) applicable in both RGB and HLS color spaces. The algorithm (operating in either RGB or HLS) automatically identifies the regions and measures the areas for the following morphological entities of interest of the RC: glomerulus, Bowman space, cells, mesangial matrix and glomerular basement membranes, and capillaries.

The quantitative analysis performed for an arbitrary RC (e.g., RC in Figure 1), in the RGB and HLS color spaces, resulted in two sets of binary images (illustrated in Figures 2–6) for measurements application and two sets of numerical results. Finally, for all the above-mentioned characteristics, a mean value/case has been calculated in RGB and HLS space, respectively.

The exploitation of the algorithm requires a preparatory stage for defining the working chromatic domains (separately for RGB and HLS spaces, respectively) for each component of the corpuscle, in accordance with the pathologist experience and specific color tints of investigated specimens, as follows:

- for Bowman space and glomerular capillaries (except intraluminal red blood cells): BS&C_RGB, BS&C_HLS;
- for nuclei: N_RGB, N_HLS;
- for mesangial matrix and glomerular basement membranes: MBM_RGB, MBM_HLS.

The algorithm, operating in RGB and, separately, in HLS, has been applied to 10 RCs for each specimen (corresponding to a case). The same number of RCs was investigated for each studied case, in order to ensure the uniformity of the approach. The number 10 was decided in accordance with the values considered by practice guidelines for the renal biopsy as defining a representative sample [54].

Student’s t-test was applied for all statistical comparisons.

<table>
<thead>
<tr>
<th>Table 1 – The main steps of the algorithm applied for the computerized analysis, in RGB and HLS color spaces</th>
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</thead>
<tbody>
<tr>
<td><strong>Sequence</strong></td>
</tr>
</tbody>
</table>
| Step 1 | Initialization and delimitation of renal corpuscle contour | • images load;  
| | | • manual drawing of RC contour. |
| Step 2 | Set chromatic domains requested by segmentation procedures | • load of the working chromatic domains defined for each component of the corpuscle (see preparatory stage):  
| | | – BS&C_RGB = [200,255] × [190,255] × [190,255];  
| | | – BS&C_HLS = ([0,43] ∪ [200,255]) × [200,255] × [0,255];  
| | | – N_RGB = [0,130] × [0,15] × [0,15];  
| | | – N_HLS = ([0,7] ∪ [249,255]) × [0,128] × ([0] ∪ [153,255]);  
| | | – MBM_RGB = [50,185] × [50, 180] × [50,180];  
| | | – MBM_HLS = [17,142] × [0,179] × [0,179]. |
| Step 3 | Color segmentation | • construction of binary images from identification of appropriately colored regions. |
| Step 4 | Binary images processing | • removal of false information (accidentally resulting from segmentation). |
| Step 5 | Measurements | • renal corpuscle area (RC_A);  
| | | • Bowman space area (BS_A);  
| | | • glomerulus area (G_A);  
| | | • total nuclei area (N_A);  
| | | • total mesangial matrix and glomerular basement membranes area (MBM_A);  
| | | • total capillaries area (C_A);  
| | | • residual (red blood cells) area (R_A). |
| Step 6 | Calculations | • relative areas (percentages) reported to RC_A: G_A/RC_A and BS_A/RC_A;  
| | | • relative areas (percentages) reported to G_A: N_A/G_A, MBM_A/G_A, C_A/G_A, R_A/C_A. |
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Figure 1 – MPGN type I: color image used as example.

Figure 2 – Binary images corresponding to Bowman space and to glomerular capillaries: (a) RGB; (b) HLS.

Figure 3 – Binary image representing Bowman space.

Figure 4 – Binary images corresponding to nuclei: (a) RGB; (b) HLS.
Validation of computerized morphometric algorithm

Three types of tests (in accordance with our previous experience reported in [54]) were considered for the accuracy evaluation of the results provided by the algorithm. Each type of test implements a criterion for checking the robustness of the algorithm with respect to the uncertain/uncontrolled actions that may affect the development of the procedure. All the three types of tests were meant to use the same digital image (e.g., Figure 1 in our approach), so that three types of errors can be created (relative to the same morphologic information) and subsequently three different quantification modalities become available for the algorithm robustness. It is worth noticing that equations (1)–(3) used below for the explanation of the tests have a generic form, as being applicable to all concrete entities of a studied RC.

Tests of type (1) – comparisons of the measurement results obtained for the interest entities by automated versus manually selection

We have manually drawn the contours of the interest entities inside an RC digital image (e.g., nuclear contours in Figure 7 in our approach), and we have measured the total area. The obtained numerical results have been compared with data obtained by automatic identification of the interest entities via the algorithm. The differences have been evaluated by means of the relative errors calculated by equation (1).

\[
er_{\text{man}(\text{morph}_i \_\text{characteristic})} = \frac{|\text{value}_{\text{manual}(\text{morph}_i \_\text{characteristic})} - \text{value}_{\text{manual}(\text{morph}_i \_\text{characteristic})}|}{\text{value}_{\text{manual}(\text{morph}_i \_\text{characteristic})}} \tag{1}\]

Tests of type (2) – perturbations of the working chromatic domains allocated to the interest entities

We have repeated the algorithm for the interest entities inside an RC digital image (e.g., mesangial matrix and glomerular basement membranes) after having applied changes (with the role of perturbations) to the chromatic domains considered during the preparatory stage and we have measured the corresponding area. The obtained numerical data have been compared to the information resulting from the algorithm run with the initial chromatic domains. The differences have been evaluated by the relative errors calculated by equation (2).

\[
er_{\text{perturbed}(\text{morph}_i \_\text{characteristic})} = \frac{|\text{value}_{\text{perturbed}(\text{morph}_i \_\text{characteristic})} - \text{value}_{\text{perturbed}(\text{morph}_i \_\text{characteristic})}|}{\text{value}_{\text{perturbed}(\text{morph}_i \_\text{characteristic})}} \tag{2}\]

Tests of type (3) – comparisons of the measurements results obtained by the use of the two different color spaces

By running the algorithm in both RGB and HLS color spaces, such a test allowed numerical comparisons of the measurement values obtained for each interest entity inside an RC digital image. The differences have been evaluated by relative errors calculated by equation (3).
The denominator of the above-mentioned formula refers to the minimum feature value, so that the relative error is maximized, and the worst case is considered in interpreting the differences between the measurements performed in RGB and HLS color spaces, respectively.

$$\text{er}(\text{morph_characteristic}) = \frac{|\text{value}_{\text{RGB}}(\text{morph_characteristic}) - \text{value}_{\text{HLS}}(\text{morph_characteristic})|}{\min[\text{value}_{\text{RGB}}(\text{morph_characteristic}), \text{value}_{\text{HLS}}(\text{morph_characteristic})]}$$

\section{Results}

\subsection*{Algorithm robustness analysis – tests outcomes}

The three types of tests previously defined in “Materials and Methods” section were performed on the RC image in Figure 1. The results reproduced below were selected from a much larger set of experimental tests, as offering relevant illustrations for the intended robustness analysis.

\subsection*{Tests of type (1)}

Tests of type (1) were applied for nuclear areas determined by manual drawing of 96 contours, as shown by Figure 7, resulting in the binary image in Figure 8; comparisons were made by using the nuclei automatically identified via the algorithm running in the RGB and HLS color spaces. The obtained area values were the following: 9851 $\mu m^2$ for manual contours, 9279 $\mu m^2$ for RGB- and 10225 $\mu m^2$ for HLS-operating algorithm. The relative errors given by equation (1) were of 5.8% and 3.8%, respectively, demonstrating a high precision of the algorithm operating in both RGB and HLS spaces, relative to the manual delineation (once the working chromatic domains are well defined).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure7.png}
\caption{Manual contours draw around the nuclei.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure8.png}
\caption{Binary image corresponding to the manual drawn contours.}
\end{figure}

\subsection*{Tests of type (2)}

Tests of type (2) were applied for the total area of mesangial matrix and glomerular basement membranes measured on the digitized image (Figure 1), by the perturbation of the R component of the RGB- and H component of the HLS-working chromatic domain.

In RGB color space, the working value of the R component (i.e., 185, in Table 1) was perturbed by $\pm 5$ units, meaning an error of $\frac{190 - 180}{256} = 3.9\%$ for the R coordinate; the resulting areas ranged between 8499 $\mu m^2$ (for value 180) and 9236 $\mu m^2$ (for value 190). Consequently, the relative error given by equation (2) has been $\frac{9236 - 8499}{9236} = 7.9\%$ for the total area of mesangial matrix and glomerular basement membranes.

In HLS color space, the working value of H component (i.e., 17, in Table 1) was perturbed by $\pm 5$ units, meaning an error of $\frac{28 - 17}{360} = 3.0\%$ for the H coordinate; the resulting areas ranged between 8798 $\mu m^2$ (for value 17) and 5014 $\mu m^2$ (for value 28). The relative error given by equation (2) has been $\frac{9236 - 8499}{9236} = 7.9\%$ for the total area of mesangial matrix and glomerular basement membranes. Subsequently, the relative error given by equation (2) has been $\frac{8798 - 5014}{8798} = 43\%$ for the total area of mesangial matrix and glomerular basement membranes.

This type of tests recommends the RGB utilization of the algorithm as more robust than HLS, applied to the peculiar chromaticity of trichrome staining used for the renal biopsy assessment. Nevertheless, we have to mention that the HLS version of algorithm returns trustable area values for a carefully selected chromatic domain, because a 5 unit perturbation of the H coordinate means an inattentive approach to the preliminary stage.

\subsection*{Tests of type (3)}

Tests of type (3) were applied for nuclei area (resulting a relative error of 2.1%), mesangial matrix and glomerular basement membranes area (resulting a relative error of 1.4%), and capillaries area (resulting a relative error of 9.6%). Such a test provides accurate numerical information characterizing the comparisons that can be roughly made by the visualization of the binary images (available from Step 4 of algorithm in Table 1), as illustrated by Figures 4a, 5a and 6a (RGB applied algorithm) versus Figures 4b, 5b and 6b (HLS applied algorithm).

\subsection*{Study group analysis – qualitative characteristics}

Ten cases diagnosed as type I MPGN had RCs
characterized by high hypercellularity due to an increase of mesangial cells number, increased amount of mesangial matrix and evident lobulation. Glomerular basement membranes had a typical double contour or rail tram appearance. Special staining allowed the identification of subendothelial deposits and immunofluorescence revealed a positive granular anti-C3 reaction and an interrupted linear pattern of anti-IgG and anti-IgM reaction.

Ten cases diagnosed as type II MPGN – dense deposit disease maintained in RCs a hypercellular appearance, basement membranes area; C_A: Total capillaries area; R_A: Residual (red blood cells) area.

Table 2 – MPGN type I: synopsis of automated measurements performed in RGB and HLS color spaces (mean values/ case, mean values/group)

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<tr>
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<td>13019.52</td>
<td>12977.7</td>
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<td>43.9</td>
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<td>8.</td>
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<td>13121.97</td>
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<td>12240.65</td>
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<td>18.7</td>
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<td></td>
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<td>16.6</td>
<td>93.2</td>
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<td>Mean values for type I MPGN</td>
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<td>29771.48</td>
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<tr>
<td>Mean values</td>
<td>13.9</td>
<td>86.1</td>
<td>10879.38</td>
<td>12353.03</td>
<td>3887.25</td>
<td>2651.40</td>
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</tr>
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</table>

MPGN: Membranoproliferative glomerulonephritis; RGB: Red-green-blue color space; HLS: Hue-lightness-saturation color space; RC_A: Renal corpuscle area; BS_A: Bowman space area; G_A: Glomerulus area; N_A: Total nuclei area; MBM_A: Total mesangial matrix and glomerular basement membranes area; C_A: Total capillaries area; R_A: Residual (red blood cells) area.

Table 3 – MPGN type II: synopsis of automated measurements performed in RGB and HLS color spaces (mean values/ case, mean values/group)

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<td>4.</td>
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<td>2708.8</td>
<td>2772.79</td>
<td>9648.06</td>
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<td>11589.97</td>
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<tr>
<td>5.</td>
<td>36284</td>
<td>2830.15</td>
<td>33453.84</td>
<td>10805.98</td>
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<td>6.</td>
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<td>5088.41</td>
<td>34356.59</td>
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<td>11476.98</td>
</tr>
</tbody>
</table>

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Study group analysis – quantitative characteristics

The automated measurements results have been summarized in Table 2 (including 10 cases of type I MPGN) and Table 3 (including 10 cases of type II MPGN – dense deposit disease).

The statistical analysis comparatively applied between the numerical data obtained for the measured morphological characteristics, for each individual color space, showed significant differences between type I MPGN and type II MPGN for Bowman space area (p<0.006) and for mesangial matrix and glomerular basement membranes area – exclusively in RGB color space (p=0.013).
glomerular cells), the number of glomerular cells/1000 cell percentage (i.e., the number of mesangial cells/number of glomerular cells), the number of glomerular cells/1000 μm² of glomerular area, the number of endothelial cells, the number of epithelial cells and epithelial cell percentage (i.e., number of epithelial cells/number of glomerular cells) [8].

The evolution of type I MPGN in children was assessed by morphometry, proving that the ratio between the mesangial matrix area and glomerular area decreases after immunosuppressive therapy, as compared to the expanded values initially recorded; a correlation between the above morphometrical features and the level of type IV collagen, type V collagen, and fibronectin has also been established [24]. These data support the idea that the reversible clinical evolution of type I MPGN is associated with mesangial matrix reduction. Another research was carried out by a computer image analysis system in order to quantitatively compare the area of the mesangial deposits from serial kidney biopsies ultrastructurally investigated in patients with MPGN [58]. The mesangial deposit area/mesangial area ratio was significantly decreased at the date of second biopsy and certain correlations were noticed between mesangial deposits, glomerular infiltrates of monocytes and/or macrophages and proteinuria [58].

It is worth mentioning that there is no available data regarding the comparison between the quantitative profile of RCs and their morphological characteristics in type I and II MPGN.

We consider that our research may provide valuable information for optimal data translation from the histopathological domain to the clinical management. The numerical values obtained in our study highlight some of the morphological changes and differences considered as difficult to detect by a simple qualitative analysis. Thus, the numerical results can be discussed in terms of their histopathological significance, leading to the following remarks.

Our quantitative data certify an amplified RCs dimension compared to the normal status [36, 59]. RCs size increase, as a MPGN specific change, is the result of concomitant modifications appeared in mesangial and membrane components, in correlation to the cellular proliferation. As a general consideration for the RCs modified structure in MPGN, the nuclear proliferation in the glomerular area represents between 34.90 and 36.49%, mesangial matrix and glomerular basement membranes comprising between 41.6% and 43.49%, with the remnant belonging to the capillary lumens, with or without their red blood cells content.

In our opinion, the identified morphological differences between RCs in type I and type II MPGN are reflecting different pathogenic mechanism and evolution. Specifically, our measurements may indicate larger RCs and glomerular size in type II MPGN, comparative to those in type I MPGN. In correlation, Bowmann space is statistically significant smaller in type II MPGN compared to that in type I MPGN (p=0.006). These data indicate an enhanced glomerular activity in type II MPGN, which may be eventually associated with a more severe disease progression. RCs

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Mean values for type II MPGN

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Mean values for type II MPGN

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MPGN: Membranoproliferative glomerulonephritis; RGB: Red-green-blue color space; HLS: Hue-lightness-saturation color space; RC_A: Renal corpuscle area; BS_A: Bowman space area; G_A: Glomerulus area; N_A: Total nuclei area; MBM_A: Total mesangial matrix and glomerular basement membranes area; C_A: Total capillaries area; R_A: Residual (red blood cells) area.
structural changes highlight the evident differences between the ratio of nuclear area (larger in type I MPGN and smaller in type II) and mesangial matrix and glomerular basement membranes area (smaller in type I MPGN and larger in type II MPGN).

Moreover, the statistically significant differences between the mesangial matrix and glomerular basement membranes areas measured in type I and II MPGN, respectively (in RGB color space, \( p=0.013 \)), represent a solid argumentation in sustaining the severity of basement membrane lesions specific for dense deposit disease, with strong consequences in RCs histarchitectony and implied physiology. The better results obtained in the RGB space may be correlated with the type (2) tests for the algorithm robustness analysis, proving a higher accuracy for RGB (versus HLS) running.

We have to underline the value of our computerized morphometrical approach, as a development of our constant interest in the computer-assisted image analysis, dedicated also to nephropathology [53]. The systematic review of references in glomerulonephritis morphometry domain led to the conclusion that there is not enough literature information regarding the high-level automated procedures, in such a manner that they could represent standards in nephropathology quantitative analysis [10–12]. Thus, personal landmarks have been implemented in order to allow the evaluation of proposed algorithm performances. The registered relative errors in algorithm validation (less than 10%), despite chromatic complexity of the images used for measurements, are strong mathematical arguments in favor of similitudes observed during the simple visual inspection of binary images obtained in the parallel RGB and HLS color spaces analysis.

Finally, we consider that the morphometrical results might be further applied as supplementary tools in the stratification of evolution and prognosis of patients diagnosed with type I and II MGN, respectively. At the current stage, our research does not explore the prognosis role of the morphometrical results by interpreting the correlations between the clinical picture and the numerically quantified pathological information. An objective of this kind is targeted by our future projects, which obviously requires, as a starting point, the principles and results of the work presented above.

Conclusions

Our study outlines, by the means of quantitative data, the differences between type I and II MPGN. Consequently, we may promote, as a hypothesis, the idea that the morphometrical characterization of RCs histological components might represent additional criteria not only for the diagnosis of MPGNs, but also for the assessment of evolution.

Conflict of interests

The authors deny any conflict of interests, funding and other personal relationship with other people or organizations related to this study.

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References

Morphological changes in membranoproliferative glomerulonephritis – a quantitative approach

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