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ORIGINAL RESEARCH

Influence of Podocyte Injury on the Development of Class IV Lupus Nephritis

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Purpose: In the kidneys, Systemic Lupus Erythematosus leads to Lupus Nephritis (LN), a form of glomerulonephritis. There is evidence that patients with LN may present activation of specific pathways for podocyte injury. This injury can occur through different mechanisms such as loss of podocyte adhesion to the glomerular basement membrane, cell death or dedifferentiation. Podocyturia with consequent podocytopenia has been described in some nephropathies such as LN, highlighting the importance of studying podocyte injuries in this condition. Evaluating in situ morphological characteristics of podocytes becomes relevant for a better understanding of the processes involved in their pathogenesis. This study investigated podocytes in different classes of LN in renal biopsies performed by the Kidney Research Center at the Federal University of Triângulo Mineiro.

Patients and Methods: Twenty control cases and 29 biopsy cases diagnosed with LN were selected, divided according to the histopathological classes of the disease. Podocyte density was assessed through immunohistochemistry for Wilms tumor 1 protein and the evaluation of foot process effacement was performed by transmission electron microscopy.

Results: Podocyte density was lower in the LN and this reduction was observed in all analyzed classes when compared to the control group. More foot process effacement was observed in the LN group, with more effacement in classes I/II and class IV compared to the control group. The class IV group showed more foot process effacement than the class III group and presented higher proteinuria levels compared to the classes I/II group. A strong, positive, and significant correlation was observed between the activity index and foot process effacement in the class IV group.

Conclusion: Podocytes play an important role in the development of LN, and possibly, injuries to these cells are more closely related to the inflammatory/diffuse proliferative cellular process developed in class IV LN.

Keywords: podocyte density, foot processes effacement, activity index, renal biopsy

Introduction

Systemic Lupus Erythematosus (SLE) is a highly heterogeneous and multisystemic chronic autoimmune inflammatory disease resulting from the production of autoantibodies against autoantigens and the in situ inflammatory response caused by the deposition of immune complexes.[1] In the kidneys, SLE leads to Lupus Nephritis (LN), a form of glomerulonephritis that constitutes one of the most severe manifestations of SLE, affecting approximately 60% of patients.[2] Despite advances in knowledge and improved treatment options, LN remains a significant cause of morbidity and mortality among SLE patients, with 10% to 30% of patients progressing to chronic kidney disease, requiring renal replacement therapy.[3]

The clinical presentation of Lupus Nephritis (LN) is highly heterogeneous, with the possibility of presenting silent urinary abnormalities or features such as hematuria, leukocyturia, cellular casts, and mild proteinuria. Moreover, more severe cases may manifest as nephrotic syndrome and/or acute nephritic syndrome or rapidly progressive renal failure.[4,5]

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Through renal biopsy, the diagnosis of LN is established utilizing the classification of LN from the International Society of Nephrology/Renal Pathology Society (ISN/RPS). This classification is based on the location of immune complex deposits in the glomeruli, the extent of glomerular involvement (mesangial, endocapillary, and extracapillary proliferative lesions), and whether the pattern of injury reflects acute inflammation (active disease) or sclerosis (chronic disease). The classification also proposes a semiquantitative assessment of activity and chronicity for classes III and IV, with a maximum score of 24 for the activity index and 12 for the chronicity index.[6–8]

The pathogenesis of LN is related to the deposition of circulating immune complexes and the in situ production of immune complexes in the kidneys, triggering the activation of the complement system through the classical pathway, as well as the activation of macrophages and neutrophils. This results in the release of reactive oxygen species and the production of pro-inflammatory cytokines, leading to tissue inflammation that causes malfunctioning of the glomerular filtration barrier, of which podocytes are a part.[9]

Podocytes are terminally differentiated cells that, in response to injury, undergo cell death due to an imbalance between adaptive responses that maintain homeostasis and cellular dysfunction.[10] Morphologically, they respond to injuries with foot process effacement, resulting in proteinuria and/or nephrotic syndrome.[11] Our previous studies have already demonstrated a reduction in podocyte density and foot process effacement in renal biopsies of other glomerular diseases such as Focal Segmental Glomerulosclerosis (FSGS), Minimal Change Disease (MCD), and Diabetic Nephropathy (DN).[12,13]

Taking into consideration that one of the greatest challenges in monitoring SLE and LN is that their course is marked by episodes of reactivation and that there is a role of podocyte injury in the pathogenesis and progression of LN, the evaluation of in situ morphological characteristics of podocytes becomes relevant for a better understanding of the processes involved in its physiopathology.

Materials and Methods

Study Subjects

Renal biopsies diagnosed with LN were performed by the Kidney Research Center at the Federal University of Triângulo Mineiro. These biopsies were performed between 1996 and 2023 and stored in the service for further retrospective studies like this one. The diagnosis was conducted according to the latest classification of LN from the International Society of Nephrology/Renal Pathology Society (ISN/RPS), including the division into classes and the activity and chronicity indices in classes III and IV.[6]

In the present study, classes I, II, III, and IV were included. Classes V and VI were excluded since Class V corresponds to Membranous Nephropathy associated with LN, and Class VI had only two cases in our service. Cases with insufficient material for the three analyses (common light microscopy, fluorescence, and electron microscopy) or lacking relevant clinical data for correlations with morphology were also excluded. Epidemiological, clinical, and laboratory data were obtained from the biopsy request form.

After applying the exclusion criteria, we obtained a total of 29 cases, which were divided into groups according to the diagnosis: Class I/II group (n=9), Class III group (n=6), and Class IV group (n=14). The control group consisted of 20 samples of kidney fragments from autopsy of adult individuals, whose cause of death was not related to renal disease or infectious diseases.

Evaluation of Podocytes

Paraffin-embedded renal biopsy fragments with a thickness of 2 μm underwent immunohistochemical techniques. For podocyte labeling, the primary antibody anti- Wilms tumor 1 protein (WT1) (Dako) was used at a dilution of 1:500. The antibody was incubated in a dark, humid chamber at room temperature for 2 hours. The technique was performed manually using the non-biotinylated Novolink polymer system (Novolink Polymer Detection System Kit, BL, UK), following the manufacturer's instructions. Subsequently, the slides were counterstained with Hematoxylin and subjected to morphometric analysis.

Digital images of glomeruli were captured using the AxionCam ICc55 digital camera (Zeiss, Germany) attached to a light microscope with a 40X objective lens. All immunolabeled podocytes were counted in each glomerulus, and the area of each glomerular tuft was measured.[12,13] The results were expressed in podocyte density.[14]

Evaluation of Foot Processes

Renal biopsy fragments fixed in Karnovsky + Ruthenium Red were processed for resin embedding and subjected to semi-thin sections of 250 nm thickness, stained with toluidine blue, to identify the best glomeruli for evaluation under the transmission electron microscope. Once the glomeruli were selected, the block was trimmed for the preparation of ultra-thin sections with a thickness of 50 to 70 nm. The grid containing the ultra-thin section was examined under the Zeiss EM-900 transmission electron microscope.

Images of all available glomerular capillaries were evaluated at a magnification of 7000x, with at least two capillaries per glomerulus. Subsequently, the images were analyzed using the semi-automatic ImageJ 1.53t program. To do this, the system was calibrated using the scale bar in the electron micrographs. Identified foot processes were manually counted and marked. Subsequently, the length of the glomerular capillary to be studied was measured. The estimation of the width of the foot process was performed using the formula FPW = (Σ length of glomerular capillary / Σ number of foot processes) x $\pi/4$,[13,15] where $\pi/4$ is a correction factor to adjust for a presumed random variation in the angle of the relative section of the axial axis of the foot process and the results were expressed in nanometers.

Statistical Analysis

For statistical analysis, a spreadsheet was created using Microsoft Excel, and data analysis was conducted using the GraphPad Prism program (version 7.0). The variables were tested to determine whether they exhibited a normal or non-normal distribution through the Kolmogorov–Smirnov test, and analysis of variance was performed. Non-parametric tests were applied, with the Mann–Whitney test and Kruskal–Wallis test followed by Dunn's post-test for the comparison between two groups and three or more groups, respectively. The Spearman test was employed for correlation analysis. Differences were considered statistically significant when the "p" value was less than 5% ($p < 0.05$).

Results

Clinical-Epidemiological Profile

After applying the exclusion criteria, 29 lupus nephritis cases were used. Among these, the majority were female (93.10%), with a mean age of 32 ± 9.55 years. The clinical-laboratory profile is detailed in Table 1.

Podocyte Alterations in Lupus Nephritis

Whereas the crucial role of podocytes in glomerular diseases, we assessed the number of podocytes through the expression of WT1 in LN cases, observing a significant reduction in podocyte density compared to the control group (Figure 1A, $p < 0.0001$). When separated by class, this reduction was also observed in all LN classes compared to the control group (Figure 1B–F, $p < 0.0001$); however, there was no statistical difference between the classes.

After we observed a reduction in podocyte density in LN cases, we sought to analyze possible ultrastructural alterations such as foot process effacement, which was assessed by measuring the foot processes width (FPW). We observed a higher FPW in the LN group compared to the control group (Figure 2A, $p = 0.0011$), indicating a greater degree of foot process effacement in this group. Concerning LN classes, we observed a higher FPW in the classes I/II group and the class IV group compared to the control group, and among the classes, there was more foot process effacement in the class IV group compared to the class III group (Figure 2B–F, $p = 0.0008$).

Proteinuria is Associated with Podocyte Alterations in Lupus Nephritis

Because that podocyte alterations clinically manifest with proteinuria, we sought to assess proteinuria in LN cases divided according to histopathological classification. We observed an increase in proteinuria levels as the classes

Table 1 Clinical and Laboratory Characteristics of Patients with Lupus Nephritis

	Lupus Nephritis	Classes I/II	Class III	Class IV
Age (Years)				
Mean±SD	32±9.55	37±9.40	27.83±6.55	30.57±9,89
Median (min-max)	31 (18–54)	35 (25–54)	28.50 (19–35)	29 (18–53)
Gender n (%)				
Male	2 (6.90)	1 (11.11)	0 (0.00)	1 (7.14)
Female	27 (93.10)	8 (88.89)	6 (100.00)	13 (92.86)
Creatinine (mg\dL)				
Mean ± SD	1.83±2.45	1.70±2.40	0.95±0.56	2.28±2.95
Median (min-max)	1.00 (0.34–11.40)	0.80 (0.55–7.62)	0.88 (0.36–1.98)	1.00 (0.34–11.4)
eGFR (mL/min/ 1.73m^2)				
Mean ± SD	77.78±41.98	85.38±39.47	96.00±41.65	65.64±42.53
Median (min-max)	81 (4.00–149.00)	98.50 (5.00–125.00)	93.50 (33.00–149.00)	64.00 (4.00–141.00)
Proteinuria (g/24h)				
Mean ± SD	2.57±2.49	1.36±1.11	2.23±2.90	3.55±2.71
Median (min-max)	1.54 (0.00–8.60)	1.30 (0.00–3.59)	1.11 (0.57–8.08)	3.0 (0.67–8.60)

Abbreviations: SD, Standard deviation; Max, Maximum; Min, Minimum; N, Number of cases; eGFR, Estimated glomerular filtration rate.

progressed, and the class IV group presented significantly higher proteinuria levels compared to the classes I/II group (Figure 3, p = 0.0491), suggesting a clinical worsening in classes with more inflammation/cellular proliferation.

Podocyte Alterations are More Prevalent in Proliferative Classes with Greater Inflammation

Considering that more inflammatory classes of the disease are associated with more morphological alterations and worse levels of proteinuria, we sought to relate the activity and chronicity indices to podocyte alterations in cases diagnosed with LN class IV.

No correlations were observed between podocyte density and foot process effacement, respectively, with the chronicity index of the disease. Regarding the activity index, when analyzing class III patients individually, there was no statistically significant correlation when correlating the activity index and its criteria with foot process effacement (p = 0.5167). On the other hand, when analyzing class IV patients individually, a strong, positive and significant correlation was observed between the activity index and foot process effacement (Figure 4A, p = 0.0189). Still, there were no significant correlations between foot process effacement and subendothelial deposits in class IV (Figure 4B, p = 0.6134). However, some important activity index criteria such as endocapillary hypercellularity (p = 0.0311, Figure 4C) and leukocyte infiltration (p = 0.0369, Figure 4D) correlated strongly, positively and significantly with foot process effacement.

Relationship Between Podocyte Damage and Immunocomplex Deposition

The LN group was divided into two groups according to immune deposits: IgG+IgM+C1q+C3 and IgA+IgG+IgM+C1q +C3 (Full-House). Most cases were classified as Full House. The control group presented higher podocyte density (p<0.0001) and lower FPW (p<0.0089) compared to the IgG+IgM+C1q+C3 and Full-House groups, but without significant difference between the groups with NL, regardless of the deposits (Figure 5).

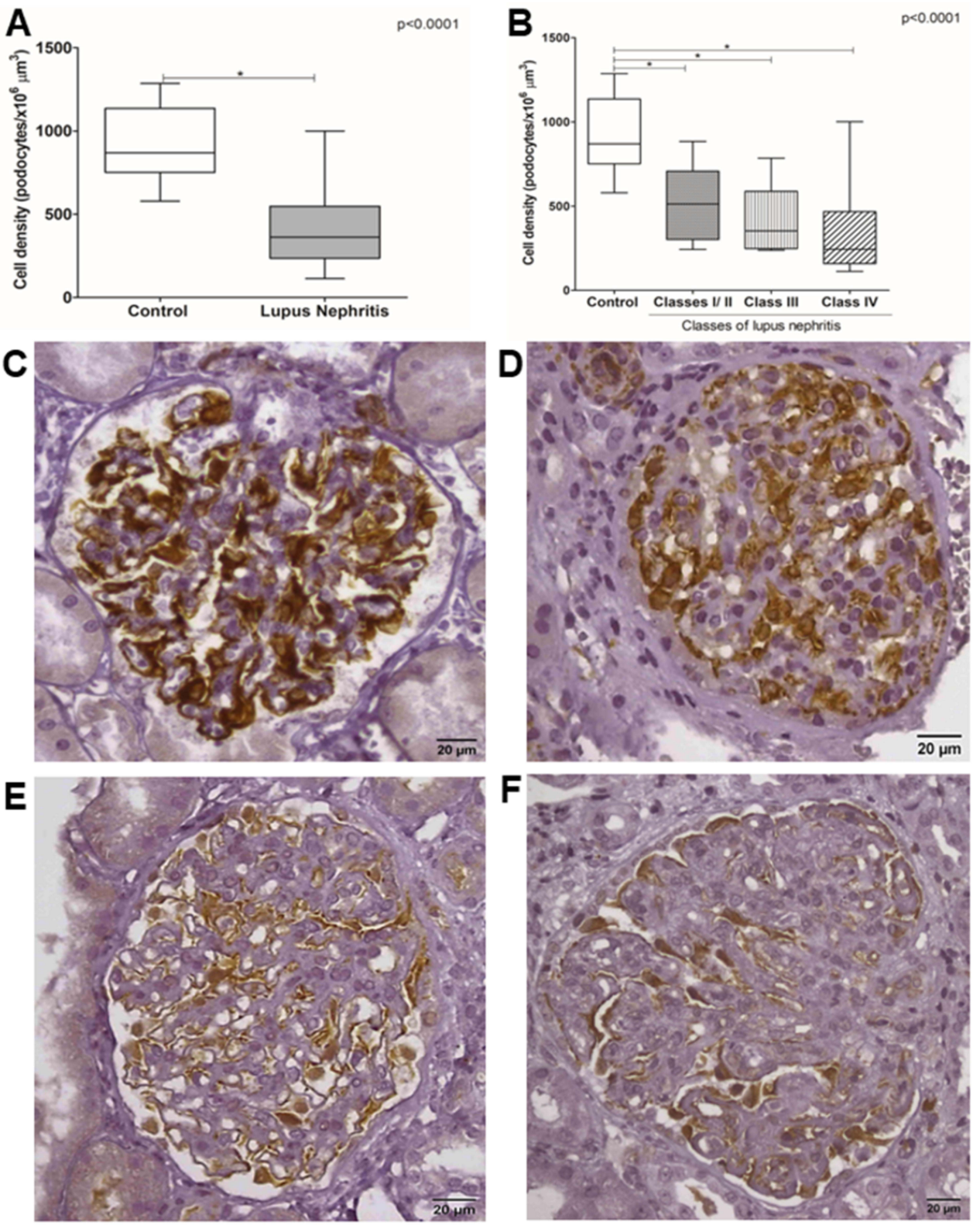

Figure 1 Analysis of podocyte density using immunohistochemical technique for WT1. **(A)** Comparison between the control group and the Lupus Nephritis group. Mann–Whitney comparison test, p<0.0001. **(B)** Comparison between control group and the classes of Lupus Nephritis. Kruskal–Wallis test followed by Dunn's multiple comparison, p<0.0001. Horizontal lines represent the medians, bars represent the 25–75 percentiles, and vertical lines represent the 10–90 percentiles. WT1 immunostaining in **(C)** control sample, **(D)** in classes II of lupus nephritis, **(E)** in classes III of lupus nephritis, and **(F)** in classes IV of lupus nephritis.

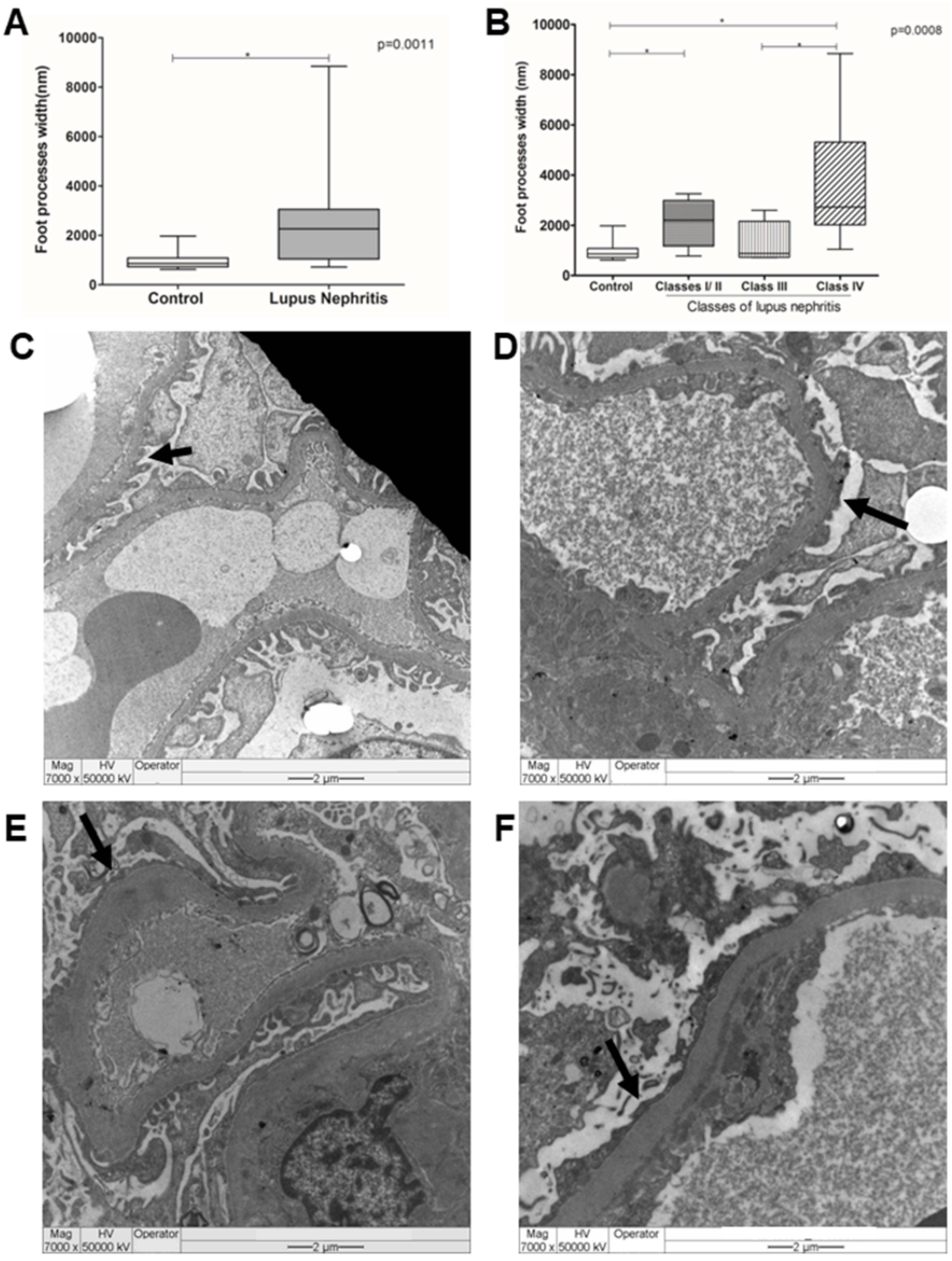

Figure 2 Analysis of podocyte foot process effacement by transmission electron microscopy. **(A)** Comparison between the control group and the lupus nephritis group. Mann–Whitney comparison test, p=0.0011. Comparison between **(B)** control group and the classes of lupus nephritis. Kruskal–Wallis test followed by Dunn's multiple comparison, p=0.0008. Horizontal lines represent the medians, bars represent the 25–75 percentiles, and vertical lines represent the 10–90 percentiles. **(C)** Image of the glomerular loop from a case-control study showing preserved podocyte processes (arrow). Foot process effacement evidenced by arrows in a case of **(D)** lupus nephritis class II, (**E**) lupus nephritis class III, and **(F)** lupus nephritis class IV. Magnification (Mag), High Voltage (HV).

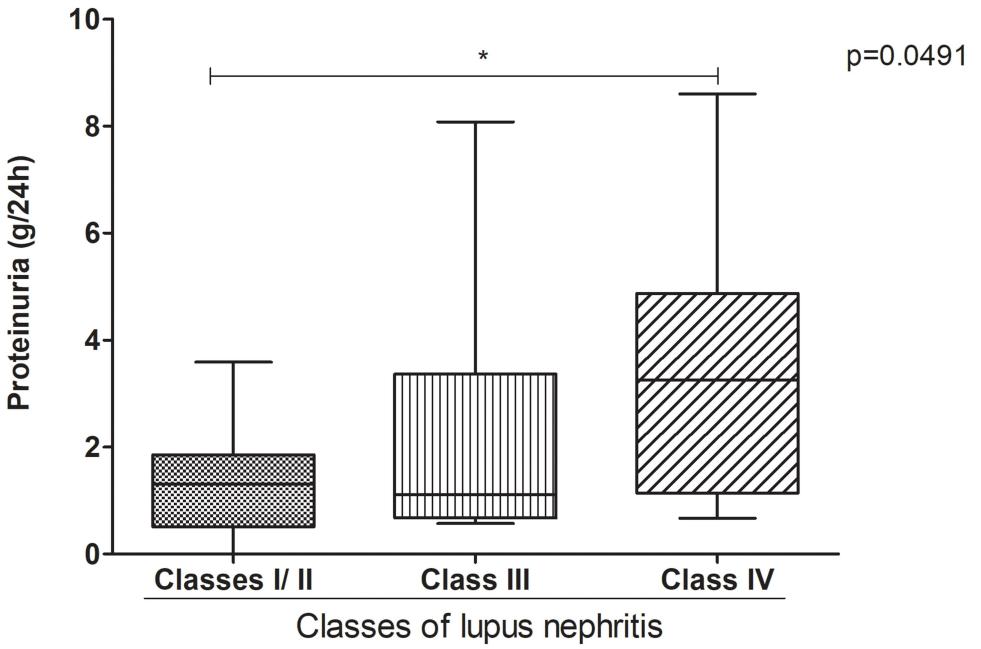

Figure 3 Analysis of proteinuria among the classes of lupus nephritis. Kruskal–Wallis test followed by Dunn's multiple comparison. Horizontal lines represent the medians, bars represent the 25–75 percentiles, and vertical lines represent the 10–90 percentiles. p=0.0491.

Discussion

Lupus Nephritis is a clinical manifestation of Systemic Lupus Erythematosus considered the most severe condition of the disease, affecting about 60% of diagnosed patients. Its pathogenesis is related to glomerulonephritis, resulting from the deposition of immune complexes and complement system activation.[9] The deposition of immune complexes in the glomerulus triggers changes in all cells in this compartment: endothelial cells, mesangial cells, and podocytes. Podocyte injury clinically results in proteinuria and/or nephrotic syndrome.[16]

In the present study, we observed a reduction in podocyte density in the LN group compared to the control group. Our group has also demonstrated a decrease in podocytes and consequent foot process effacement in FSGS, MCD and DN. We believe that this reduction is a result of podocyte injury due to inflammation caused by the deposition of immune complexes.[12,13]

Podocytes have an intrinsic system that supports injuries, but they can be damaged when stresses exceed this capacity, leading to cell death. As a terminally differentiated cell, this results in a reduction in numbers, as observed in the present study. These cells can commonly appear in the urine, a phenomenon referred to as podocyturia. In recent years, the evaluation of podocyturia has emerged as an indirect tool in the analysis of podocyte injury in some cases of glomerular diseases[15] including cases of LN.[17] This loss of podocytes appears to occur through apoptosis, resulting in in situ podocytopenia.[18] In LN, podocyte apoptosis is related to the activation of the Th17 profile and the production of IL-17, leading to changes in their cytoskeleton with loss of foot processes, increased motility, decreased expression of proteins maintaining cellular homeostasis, increased oxidative stress, and activation of inflammasomes and caspases.[19–21]

We observed a higher prevalence of class IV cases, which is consistent with previous studies that have shown a higher prevalence of class IV LN.[22,23] Class IV, known as diffuse LN, is characterized by diffuse, segmental, or global endo- or extracapillary glomerulonephritis, active or inactive, involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial changes.[8] It has the most severe prognosis and is associated with a higher risk of progression to end-stage renal disease.[24] Thus, renal biopsy is indicated, which may justify the increased prevalence of this class in studies using the morphological classification of LN.

When evaluating the number of podocytes and foot process effacement in LN classes, we did not observe a difference in the number of podocytes between classes. However, class IV showed greater foot process effacement. Podocytes in LN can be targeted by autoantibodies that react directly with podocyte proteins, such as α-actinin-4,[25] and it is also

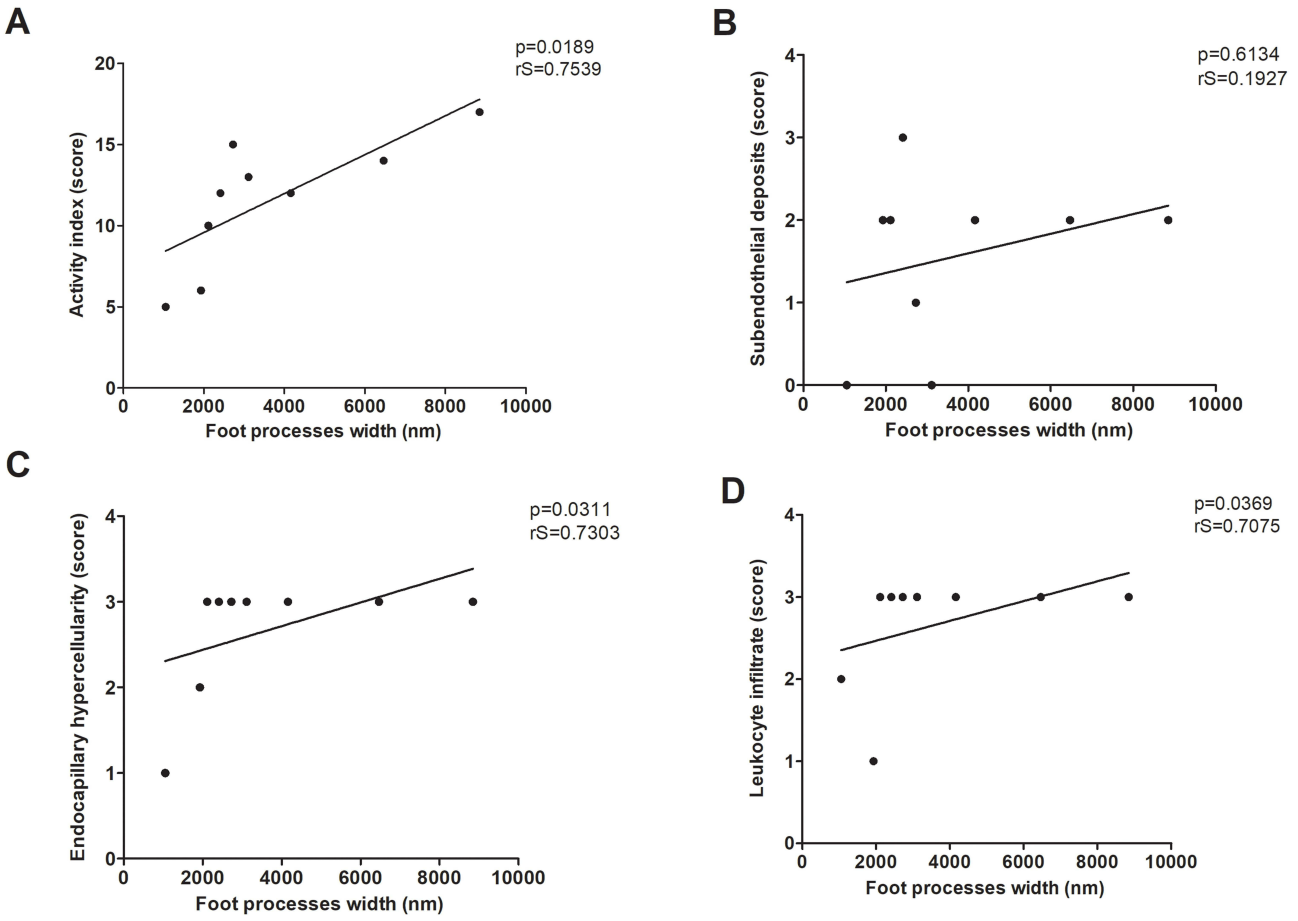

Figure 4 Assessment of foot process effacement and lupus nephritis class IV activity. Correlation between **(A)** activity index, **(B)** subendothelial deposits, **(C)** endocapillary hypercellularity, **(D)** leukocyte infiltration and foot process effacement in lupus nephritis class IV. Spearman correlation coefficient. Nanometer (nm).

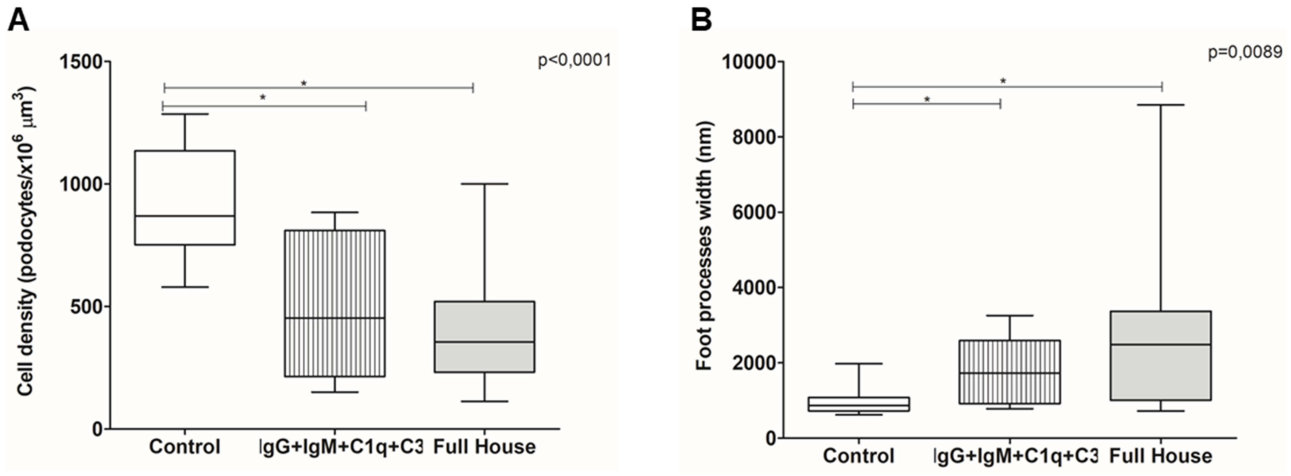

Figure 5 Podocyte damage and immunocomplex deposition. Comparison of **(A)** podocyte density and **(B)** foot process effacement between the control group and the IgG +IgM+C1q+C3 and IgA+IgG+IgM+C1q+C3 (Full-House) groups. Kruskal–Wallis test followed by Dunn's multiple comparison. Horizontal lines represent the medians, bars represent the 25–75 percentiles, and vertical lines represent the 10–90 percentiles. Nanometer (nm).

believed that cell-mediated immunity may play a significant role in podocyte injury, especially in class IV LN, where a large number of macrophages, Th1 cells, and CD40+ cells are observed.[26] As a result, podocytes respond with reorganization of the podocyte cytoskeleton, morphologically culminating in foot process effacement.

Among the classes, higher proteinuria was observed in class IV, which was the class that showed greater foot process effacement. However, we did not find a correlation between proteinuria and foot process effacement, as found in studies of LN[27,28] and in other glomerular diseases such as FSGS[12] and IgA nephropathy.[29] In a well-established model of proteinuria, foot process effacement preceded proteinúria.[30] Some studies that also did not find a relationship between foot process effacement and proteinuria suggested that proteinuria depends mainly on the nature of the underlying disease and not necessarily on foot process effacement.[15,31]

Considering that class IV presented more podocyte alterations, we made correlations between foot process effacement and the activity index and its criteria (subendothelial deposits, endocapillary hypercellularity, and leukocyte infiltration). We observed a strong, positive, and significant correlation between foot process effacement and the activity index, as well as endocapillary hypercellularity and leukocyte infiltration. We believe that the "cross-talk" between podocytes and endothelium is responsible for these correlations.[32] Thus, immune complex deposition leads to the production of mediators and growth factors that will alter the podocyte cytoskeleton, resulting in foot process effacement.[33]

Podocytes produce pro-inflammatory cytokines and chemokines that are involved in the recruitment, maturation, and activation of immune cells in the context of LN, justifying the correlation with inflammatory infiltrate. It has been demonstrated that in response to inflammatory stimuli, there is a significant increase in levels of IL-6, IL-8, VEGF, and M-CSF that podocytes express at basal levels for homeostasis maintenance, as well as new production of IP-10 and IL-10. In response to the inflammatory environment, there is an increase in intracellular calcium levels in podocytes, leading to actin cytoskeleton dysregulation resulting in effacement.[34]

We believe that during the development of LN, podocytes are targets of adaptive immunity as they can express various target antigens[25,35] and innate immunity since they express toll-like receptor during the disease development.[36] However, it is believed that podocytes also have a direct involvement as an immunological actor in the process of LN, as they can communicate with other cells of the immune system. Podocytes can act as antigen-presenting cells[37] and it has been demonstrated that the molecule B7-1 (CD80), which induces T cell co-stimulation, is expressed in podocytes in various models of proteinuric renal diseases, including LN contributing to adaptive immunity.[38]

It was observed a reduction in podocyte density and increased foot process effacement in the IgG+IgM+C1q+C3 and Full-House groups. The immunocomplexes deposited in the glomerulus promote activation of the classical complement pathway and of macrophages and neutrophils, through the interaction between the surface Fc receptors of phagocytic cells and the complex immunoglobulins. As a consequence of the activation and local recruitment of neutrophils, there is the release of reactive oxygen species (ROS), production of pro-inflammatory cytokines, and amplification of the immunoinflammatory response in renal tissue.[9] Furthermore, the production of ROS can further damage podocytes in LN, and the use of nitric oxide synthesis inhibitors has shown a protective effect against proteinuria.[39]

In summary, our study suggests that a more detailed investigation of podocytes, associated with morphological findings from renal biopsy, may be useful in relating to higher LN activity. Since patients with more foot process effacement and consequently higher levels of proteinuria present with LN class IV, the extent of foot process effacement is possibly more related to diffuse inflammatory/cellular proliferation rather than focal, as in LN class III. Most nephropathies result in podocyte damage; however, the focus of this work is to understand the role of podocytes in a nephropathy that is not a primary podocytopathy, such as LN. Therefore, understanding the pathogenesis of podocyte injury in the immune microenvironment in LN could contribute to the development of specific therapies for protecting glomerular integrity and, consequently, renal function.

Study Limitations

Among the limitations of the study, we may mention the small number of cases of LN classes I and II, as these cases generally do not receive an indication for renal biopsy, which is more common in patients with clear altered clinical-laboratory data. Consequently, the most frequently diagnosed classes are those associated with more evident renal damage, ie, classes III, IV, and V. Another factor contributing to the small number of cases included in the study was the

difficulty in obtaining clinical-laboratory data from patients, which led to the exclusion of many cases. Additionally, there is a limitation because it is a morphological study, as we have morphological data to compare with clinical data only at the time of the biopsy. Any morphological study presents this same limitation.

Conclusion

Podocytes play an important role in the development and progression of LN, especially in class IV. This class showed more foot process effacement, and this injury is related to the activity of the disease.

Ethics Approval

The present study was approved by the Ethics and Research Committee of the Federal University of Triângulo Mineiro with the number CAAE 61450322.8.0000.5154 in 26/08/2016. All kidney samples were archived, and cases were identified by codes with letters and numbers to ensure that individuals were anonymized. These biopsies were performed between 1996 and 2021 and stored in the service for further retrospective studies like this one. Because it is a retrospective study, ethics committee waived the requirement for informed consent. The guidelines outlined in the Declaration of Helsinki were followed.

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Disclosure

The authors report no conflicts of interest in this work.

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