UNIVERSIDADE FEDERAL DO TRIÂNGULO MINEIRO
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MARIA LUIZA GONÇALVES DOS REIS MONTEIRO

NEFROPATIA POR IGA PRIMÁRIA: RELAÇÃO ENTRE DADOS CLÍNICOS E PARÂMETROS MORFOLÓGICOS, PAPEL DOS SUBTIPOS DE GLOMERULOESCLEROSE SEGMENTAR E DAS ALTERAÇÕES ULTRAESTRUTURAIS PODOCITÁRIAS

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Orientadora: Prof. Dra. Marlene Antônia dos Reis

Co-orientadora: Profa. Dra. Juliana Reis Machado

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Defesa de tese e exame final de **MARIA LUIZA GONÇALVES DOS REIS MONTEIRO**, candidato (a) ao grau de Doutor (a) em Ciências – Patologia Humana.

A Comissão Examinadora comunica à Coordenadoria do PGCS/UFTM, para os devidos fins, que procedeu, nesta data, ao exame da Tese de Doutorado **"ASSOCIAÇÃO CLÍNICO-MORFOLÓGICA NA NEFROPATIA POR IgA PRIMÁRIA: RELAÇÃO ENTRE DADOS CLÍNICOS E PARÂMETROS MORFOLÓGICOS, PAPEL DOS SUBTIPOS DE GLOMERULOESCLEROSE SEGMENTAR E DAS ALTERAÇÕES ULTRAESTRUTURAIS PODOCITÁRIAS"** do Programa de Pós-Graduação em Ciências da Saúde, bem como ao exame final.

O (a) candidato (a) foi considerado (a) APROVADA.

A COMISSÃO:

Dra. Marlene Antônia dos Reis

Dr. Adriano Mota Loyola

Dr. Sérgio Vitorino Cardoso

Dra. Helenice Gobbi

Dra. Fernanda Rodrigues Helmo

Dedico esta tese
aos meus queridos pais, Carlos e Dalma
às minhas irmãs de corpo e de alma, Fabiana e Letícia
e ao meu amado companheiro e marido, Marco.

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“Cada dia que amanhece assemelha-se a uma página em branco,
na qual gravamos os nossos pensamentos, ações e atitudes.
Na essência, cada dia é a preparação de nosso próprio amanhã.”
Francisco Cândido Xavier (1910-2002)

RESUMO

Introdução: Nefropatia por IgA (NIgA) é a glomerulonefrite primária mais comum, apresenta ampla variação de manifestações clínicas e histológicas. Este estudo objetivou avaliar a sensibilidade, especificidade e acurácia dos dados clínicos no momento da biópsia na previsão dos parâmetros da classificação de Oxford e investigar a influência na apresentação clínica dos subtipos de esclerose segmentar (GESF), lesão podocitária, apagamento dos pedicelos (AP) e evidências de morte podocitária. **Material e métodos**: Estudo transversal com biópsias de 103 pacientes com NIgA, que foram submetidas a microscopia de luz, imunofluorescência, imuno-histoquímica para WT1 e microscopia eletrônica de transmissão (MET). A classificação de Oxford foi atualizada e as lesões de GESF foram subclassificadas. Foram utilizadas curvas ROC, regressão logística univariada e multivariada. Células marcadas com WT1 em alças glomerulares foram contadas como podócitos e a área glomerular foi medida para obter a densidade de podócitos (DP). O número de diafragmas de filtração (DF) foi dividido pelo comprimento (µm) da alça, resultando em densidade de DF/µm. O perímetro da alça coberto por AP ou áreas desnudas foi dividido pelo perímetro total, resultando em Índice de Apagamento (IA). **Resultados:** Na classificação de Oxford, a maioria dos pacientes apresentava M0, E0, S1, T2 e C0. A hipertensão aumenta a chance de M1 em 2,54x (p=0,02). Para cada unidade de aumento de creatinina, 2,6x mais chances de E1 (p = 0,001). S1 é previsto pela proteinúria com sensibilidade de 75% e especificidade de 90,9% (p<0,0001). Para cada unidade de aumento da TFG, há redução de 6% na chance de T2 em relação a T0 (p=0,0001). Se houver hipertensão, há 5 vezes mais chances de T2 do que T0 (p=0,01). Para cada unidade de aumento de creatinina, há 2,8 vezes mais chances de C (p=0,003). A creatinina também apresentou sensibilidade de 75,8% e especificidade de 75% para predição de C (p=0,002). Inversamente, para cada unidade de TFG, a chance de C é reduzida em 4% (p=0,007). A proteinúria foi o único parâmetro clínico com diferença significativa entre os grupos S0 e S1 (p<0,0001). Os subtipos de GESF relacionados à proteinúria foram celular (p=0,03) e peri-hilar (p=0,02). A DP foi menor nos casos com proteinúria nefrótica (p=0,03; r=-0,22). Podócitos destacados foram encontrados em todos os grupos de morte de podócitos, com proporção significativamente menor nos

casos de autofagia (p=0,03). Na maioria dos casos com podócitos destacados, houve morte de podócitos, especialmente autofagia e necrose. Nenhum podócito apoptótico foi encontrado. Os pseudocistos não se correlacionaram com o destacamento de podócitos (p=0,49). Os casos com autofagia apresentaram frequência significativamente menor de hematúria (p=0,03). A densidade de DF e o IA não se correlacionaram com dados clínicos ou morfológicos. **Conclusão:** Os parâmetros de classificação de Oxford corresponderam a alguns dados clínicos, o que abre a possibilidade de no futuro predizer-se os dados morfológicos a partir dos dados clínicos. Lesões de GESF não especificamente relacionadas a podocitopatias podem influenciar os parâmetros clínicos. A proteinúria correlacionou-se com a DP e não com o AP, o que reforça a teoria de que o apagamento é um mecanismo adaptativo. Os podócitos destacados estão relacionados à lesão podocitária e não aos pseudocistos, o que reforça a hipótese de que, na NIgA, a perda de podócitos está relacionada à lesão celular e não apenas a fatores mecânicos. Autofagia parece ser um mecanismo de proteção com menor proporção de podócitos destacados e menos casos de hematúria. Essas características podem auxiliar na avaliação da gravidade na rotina diagnóstica.

PALAVRAS CHAVE

Nefropatia por IgA. Glomeruloesclerose segmentar. Podócitos. Morte celular. Autofagia. Microscopia Eletrônica de Transmissão. Proteinúria.

ABSTRACT

Introduction: IgA nephropathy (IgAN) is the most common primary glomerulonephritis, has a broad range of histological and clinical manifestations. This study aims to assess sensitivity, specificity and accuracy of clinical data at the time of biopsy in predicting Oxford classification parameters and investigate if subtypes of segmental sclerosis (FSGS), podocyte injury, foot process effacement (FPE) and evidences of cell death influence clinical presentation. **Material and methods**: Transversal study with 103 IgAN patients. Renal samples underwent light microscopy, immunofluorescence, immunohistochemistry for WT1 and transmission electron microscopy (TEM). Oxford classification was updated and FSGS lesions were subclassified. ROC curves, univariate and multivariate logistic regression were used. WT1-labeled cells in glomerular loops were counted as podocytes and glomerular area was measured to get podocyte density (PD). The length (µm) of the loop divided the number of slit diaphragms (SD), resulting in density of SD/µm. The perimeter of the loop covered by FPE or denuded areas were divided by total perimeter, resulting in Effacement Index (EI). **Results:** In Oxford classification, the majority of patients had M0, E0, S1, T2 and C0. Hypertension increases the chance of M1 in 2.54x ($p=0.02$). For each unit of increased creatinine, 2.6x more chances of E1 ($p=0.001$). S1 is predicted by proteinuria with 75% sensitivity and 90.9% specificity ($p < 0.0001$). For each unit of increase in GFR, there is a reduction of 6% in the chance of T2 in relation to T0 ($p=0.0001$). If hypertension, there is 5x more chances of T2 than T0 ($p=0.01$). For each unit of increase in creatinine, there are 2.8x more chances of C ($p=0.003$). Creatinine also showed 75.8% sensitivity and 75% specificity for prediction of C ($p=0.002$). Inversely, for each unit of GFR, the chance of C is reduced by 4% ($p=0.007$). Proteinuria was the only clinical parameter with significative difference, between S0 and S1 groups ($p < 0.0001$). FSGS subtypes related to proteinuria were cellular ($p=0.03$) and peri-hilar ($p=0.02$). PD was lower in cases with nephrotic proteinuria ($p= 0.03$; $r=-0.22$). Detached podocytes were found in all groups of podocyte death, with significantly lower proportion in cases with autophagy ($p=0.03$). In most cases with detached podocytes, there were podocyte death, specially autophagy and necrosis. No apoptotic podocyte was found. Pseudocysts were not correlated with podocyte detachment ($p=0.49$). Cases with autophagy, had significantly lower frequency

of hematuria (p=0.03). SD density and EI did not correlate with clinical or morphological data. **Conclusion:** Oxford classification parameters corresponded to some clinical data, making it possible to predict, in the future, morphological data from clinical parameters. FSGS lesions not specifically related to podocytopathies may also influence clinical parameters. Proteinuria correlated with PD and not with FPE, which reinforces the theory that this FP change is an adaptive mechanism. Detached podocytes were related to podocyte injury and not with pseudocysts, which reinforces the hypothesis that in IgAN, podocyte loss is related to cell injury and not just mechanical factors. Autophagy seems to be a protection mechanism with a smaller proportion of detached podocytes and fewer cases with hematuria. These characteristics may add to severity assessment in diagnostic routine.

KEY WORDS

IgA Nephropathy. Segmental glomerulosclerosis. Podocytes. Cell death. Autophagy. Transmission Electron Microscopy. Proteinuria.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA- Análise de Variância

AP- Apagamento dos Pedicelos

AUROC- Area Under the Curve

BP- Blood Pressure

C- Crescentes

CAPES- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CKD-EPI- Chronic Kidney Disease Epidemiology Collaboration

CNPq- Conselho Nacional de Desenvolvimento Científico e Tecnológico

DF- Diafragmas de Filtração

DNA- Desoxirribonucleic Acid

DP- Densidade de Podócitos

E- Hipercelularidade Endocapilar

EI- Effacement Index

ER- Endoplasmic Reticulum

FAPEMIG- Fundação de Amparo à Pesquisa do Estado de Minas Gerais

FITC- Fluorescein Isothiocyanate

FSGS- Focal and Segmental Glomerulosclerosis

FPE- Foot Process Effacement

FUNEPU- Fundação de Ensino e Pesquisa de Uberaba

g/24h- gramas por 24 horas

GBM- Glomerular Basement Membrane

GESF- Glomeruloesclerose Segmentar E Global

GFR- Glomerular Filtration Rate

HE- Hematoxilina e Eosina

IA- Índice de Apagamento

IF- Immunofluorescence

IgAN- IgA Nephropathy

IIgANN/ RPS-International IgAN Network and The Renal Pathology Society

LM- Light Microscopy

M- Hipercelularidade Mesangial

MAP-Mean Arterial Pressure

MBG- Membrana Basal Glomerular

MC- Mitotic Catastrophe

MET- Microscopia Eletrônica de Transmissão

ml/min- mililitros por minuto

ml/min/1.73 m^2- mililitros por minuto por 1,73 metros quadrados

NIgA- Nefropatia por IgA

NOS- Not Otherwise Specified (Sem Características Específicas)

OR- Odds Ratio

p- p valor

PAMS- Coloração de Prata Metenamina

PBS- Phosphate Buffered Saline

PD- Podocyte Density

RAAS- Renin- Angiotensin-Aldosterone System

ROC- Receiver Operating Characteristic

S- Esclerose Segmentar

SD- Slit Diaphragms

T- Atrofia Tubular e Fibrose Intersticial

TEM- Transmission Electron Microscopy

TFG- Taxa de Filtração Glomerular

TUNEL- Terminal Deoxynucleotidyl Transferase Dutp Nick End Labeling

UFTM- Universidade Federal Do Triângulo Mineiro

UK- United Kingdown

WT1- Wilms Tumor 1

LISTA DE SÍMBOLOS

F- ANOVA

χ2- Chi-Square

H- Kruskal-Wallis test

U- Mann Whitney test

μm^3- micrômetro cúbico

rS- Spearman's test

t- Student´s t test

SUMÁRIO

1 INTRODUÇÃO

O primeiro diagnóstico da nefropatia por IgA (NIgA) foi realizado por Jean Berger em 1968, que analisou biopsias renais com a técnica de imuno-histoquímica e observou a presença de imunocomplexos de IgA e IgG na região mesangial glomerular (BERGER; HINGLAIS, 1968). A doença é caracterizada pelo depósito predominante de imunocomplexos de IgA, podendo estar ou não associados a co-depósitos (SEEDAT et al., 1988). Sua patogênese inicia-se com a produção de IgA hipoglicosilada, que o organismo reconhece como antígeno e produz anticorpos que irão se ligar a essas moléculas, com formação de imunocomplexos que se depositam no mesângio (D'AMICO, 2000). Assim, atualmente a NIgA é considerada uma doença auto-imune (WYATT, 2013).

Os depósitos de IgA podem ser encontrados no mesângio ou nas alças capilares, principalmente no espaço subendotelial (BELLUR et al., 2011), situação associada a pior prognóstico (D'AMICO, 2004). Esses depósitos são reconhecidos pelas técnicas de imunofluorescência ou imunoperoxidase. Além das moléculas de IgA, pode-se encontrar outros depósitos, como imunoglobulinas (IgG e IgM) e partículas do complemento (C3) (VALENTJIN et al., 1984). Estudos mostram que co-depósitos de IgG podem estar relacionados a pior prognóstico quando comparados a depósitos isolados de IgA (D'AMICO, 2004; FURNESS, 2001). A presença de depósitos de C3 pode estar relacionada a maior dano renal quando associada a redução dos seus níveis séricos (KIM et al., 2012).

A relevância desta doença é tal que atualmente corresponde à glomerulonefrite primária mais comum no mundo, com taxas crescentes de diagnóstico (SUZUKI et al., 2009).

Há grande variação de manifestações histológicas e clínicas, desde glomérulos morfologicamente normais até totalmente esclerosados e com manifestações clínicas que variam de hematúria isolada até o estágio de rim terminal (D'AMICO, 1985).

Existem evidências da possibilidade de predizer a progressão da doença a partir da análise morfológica do parênquima renal no momento da biopsia associado a dados clínicos como taxa de filtração glomerular, proteinúria e pressão arterial

sistêmica (BARTOSIK et al., 2001). Dessa forma, há a possibilidade de intervenções clínicas que possam reduzir os riscos de desenvolver danos renais permanentes e aumentar as chances de evitar a progressão da doença ao estágio de rim terminal (BARBOUR et al., 2016; BARTOSIK et al., 2001). Caracteristicamente, em cerca de 20 anos, mais de um terço dos pacientes atingem a doença em estágio terminal, o que contribui para um aumento da população de pacientes dependentes de diálise (ROUFOSSE, COOK, 2009).

Em 2009, a classificação de Oxford para a NIgA foi publicada com o objetivo de uniformizar a análise dos padrões morfológicos por meio da escolha de quatro parâmetros: hipercelularidade mesangial (M), hipercelularidade endocapilar (E), esclerose segmentar (S) e atrofia tubular/fibrose intersticial (T). A partir da nomenclatura em inglês, foi criado o mnemônico MEST (ROBERTS et al., 2009).

Hipercelularidade mesangial é caracterizada como a presença de quatro ou mais células por eixo mesangial, sendo que se presente em menos de 50% dos glomérulos classifica-se como M0 e em mais de 50%, M1; a hipercelularidade endocapilar é classificada como E0 se ausente e E1 se presente; a glomeruloesclerose segmentar tem sua classificação como S0 se ausente e S1 se presente; a atrofia tubular/fibrose intersticial é determinada pela porcentagem de comprometimento da região cortical do rim, sendo que T0 abrange de 0-25%, T1 de 25-50% e T2 se presente em mais de 50% da área (ROBERTS et al., 2009; KAWAMURA et al., 2013).

Em 2017, a classificação de Oxford foi atualizada após a divulgação dos resultados dos Grupos de Trabalho de crescentes e de esclerose segmentar, dois dos seis grupos resultantes de um esforço conjunto da “International IgAN Network and the Renal Pathology Society” (IIgANN/RPS) (ROBERTS; SOARES, 2018). O primeiro grupo adicionou o parâmetro C (crescentes) à classificação e o mnemônico foi modificado para MEST-C. O escore C0 representa a ausência de crescentes, C1 corresponde a crescentes em menos de 25% dos glomérulos e C2 se crescentes em mais de 25% dos glomérulos (TRIMARCHI et al., 2017).

O grupo de trabalho de esclerose segmentar recomendou que as lesões de glomeruloesclerose focal e segmentar, S1 na classificação de Oxford, com

características morfológicas tipicamente associadas a lesões de podocitopatia – lesão de ponta ou hipertrofia de podócitos - deveriam ser especificadas em relatórios de biópsia renal (TRIMARCHI et al., 2017), uma vez que tais subtipos de glomeruloesclerose segmentar podem estar relacionados a manifestações clínicas e interferir no prognóstico da doença (BELLUR et al., 2017).

Como suporte a essa recomendação, há vários trabalhos realizados após a Classificação de Oxford de 2009 que sugeriram que as diversas apresentações morfológicas de lesões de glomeruloesclerose segmentar podem estar relacionadas com diferentes etiologias (NASRI, MUBARAK, 2014; MUBARAK, NASRI, 2013; MAEDA et al., 2013; BELLUR et al., 2017). Esses achados são mais consistentes quando as lesões de glomeruloesclerose segmentar são caracterizadas por lesão de ponta (*tip lesion*) ou hipertrofia de podócitos, pois parecem estar relacionados a maior grau de proteinúria e evolução mais rápida para perda da função renal em casos de NIgA.

A glomeruloesclerose segmentar e focal pode acontecer devido a doenças glomerulares que afetam principalmente os podócitos, conhecidas como podocitopatias, bem como devido a fenômenos secundários à cicatrização de muitos tipos de lesões em outras doenças glomerulares (KIM; HAN, 2016), incluindo NIgA. Diferentes características histológicas podem refletir causas subjacentes de lesões escleróticas e há pelo menos três vias pelas quais a glomeruloesclerose segmentar pode ocorrer em NIgA: cicatrização pós-inflamatória, alterações hemodinâmicas compensatórias após redução de néfrons e finalmente dano podocitário primário, provavelmente devido a imunocomplexos de IgA1 na região mesangial (KAROUI et al., 2011).

Os subtipos de esclerose segmentar propostos pelo Grupo de Trabalho IIgANN/ RPS são: esclerose peri-hilar, hialinose, lesão esclerosante segmentar sem características específicas (NOS), adesão capsular sem esclerose, lesões colapsantes, lesão de ponta, hipertrofia podocitária, gotículas de reabsorção nos podócitos e células espumosas endocapilares (BELLUR et al., 2017). No entanto, até o momento, a classificação de Oxford não determina a subclassificação sistemática das lesões de S1, uma vez que ainda faltam estudos em outros centros

que comprovem a eficácia e significância dessa abordagem. Um dos objetivos deste estudo é contribuir para essa discussão com uma coorte brasileira.

Novos modelos para prever a progressão da doença envolvendo alguns dos parâmetros da classificação de Oxford e dados clínicos (TFG e proteinúria) foram propostos (TANAKA et al., 2013). Inclusive um dos Grupos de Trabalho IIgANN/ RPS é a modelagem probabilística, que está trabalhando para otimizar a previsão da progressão da doença a partir de dados morfológicos e clínicos no momento da biópsia (BARTOSIK et al., 2001). Além disso, tem havido extensas buscas por métodos diagnósticos não invasivos na NIgA, como a identificação de miRNA na urina de ratos, embora em fase de pesquisa (MIN et al., 2018) e há outro Grupo de Trabalho da IIgANN/ RPS que está definindo biomarcadores na NIgA (SOARES, ROBERTS, 2018).

Como anteriormente explicitado, os conceitos clássicos antigos relacionavam a patogênese da NIgA quase exclusivamente ao mesângio, mas um corpo crescente de evidências provou que o estresse do mesângio pode afetar o comportamento dos podócitos (TRIMARCHI et al., 2019).

Certamente, as lesões podocitárias também influenciam na apresentação clínica e no prognóstico (HISHIKI et al., 2001). Entre os parâmetros clínicos, a proteinúria tem sido especialmente correlacionada com dano ou perda podocitária, evidenciada por alterações na expressão de marcadores específicos de podócitos (TIAN et al., 2007; LAI et al., 2009; GAGLIARDINI et al., 2003), necrose seguida de destacamento de podócitos da membrana basal glomerular (MBG) (NG WL et al., 1984), bem como podocitopenia (HISHIKI et al., 2001; LEMLEY et al., 2002).

Os podócitos são células com estrutura complexa e uma posição especial fora do capilar glomerular, em contato íntimo com o ultrafiltrado e são fixados na MBG apenas pelos seus pedicelos, ou seja, flutuam, poeticamente, no espaço urinário (KRIZ et al., 2013). Como suas características foram filogeneticamente conservadas ao longo da seleção natural, pode-se presumir que essa estrutura é indispensável para a suas funções, incluindo o fato relevante de sua incapacidade de replicação *in situ* (KRIZ et al., 2013).

Esse conjunto de aspectos morfofuncionais tornam os podócitos constantemente expostos a diversos tipos de estresse, com risco de desprendimento (KRIZ et al., 2013). Quando desafiados, sofrem alterações estruturais como o apagamento dos pedicelos, formação de pseudocistos, acúmulo citoplasmático de vesículas (KRIZ; GRETZ; LEMLEY, 1998) entre muitos outros.

Para avaliar a arquitetura dos podócitos, as microscopias eletrônicas de varredura e transmissão (MET) ainda são os métodos padrão-ouro (INOKUCHI et al., 1993; INOKUCHI et al., 1996; KIM et al., 2001; KRIZ; GRETZ; LEMLEY, 1998; NAGATA; KRIZ, 1992). De fato, o conhecimento atual e a compreensão da dinâmica dos processos de lesão e desprendimento de podócitos são amplamente baseados na MET (KRIZ; GRETZ; LEMLEY, 1998; KRIZ; LEHIR, 2005; KRIZ et al., 2013).

A relação entre proteinúria nefrótica e alterações ultraestruturais glomerulares ainda não está totalmente elucidada na NIgA. No entanto, existem casos com síndrome nefrótica como única manifestação da doença, com glomérulos pouco alterados à microscopia de luz, mas com apagamento difuso dos pedicelos na MET, demonstrando a relevância da análise ultraestrutural em casos de NIgA. (TEWARI et al., 2015).

Já foi relatado destacamento de podócitos da MBG em pacientes com NIgA (ROHIT et al., 2015). Fatores mecânicos parecem ter papel fundamental no destacamento de podócitos (BURFORD et al., 2017), pois para a função de filtração glomerular, é necessária uma pressão transcapilar, no entanto tal pressão pode tornar-se estresse para as células epiteliais se aumentada acima dos níveis fisiológicos (NAGATA, 2016). Além disso, áreas circunscritas de membrana basal glomerular desnuda, sem pedicelos, podem originar fluxos descontrolados de ultrafiltrado, iniciando a formação de pseudocistos (KRIZ; LEMLEY, 2017) O aumento do fluxo local de ultrafiltrado nas áreas de pseudocistos está associado a um alto estresse de cisalhamento e talvez essa alteração mecânica possa ser crucial para o destacamento de podócitos (BURFORD et al., 2017).

Além disso, a morte celular de podócitos por anoiquia, autofagia ou catástrofe mitótica (CM) são possíveis causas de destacamento de podócitos *in vivo* (HARTLEBEN; WANNER; HUBER, 2014; LIAPIS et al., 2013). De fato, apagamento

de pedicelos é frequentemente associado a podócitos binucleados *in vivo*, (NAGATA et al., 1995; KRIZ et al., 1995) e esse tipo de lesão celular é frequentemente visto em podócitos na urina, portanto o destacamento pode ser causado por propriedades intrínsecas desconhecidas em resposta à lesão (LIAPIS et al., 2013). Assim, atualmente entende-se que o destacamento de podócitos pode ser causado por respostas celulares biológicas devido a lesões e também por forças mecânicas quando o ultrafiltrado através da MBG tende a elevar o corpo celular (NAGATA, 2016). O conhecimento desses mecanismos de destacamento é relevante, pois se correlaciona com a progressão patológica da doença em humanos e animais experimentais (FUKUDA et al., 2012; HARA; YANAGIHARA; KIHARA, 2007), sugerindo que está subjacente à podocitopenia e à progressão para glomerulosclerose.

Uma das maneiras de se quantificar a podocitopenia é a contagem do número de podócitos *in situ* com marcador podocitário na imuno-histoquímica (VENKATAREDDY et al., 2014), como o WT1. Este é um fator de transcrição de expressão nuclear encontrado nos podócitos maduros e que regula a expressão de podocina (PALMER et al., 2001) e nefrina (GUO et al., 2004).

O entendimento moderno defende que o apagamento dos pedicelos é uma mudança adaptativa final devido a desafios mecânicos que levam à expansão da MBG (ISAO, 2002). A primeira adaptação seria a substituição dos diafragmas de filtração por estruturas mais próximas, denominada junções ocludentes (KRIZ, 2013). O diafragma de filtração é a principal barreira que limita a proteinúria (NAGATA, 2016). As junções ocludentes retrairiam os pedicelos e promoveriam a ligação do corpo celular à MBG (KRIZ et al., 2013). Essa resposta do fechamento das fendas parece ser a mais crítica para a preservação dos podócitos, pois acaba levando ao apagamento dos pedicelos, o que implica melhor adesão à MBG, impedindo a podocitopenia. Caso contrário, o podócito continuaria sujeito a tensões de cisalhamento e finalmente se desprenderia (KRIZ; LEMLEY, 2015). Os estágios iniciais do apagamento dos pedicelos são reversíveis, mas eventualmente atingem um ponto de não retorno a partir do qual essa adaptação participa de outro mecanismo de proteção, a fim de manter a lesão em um único local do glomérulo,

gerando uma cicatriz segmentar conhecida como glomerulosclerose (KRIZ; LEMLEY, 2017).

Além da avaliação das lesões podocitárias e do apagamento dos pedicelos, a análise morfológica das evidências de morte celular é essencial para identificar quais mecanismos de morte contribuem para a perda de podócitos e suas consequências clínicas. Os mecanismos de morte celular já descritos nos podócitos incluem apoptose, anoiquia, autofagia, entose, necrose, necroptose e CM (LIAPIS et al., 2013), sendo a MET o padrão-ouro para identificá-los, principalmente por características patológicas nucleares (KRIZ et al., 2013). Destacam-se os mecanismos de necrose, autofagia, CM e apoptose.

As características morfológicas da necrose incluem oncose, caracterizada por edema com aumento do volume celular, ruptura da membrana citoplasmática e perda da eletrondensidade no citoplasma; com lise nuclear (LIAPIS et al., 2013). Já a autofagia é caracterizada pela formação de autofagossomos, vacúolos com membrana dupla que sequestram organelas grandes como mitocôndrias e ribossomos (KURZ; TERMAN; BRUNK, 2017; KLIONSKY; SCOTT, 2000). A autofagia tem um papel aparentemente paradoxal na morte celular (KROEMER; LEVINE, 2008; SCARLATTI et al., 2009), pois é um processo citoprotetor, mas também está associado à morte celular através de suas relações com apoptose e necrose (COLLEL et al., 2007; EISENBERG-LERNER et al., 2009; LOOS; ENGELBRECHT, 2009).

Células binucleadas ou podócitos com micronúcleos indicam aneuploidia, implicando a CM como causa de perda de podócitos (LIAPIS et al., 2013). Essa perda ocorreria por destacamento devido ao aumento de volume da estrutura celular e alterações complexas (SWANSON et al., 1995), o que a torna mais suscetível a qualquer estresse mecânico (MARSHALL; SHANKLAND, 2007). A binucleação ocorre quando um estímulo lesivo suplanta o bloqueio natural do ciclo celular de podócitos (MARSHALL; SHANKLAND, 2007) e a célula inicia a divisão (LASAGNI et al., 2013). Devido ao seu complexo citoesqueleto, os podócitos não conseguem concluir a citocinese e sofrerão mitose abortiva ou resultarão em células binucleadas (KRIZ et al., 2013).

Dados morfológicos que sugerem morte celular apoptótica seriam remanescentes nucleares de cromatina fragmentada ou condensada, e raramente são vistos em podócitos (KRIZ et al., 2013). Embora ensaios enzimáticos (caspase) e bioquímicos de fragmentação do DNA (ensaio TUNEL) sejam frequentemente usados para demonstrar evidências de apoptose, eles definitivamente não separam a apoptose de outros tipos de morte celular (LIAPIS et al., 2013) e a MET permanece o método padrão ouro para demonstrar apoptose (KRIZ, 2013).

Assim, um dos nossos objetivos está alinhado com os esforços dos Grupos de Trabalho IIgANN/ RPS, uma vez que procuramos avaliar a possibilidade de prever parâmetros de classificação de Oxford a partir de dados clínico-laboratoriais e investigar se diferentes subtipos de glomeruloesclerose segmentar podem influenciar a apresentação clínica em uma coorte brasileira.

Outro objetivo deste estudo é avaliar, em pacientes com NIgA, a associação entre anormalidades quantitativas e qualitativas de podócitos, reconhecíveis morfologicamente pela MET, incluindo características de morte celular; densidade de podócitos reconhecível por marcação imuno-histoquímica por WT1; e parâmetros clínicos.

2 JUSTIFICATIVA

A NIgA é a glomerulopatia primária crônica mais comum no mundo. Por ser uma doença muito heterogênea, tanto clínica quanto morfologicamente, com risco variável de perda da função renal, torna-se um desafio a busca por fatores que possam predizer a evolução clínica, sendo que atualmente não há uma ferramenta estabelecida (REICH et al., 2007; TANAKA et al., 2013; LE W et al., 2012; BARBOUR; REICH, 2012).

A histopatologia é uma das ferramentas importantes para auxiliar na estratificação clínica dos pacientes e monitorar as terapêuticas empregadas. No entanto, tem havido controvérsias se os dados histopatológicos seriam superiores aos dados clínicos na estratificação de risco da NIgA (HAAS; REICH, 2012).

Acreditamos que a associação entre os parâmetros histológicos da classificação de Oxford com os dados clínicos no momento da biópsia pode melhorar a acurácia da estratificação de risco dos pacientes, favorecendo um manejo terapêutico mais precoce e eficiente.

Assim, diante da atualização da Classificação de Oxford em 2017, propomos avaliar se as diferentes apresentações morfológicas de glomeruloesclerose segmentar no contexto da NIgA tem relação com a apresentação clínica no momento da biópsia. Esses resultados, poderão contribuir para criar novo entendimento acerca do escore S da classificação de Oxford e seu papel nas manifestações clínicas e definição de prognóstico.

Além disso, a lesão podocitária, que ocasionalmente culmina com a perda de podócitos e consequente podocitopenia, pode gerar proteinúria. Sabe-se que proteinúria está relacionada com evolução para doença renal terminal (CHENG; HARRIS, 2010). Sendo assim, é importante conhecer os mecanismos de lesão e destacamento podocitário na NIgA, especialmente os tipos de morte celular envolvidos na podocitopenia (LIAPIS et al., 2013; TEWARI et al., 2015).

3 OBJETIVOS

OBJETIVOS GERAIS

Avaliar, em uma série de casos de NIgA, a possibilidade de prever parâmetros da classificação de Oxford a partir de dados clínico-laboratoriais; investigar se diferentes subtipos de esclerose segmentar podem influenciar a apresentação clínica; e se há correlação dos parâmetros clínicos com anormalidades quantitativas e qualitativas de podócitos na MET, incluindo evidências de morte celular e com a densidade dos podócitos na imunomarcação por WT1.

OBJETIVOS ESPECÍFICOS

1) Caracterizar clínico-epidemiologicamente os pacientes do Serviço de Nefropatologia da Universidade Federal do Triângulo Mineiro com diagnóstico de Nefropatia por IgA primária entre os anos de 2010 a 2016

2) Realizar a análise histopatológica das biópsias renais na microscopia de luz conforme a Classificação de Oxford atualizada em 2017

3) Subclassificar as lesões de glomeruloesclerose segmentar

4) Investigar possível correlação entre os dados clínicos no momento da biópsia e a presença e o subtipo de glomeruloesclerose segmentar, em busca de diferentes padrões de apresentação clínica

5) Descrever alterações ultraestruturais no citoplasma e núcleo de podócitos, que possam refletir lesão podocitária e, em última instância, morte celular

6) Quantificar o número de diafragmas de filtração/ fendas de filtração (diafragmas *slit*) e a aferição do comprimento da MBG para calcular a densidade de fendas de filtração na MET e o índice de apagamento

7) Quantificar o número de podócitos e a área do tufo capilar glomerular nos casos imunomarcados por WT1 para calcular a densidade de podócitos

8) Associar as alterações morfológicas ultraestruturais com os dados clínicos e demais dados morfológicos obtidos na microscopia de luz e na imuno-histoquímica

4 Artigo 1

Título: Is it possible to predict parameters of the Oxford classification of primary IgA nephropathy from clinical laboratory data? Focus on the role of segmental glomerulosclerosis subtypes

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Is it possible to predict parameters of the Oxford classification of primary IgA Nephropathy from clinical laboratory data? Focus on the role of segmental glomerulosclerosis subtypes

Maria Luiza Gonçalves dos Reis Monteiro [a, 1] ✉, Matheus Rodrigues Vieira [a, 1] ✉, Lívia Helena Morais Pereira [a] ✉, Liliane Silvano Araújo [a] ✉, Crislaine Aparecida Silva [a] ✉, Lúcio Borges Araújo [b] ✉, Laura Penna Rocha [a] ✉, Marlene Antônia dos Reis [a] ✉, Juliana Reis Machado [a] ✉

[a] Federal University of Triângulo Mineiro, Praça Manoel Terra, 330, 38015-050, Uberaba, MG, Brazil

[b] Federal University of Uberlândia, Av. João Naves de Ávila, 120, Santa Mônica, Uberlândia, MG, 38408-100, Brazil

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Is it possible to predict parameters of the Oxford classification of primary IgA Nephropathy from clinical laboratory data? Focus on the role of segmental glomerulosclerosis subtypes

Maria Luiza Gonçalves dos Reis Monteiro[a,1], Matheus Rodrigues Vieira[a,1], Lívia Helena Morais Pereira[a], Liliane Silvano Araújo[a], Crislaine Aparecida Silva[a], Lúcio Borges Araújo[b], Laura Penna Rocha[a], Marlene Antônia dos Reis[a], Juliana Reis Machado[a,*]

[a] Federal University of Triângulo Mineiro, Praça Manoel Terra, 330, 38015-050, Uberaba, MG, Brazil
[b] Federal University of Uberlândia, Av. João Naves de Ávila, 120, Santa Mônica, Uberlândia, MG, 38408-100, Brazil

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ABSTRACT

Introduction: IgA nephropathy (IgAN) is the most common primary glomerulonephritis in the world and has a broad range of histological and clinical manifestations, ranging from morphologically normal to globally sclerotic glomeruli with clinical manifestations varying from isolated hematuria to end stage renal disease. This study aims to assess sensitivity, specificity and accuracy of clinical data at the time of biopsy in predicting 2017 updated Oxford classification parameters and to investigate if subtypes of segmental sclerosis (FSGS) influence clinical presentation.

Material and methods: Renal biopsies from 103 patients with IgAN were analyzed. Oxford classification was updated and FSGS lesions were subclassified. ROC curves, univariate and multivariate logistic regression were used.

Results: In Oxford classification, the majority of patients had mesangial hypercellularity in less than a half of glomeruli (M0), did not have endocapillary hypercellularity (E0), had segmental glomerulosclerosis (S1), had interstitial fibrosis and tubular atrophy in more than a half of the sample (T2) and had no crescents (C0). Hypertension increases the chance of M1 in 2.54x ($p = 0.02$). For each unit of increased creatinine, 2.6x more chances of E1 ($p = 0.001$). S1 is predicted by proteinuria with 75% sensitivity and 90.9% specificity ($p < 0.0001$). For each unit of increase in GFR, there is a reduction of 6% in the chance of T2 in relation to T0 ($p = 0.0001$). If hypertension, there is 5x more chances of T2 than T0 ($p = 0.01$). For each unit of increase in creatinine, there are 2.8x more chances of crescents- C ($p = 0.003$). Creatinine also showed 75.8% sensitivity and 75% specificity for prediction of C ($p = 0.002$). Inversely, for each unit of GFR, the chance of C is reduced by 4% ($p = 0.007$). Other clinical data related with C are hypertension ($p = 0.03$) and proteinuria ($p = 0.02$). To determine the role of FSGS subtypes in clinical presentation, we divided patients in S0 and S1 groups. Proteinuria was the only clinical parameter with significative difference, respectively, 0.3 (0–2.1) and 1.6 (0.02–16.2) g/24 h ($p < 0.0001$). FSGS subtypes related to proteinuria were cellular ($p = 0.03$) and peri-hilar ($p = 0.02$). Subtypes classically related to podocytopathies showed no correlation with clinical data.

Conclusion: In the future, with noninvasive methods for diagnosis of IgAN, it will be essential to predict Oxford classification parameters using clinical laboratory data for establishment of prognosis and therapeutics. We showed that Oxford classification parameters correspond to some clinical laboratory data, making this approach possible. FSGS lesions not specifically related to podocytopathies may also influence clinical parameters that affect renal disease progression.

* Corresponding author.
E-mail addresses: marialuizapatologia@gmail.com (M.L.G.d.R. Monteiro), matheusmed77@gmail.com (M.R. Vieira), liviahmp@hotmail.com (L.H.M. Pereira), lili_silvano@yahoo.com.br (L.S. Araújo), crislaine.0604@gmail.com (C.A. Silva), lucio.araujo@ufu.br (L.B. Araújo), laurapenna1@hotmail.com (L.P. Rocha), mareispatologia@gmail.com (M.A.d. Reis), julianareismachado@hotmail.com (J.R. Machado).
[1] These authors contributed equally to this work.

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1. Introduction

Jean Berger performed the first diagnosis of IgA nephropathy in 1968 [1]. This disease is characterized by the predominant deposition of IgA immunocomplexes, which may or may not be associated with co-deposits [2]. Pathogenesis is related to abnormal hypoglycosylated IgA recognized by the immune system as an antigen, with production of antibodies that will bind to these molecules and form immunocomplexes which deposit in mesangium [3].

The relevance of this disease is such that it corresponds to the most common primary glomerulonephritis in the world, with increasing rates of diagnosis [4]. Its complexity is also significant, as IgA nephropathy has a broad range of histological and clinical manifestations, ranging from morphologically normal to global sclerotic glomeruli with clinical manifestations varying from isolated hematuria to end stage renal disease [5].

In order to standardize analysis of morphological patterns, in 2009, Oxford classification for IgA nephropathy was published with the following parameters: mesangial hypercelularity (M), endocapillary hypercelularity (E), segmental glomerulosclerosis (S) and tubular atrophy/ interstitial fibrosis (T), resulting in the mnemonic MEST [6].

In 2017, an update of this classification was published, after disclosure of results from the Working Groups of crescents and of segmental sclerosis, two of the six groups resultants of a joint endeavor of International IgAN Network and the Renal Pathology Society (IIgANN/RPS) [7]. The former group added the C parameter to the classification, transforming the mnemonic into MEST-C (Fig. 1). The latter group recommended that lesions of FSGS (focal and segmental glomerulosclerosis), S1 in Oxford classification, with morphological features typically associated with podocitopathies- tip lesions or podocyte hypertrophy- should be specified in renal biopsy reports [8], as certain types of FSGS subtypes may be related to clinical manifestations and interfere with disease prognosis [9].

FSGS is a term used to describe not only a glomerular disease which primarily affects podocytes, known as podocitopathies, but secondary phenomena of scarring due to many types of injury in other glomerular diseases [10], including IgAN. Different histologic features may reflect underlying causes of sclerotic lesions and it could be hypothesized at least three pathways by which segmental glomerulosclerosis may occur in IgAN: post-inflammatory scarring, compensatory hemodynamic changes following nephron reduction and finally primary podocyte damage, probably due to immunocomplexes of IgA1 or mediators from mesangial region [11].

Segmental sclerosis subtypes proposed by the IIgANN/RPS Working Group are perihilar sclerosis, hyalinosis, segmental sclerosing lesion with no specific features (NOS), capsular adhesion without sclerosis, collapsing lesions, tip lesion, podocyte hypertrophy, resorption droplets within podocytes, and endocapillary foam cells [9]. Nevertheless, to date, Oxford classification still does not determine the systematic subclassification of S1 lesions, as there is still a lack of studies in other centers that prove the effectiveness and significance of such approach. One of the goals of this study is to contribute to this discussion with a Brazilian cohort.

Therefore, in line with the efforts of IIgANN/RPS Working Groups, we set out to evaluate the possibility of predicting Oxford classification parameters from clinical-laboratory data and to investigate whether different subtypes of segmental sclerosis can influence clinical presentation in a Brazilian cohort of patients.

2. Material and methods

2.1. Study design

Patients included in the study underwent renal biopsy and the material was analyzed in the Department of Nephropathology of the Federal University of Triangulo Mineiro (UFTM) from 2010 to 2016. All patients signed the Free and Informed Consent Form and UFTM Ethics Committee approved the research with the protocol number 46369815.0.0000.5154. Patients were from both genders, adolescents (person aged 10 to 19 years, inclusive) and adults, with diagnosis of IgAN based in a representative sample (minimum of 8 glomeruli) for light microscopy (LM- Fig. 1A) and material for immunofluorescence (IF- Fig. 1B) and electron microscopy (TEM) analysis. Pathologic diagnosis of IgAN was based on finding of IgA-dominant mesangial or mesangial-capillary immune deposits through immunofluorescence (IF) microscopy.

Exclusion criteria were absence of IF or TEM sample, non-representative biopsy samples, biopsies from transplanted kidneys, incomplete clinical data, cases of secondary IgA deposition, inconclusive diagnoses and presence of concomitant diseases.

Slides were reviewed by a single nephropathologist, blinded to clinical data, who updated Oxford classification and subclassified segmental sclerosing lesions, ensuring consistency of the results.

2.2. Kidney biopsy evaluation

Each renal biopsy was prepared for light microscopy by cutting paraffin blocks into 3 μm sections and staining 2 slides for hematoxylin and eosin (HE), 2 slides for periodic acid silver methenamine stain (PAMS), 2 slides for Sirius Red and 2 slides for Masson´s trichrome. Each slide contained three sections. Materials used for IF were frozen in liquid nitrogen, sectioned with 6 μm thickness and marked with fluorescein isothiocyanate (FITC)-conjugated antibodies specific for human IgG, IgM, IgA, C1q, C3, Kappa, Lambda and fibrinogen. Tissue for TEM was saved for future study. TEM analysis was not performed in this study.

In order to determine the role of segmental glomerulosclerosis subtypes in clinical presentation at the time of biopsy, we first divided patients into two groups according to the presence (S1) or not (S0) of segmental sclerosis.

The method chosen for subclassification of FSGS lesions was the same as that used by Segmental Sclerosis IIgANN/RPS Working Group [10], which includes but is not restricted to Columbia's classification. If more than one subtype was found in a single glomerulus, all lesions presented were recorded. Podocyte hypertrophy was considered if present anywhere in a glomerulus with segmental sclerosis.

2.3. Definitions of clinical parameters

Hypertension was characterized by systolic blood pressure (BP) greater than 140 mmHg and diastolic BP greater than 90 mmHg. Hematuria was characterized if there were more than ten thousand red blood cells per field or presence of at least 1+ of red blood cells in urinalysis. Glomerular filtration rate (GFR) was calculated by CKD-EPI formula.

2.4. Statistical analysis

A Microsoft Excel table was prepared with clinical, laboratorial and epidemiological data from biopsy reports and morphological characteristics from renal fragments. Descriptive statistics were presented as median, 25 and 75 percentiles, mean and standard deviation. Statistical analysis was performed in programs GraphPad Prism (version 7.0) and Bioestat (version 5.0). Kolmogorov-Smirnov test was used to evaluate data normality. In cases of normal distribution and similar variances, ANOVA (F) parametric test followed by Tukey post-test and Student´s *t*-test (t) were used. In cases with non-normal distribution, Kruskal-Wallis test (H) followed by Dunn post-test and Mann Whitney test (U) were used. In contingency tables analysis, Fisher's exact-test was used ($\chi 2$). ROC curves, univariate and multivariate regressions were performed to define sensitivity, specificity and accuracy of each clinical parameter in predicting histological aspects that reflect prognosis. A significance of

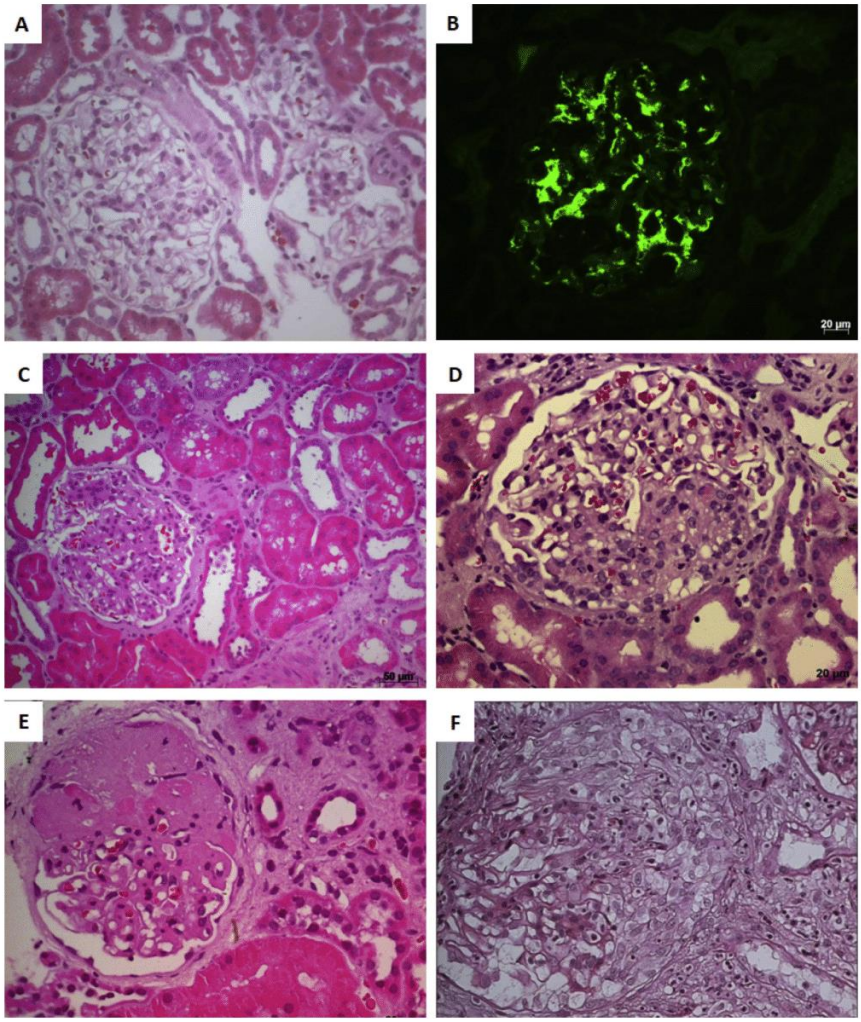

Fig. 1. Microscopic aspects of glomeruli related to IgAN diagnosis and Oxford classification. A) Normal glomerulus- light microscopy (HE) 20× 2 obj.; (B) IF IgA 40× 1 obj.; (C) Mesangial hypercellularity- M (HE) 20× 1,25 obj.; (D) Endocapillary hypercellularity-E (HE) 40× 1 obj.; (E) Segmental sclerosis (FSGS)-S (HE) 40 × 1,25 obj.; (F) Crescent- C (PS) 40× 1,25 obj.

($p < 0.05$) was adopted for all tests.

3. Results

Two hundred seventy two cases were analyzed between 2010 and 2016.

At first, exclusion criteria were absence of material for immunofluorescence analysis or light microscopy (n = 23); incomplete clinical data (n = 37) and transplanted patients (n = 9). Then cases with inconclusive diagnosis- focal, segmental or not predominant IgA deposits in IF analysis, which were suspicious but did not allowed a definite diagnosis of IgA nephropathy (n = 52) and secondary IgA nephropathy (n = 6) were excluded. The presence of concomitant diseases such as diabetes, overlapping glomerulopathies, neoplasia, among others (n = 9); and non-representative sample, with less than eight glomeruli (n = 33) were also exclusion criteria. After exclusions, a group of 103 patients was obtained (Flowchart 1 below).

3.1. Clinical-epidemiological aspects

Most patients were Caucasians (65.02%), male (64.08%) and the mean age at the time of biopsy was 38.81 ± 12.20 years (25th-75th percentiles were 31 and 48.75 years, with six patients between 11 and 18 years old).

Most patients presented hematuria (82.52%). The median proteinuria was 1.5 g/24 h (25th-75th percentiles were 0.72 and 2.82 g/24 h), with 63.11% presenting severe proteinuria (greater than 1 g/ 24 h) and 17.47% presented nephrotic levels of proteinuria. Glomerular filtration rate (GFR) had an average of 78.28 ± 37.73 mL/min/1.73 m^2 (25th-75th percentiles were 40.43 and 95.33 mL/min/1.73 m^2). Hypertension was present in49.51%of patients.

3.2. Morphological aspects and its relation with clinical presentation

According to Oxford classification, 48.42% of the patients were M1 (Fig. 1C); 35.78% E1 (Fig. 1D); 74.75% S1 (Fig. 1E); 34.73% T1 or T2, 8.42% C1 or C2 (Fig. 1F).

In order to investigate the possibility of predicting Oxford

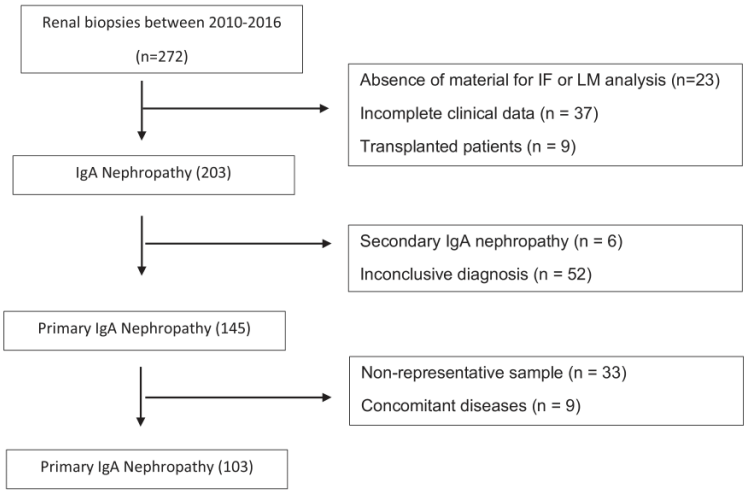

Chart 1. Exclusion factors flowchart.

classification parameters from clinical-laboratory data we performed several tests that showed each morphological parameter was related with at least one clinical laboratorial aspect.

Regarding hypercelularity parameters, M1 was associated with hypertension in univariate analysis, as hypertension increases the chance of having M1 in 2.54 times ($p = 0.024$; OR: 2.54). On the other hand, E1 was better associated with creatinine, as for each unit of increase in creatinine, there are 2.60 times more chances to have E1 ($p = 0.0018$; OR: 2.60).

As expected, S1 could be safely predicted by proteinuria, with good sensitivity (75%) and specificity (90.9%) by ROC curve with area under the curve (AUROC) of 0.86 and cutoff value of 0.61 g/day ($p < 0.0001$) (Fig. 2).

The three-tiered T parameter correlated with GFR in multivariate analysis, as for each unit of increase in GFR, there is a reduction of 6% in the chance of belonging to T2 group in relation to T0 ($p = 0.0001$, OR: 0.94). Besides, for each unit of increase in creatinine, it is 14.12 times more likely to belong to T2 than T0. T parameter is also associated with hypertension, as there is 5.08 times more chances to belong to T2 than to T0 ($p = 0.019$; OR: 5.08) if the patient is hypertensive.

Crescents were also related to several parameters, but those that stand out most are associated to renal function: for each unit of increase in creatinine, there are 2.89 times more chances to have crescents ($p = 0.0031$; OR: 2.89). Creatinine also showed good sensitivity (75.8%) and specificity (75%) for prediction of parameter C by ROC curve with AUROC of 0.7752 and cutoff value of 1.7 mg/dl ($p = 0.002$) (Fig. 3). On the other hand, GFR had an inverse relation, as for each unit of GFR, the chance of crescents is reduced by 4% ($p = 0.0079$; OR: 0.94). Other clinical data related with crescents include hypertension as if it is present, there is 9.62 times greater chance of having crescents ($p = 0.033$; OR: 9.62); and proteinuria as for each unit of increase in proteinuria, there are 1.24 more chances of having crescents ($p = 0.027$; OR: 1.24).

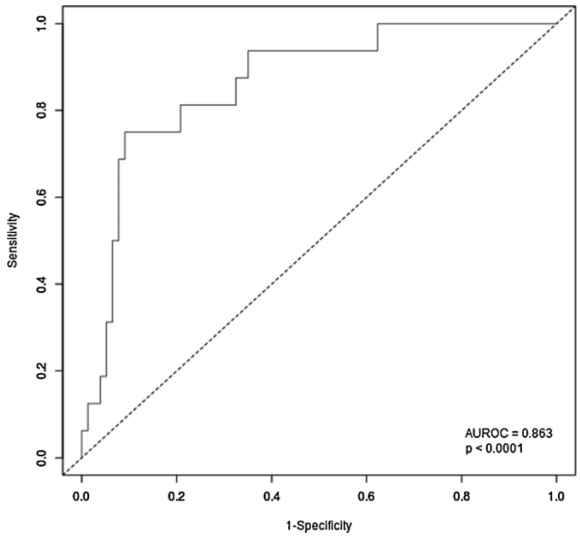

Fig. 2. Receiver operating characteristic (ROC) curve of the sensitivity plotted against 1- specificity of proteinuria for diagnosis of presence of segmental sclerosis - S1.

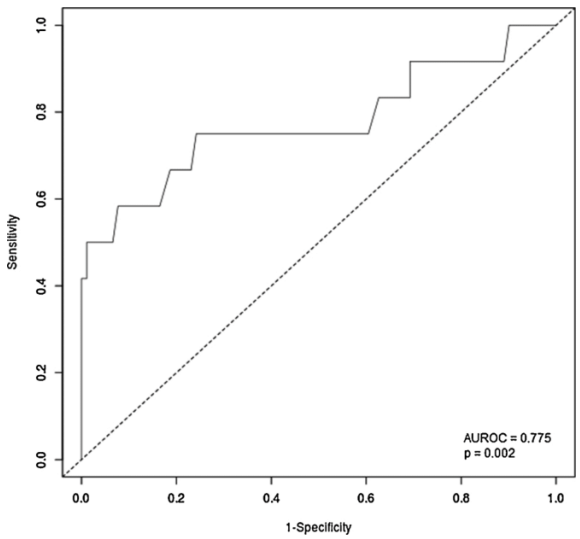

Fig. 3. Receiver operating characteristic (ROC) curve of the sensitivity plotted against 1- specificity of creatinine for diagnosis of presence of crescents.

Table 1
Clinical-epidemiological and morphological characteristics of the cases studied in the time of biopsy divided by S parameter.

	S1 (n = 77)	S0 (n = 26)	p value
Age (years)	37 (15-66)	47 (18-72)	0.1230
Females	27 (35.06%)	8 (50%)	0.2728
Race (black, brown, white)	6; 21; 50	0; 5;11	0.5089
Proteinuria	1.62 (0.02-16.2)	0.388 (0-2.1)	< **0.0001**
Arterial hypertension (present)	44 (57.14%)	7 (43.75%)	0.4111
Hematuria (present)	61 (79.22%)	15 (93.75%)	0.2880
GFR (ml/min/1.73 m^2)	63.1 (17.3-136.4)	65.05 (8.6-154.2)	0.8307
M1	42 (54.54%)	4 (25%)	**0.0523**
E1	32 (41.55%)	2 (125 %)	**0.0435**
T1 or T2	31 (40.25%)	2 (12.5%)	**0.0445**
C1 or C2	7 (9.09%)	1 (6.25%)	> 0.9999

3.3. The role of FSGS subtypes in clinical presentation

Both S0 and S1 groups were matched for clinical-epidemiological data, as there was no statistically significant difference between groups regarding age, sex, ethnicity (there was a predominance of non-blacks in both groups, representing 100% of group S0 and 92.20% of group S1, p = 0.5089), systemic arterial hypertension, hematuria and glomerular filtration rate (Table 1).

Proteinuria was the only clinical parameter with significative difference between groups, as the median proteinuria in S1 group was 1.62 (25th–75th percentiles were 0.89 and 2.86) g/24 h and in S0 the median was 0.388 (25th–75th percentiles were 0.15 and 0.66) g/24 h (p < 0.0001) (Table 1).

In contrast, most morphological characteristics were distinctive among groups (Table 1): the median percentage of glomeruli with global sclerosis (p = 0.0034), the percentage of patients classified as M1 (p = 0.0523), as E1 (p = 0.0435) and as T1 or T2 (p = 0.0445). In contrast, there was no significant difference in the percentage of patients classified as C1 or C2 (p > 0.9999).

We further divided S1 patients in morphological subtypes (Table 2) and FSGS not otherwise specified was the most prevalent, with 59.74% of cases. Next comes adhesion without sclerosis (Fig. 4A), that was found in 37.66% of cases; tip lesion (Fig. 4B) in 16.88% of cases; perihilar sclerosis in 10.38% of cases; hyalinosis in 22.07% of cases; podocyte hypertrophy in 27.27% of cases; FSGS cellular variant (Fig. 4C) in 9.09% of cases and podocyte reabsorption droplets (Fig. 4D) in 7.8% of cases. Endocapillary foam cells in sclerosis were found in 1.3% of cases and there were no collapsing lesions.

Finally, we correlated each recognized subtype with clinical data of hypertension, hematuria, GFR and proteinuria, however, statistically significant difference was found only between proteinuria and two FSGS subtypes: cellular (p = 0.0398) and peri-hilar (p = 0.0258). Surprisingly, subtypes classically related to podocytopathies showed no correlation with any clinical data alone in our cohort, nor with proteinuria: tip lesion (p = 0.6202) and podocyte hypertrophy (p = 0.5317).

Table 2
Frequency of FSGS subtypes.

FSGS subtypes	n (%)
Not otherwise specified	46 (59.74%)
Adhesion without sclerosis	29 (37.66%)
Podocyte hypertrophy	21 (27.27%)
Hialinosis	17 (22.07%)
Tip lesion	13 (16.88%)
Perihilar sclerosis	8 (10.38%)
Cellular variant	7 (9.09%)
Podocyte reabsorption droplets	6 (7.8%)
Foam cells	1(1.3%)
Collapsing	0

4. Discussion

We used clinical data at the time of renal biopsy to predict parameters of the Oxford Classification. New models for predicting disease progression involving some of the parameters of the Oxford classification and clinical data (GFR and proteinuria) have been proposed [12]. One of IIgANN/RPS Working Groups is the probabilistic modeling, which is working to optimize prediction of disease progression from morphological and clinical data at the time of biopsy [13]. This is particularly important as end stage kidney disease occurs in about a third of patients after 20 years of disease [14].

Beyond that, there have been extensive searches for non-invasive diagnostic methods of IgA nephropathy, such as the identification of miRNA in urine of rats, although in research phase [15]. In addition, another IIgANN/RPS Working Group is defining biomarkers in IgAN. Two recently described biomarkers involved in IgAN pathogenesis are FcαRI (CD68), present in myeloid cell line and involved in IgA immunocomplexes formation and transferrin receptor (CD71), present in mesangial cells, involved in immunocomplexes deposition [16].

In our study, through statistical analysis of univariate and multivariate logistic regression associated with ROC curve, we established a relationship between morphological changes in renal biopsy and different clinical manifestations.

In univariate analysis, we found a significant relationship between mesangial hypercellularity (M) and hypertension. Thus, the presence of hypertension increases the probability of the patient to belong to M1 group. On the other hand, a prognostic study of renal function found no significant difference in mean arterial pressure (MAP) values between groups M0 and M1 [17]. Hypertension is related to increased intravascular volume and inadequate control of renin-angiotensin-aldosterone system (RAAS) [18]. Therefore, increased levels of circulating angiotensin will bind to receptors in mesangial cells, leading to cell proliferation [19], which may explain the possible relationship between mesangial hypercelularity and hypertension.

Creatinine values presented a direct and strong relationship with endocapillary hypercellularity, associated with a greater chance of belonging to E1 group. This relationship is probably related to obliteration of glomerular capillaries in cases of endocapillary hypercelularity, which disturbs blood flow and reflects in loss of renal filtration function, with increased serum creatinine levels.

Although in Oxford cohort itself and other similar studies this relation between E1 and renal dysfunction was not find and E1 have only been correlated with response to immunosuppression, recent studies in adults and children have indeed shown worse prognosis in the presence of endocapillary hypercelularity in IgAN due to the association of E1 with renal function.

In a study conducted in the UK with 147 adults without immunosuppression, E1 was one of the predictors of evolution for ESRD [20]. A Brazilian study with 54 children followed for 90 ± 60 months showed that variables associated with renal survival at the time of first renal biopsy were only proteinuria and endocapillary hypercelularity [21].

In our study, proteinuria showed good specificity (90.9%) and sensitivity (75%) to predict parameter S1. Other studies also found a positive relationship between S1 parameter and levels of proteinuria [22]. Segmental glomerulosclerosis represents an irreversible secondary lesion in glomeruli. This change leads to damage of the filtration barrier, triggering proteinuria, which is one of the clinical data that best correlate to renal prognosis, and interferes in treatment decisions [17]. Thus, proteinuria is a good indicator for the presence of S1. Proteinuria was present in most cases in our study (85.43%), maybe due to biopsy indications that include the presence of persistent proteinuria in more

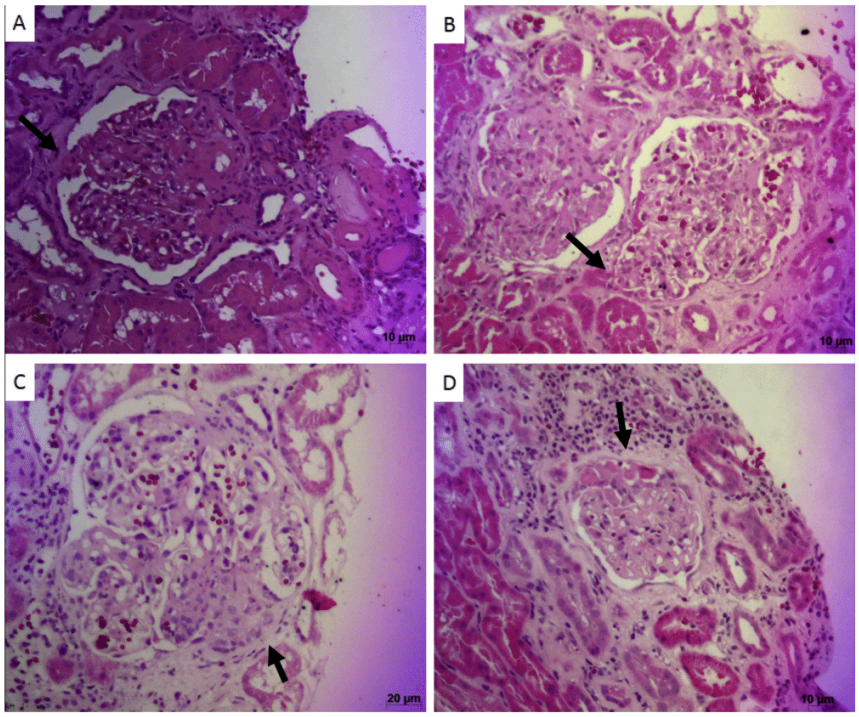

Fig. 4. Some examples of segmental sclerosis subtypes. (A) Adhesion without sclerosis- arrow (HE) 20× 1 obj.; (B) Tip lesion- arrow (HE) 20 × 1 obj.; (C) Cellular variant (HE) 40× 1 obj.; (D) Podocyte reabsorption droplets- arrow (HE) 20× 1 obj.

advanced degrees of disease.

Regarding prediction of parameter T from clinical data, it was found that the presence of hypertension increases the chances of belonging to T2 group than T0. The most commonly used prognostic evaluation considers high levels of MAP as a factor of worse prognosis. Not coincidentally, in histopathological analysis, the presence of tubular atrophy/interstitial fibrosis (T) is also used for prognostic evaluation [17].

Still regarding the parameter T, higher creatinine rates increased the patient's chances of being in T2 group compared to T0, in other words, having interstitial fibrosis and tubular atrophy, the so-called tubulointerstitial repercussions, in more than 50% of the area sampled in biopsy. Similarly, in multivariate analysis, GFR presented an inverse relation to T parameter, that is, the greater GFR the lower the chance of belonging to T2 group compared to T0. Similar findings have been reported in other publications, with a significant relationship between GFR and creatinine ratio with parameter T [22]. Thus, lower creatinine rates and increased GFR are related to reduction of tubulointerstitial repercussions, being a good indicator for parameter T.

The presence of crescents in glomeruli is correlated with worse prognosis independent of clinical data at the time of biopsy and of other MEST parameters of Oxford classification [23], being a more severe histological lesion, which affects renal function. In our study, values of creatinine, GFR, proteinuria and hypertension presented a positive and significant correlation with presence of crescents. An elegant study dealing with risk prediction in IgAN also correlated crescents with the presence of hypertension and proteinuria, which are poor prognostic parameters [17].

Therefore, the exciting discoveries of molecular mechanisms and biomarkers involved in IgAN and the description of probabilistic models to evaluate disease progression may in the future allow a non-invasive diagnosis of this glomerulopathy. In this context, it will be extremely important to have available methods to predict histological parameters related to poor clinical evolution.

Our findings endorse evidences that points to a close relationship between clinical and histopathological parameters to an extent that, in the future, could be used as a diagnostic approach.

For a better characterization of these relations, there have been several searches about the role of FSGS subtypes, especially those with morphology suggestive of Podocitopathies, in clinical presentation and evolution of the disease.

In our cases, proteinuria was the clinical data related to the presence of segmental sclerosis (S1) and it is also known to be associated with progression to chronic kidney disease [24].

Not coincidentally, by separating S1 group into subtypes, we found that, among clinical parameters, there is a positive and significant correlation only with proteinuria and in two subtypes, FSGS cellular variant and peri-hilar, characteristically not related to Podocitopathies.

Previous researches showed that subtypes of glomerulosclerosis apparently related to proteinuria are tip lesion and podocyte hypertrophy [9]. This difference may be related to different inclusion factors of the studies, as in Oxford Classification cohort [9] it was established minimum levels of proteinuria and GFR, which was not done in our study. These differences may also be due to variations in the presentation of IgAN around the world, possibly due to ethnic factors [25].

Our study points to the need to continue investigating the relationships between subtypes of segmental sclerosis and clinical presentation.

5. Conclusions

We believe that in the future, with the discovery of noninvasive methods for diagnosis of primary IgA Nephropathy, it will be essential to predict parameters of Oxford classification using clinical-laboratory data. Thus, non-invasively, it would be possible to establish prognosis and the best therapeutic strategy. We demonstrated in this study that all

parameters of the Oxford classification correspond to some clinical-laboratory data, which makes this approach possible.

Our results point to evidence that lesions of segmental sclerosis not specifically related to podocytopathies may also influence clinical parameters that affect the evolution of renal disease, such as proteinuria. Thus, we advocate the incorporation of the subclassification method of the S parameter into the routine of renal biopsy evaluation in order to contribute to further clarification of pathophysiology. The future will determine a consensus about the role of each subtype in the evolution of patients and if there is sense in maintaining this method.

The variety of segmental glomerulosclerosis subtypes that can be found in each glomerulus and in each renal biopsy is still a challenge in establishing the degree of influence of each subtype on clinical manifestations alone. Available studies are rare, requiring further evaluation in different populations to confirm and reinforce our results.

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5 Artigo 2

Título: Morphological analysis of podocitary injury and death in primary IgA nephropathy: far beyond foot process effacement

MORPHOLOGICAL ANALYSIS OF PODOCITARY INJURY AND DEATH IN PRIMARY IgA NEPHROPATHY: FAR BEYOND FOOT PROCESS EFFACEMENT

ABSTRACT

BACKGROUND: Podocytes are glomerular epithelial cells constantly exposed to stress, with risk of detachment. Transmission electron microscopy (TEM) is still the gold standard to access podocyte architecture. The relationship between nephrotic proteinuria and glomerular ultrastructural changes is not fully elucidated in IgA nephropathy (IgAN). Evaluation of podocyte injury, foot process effacement (FPE) and evidences of cell death are essential to understand podocyte loss and its clinical consequences. **AIM OF THE STUDY:** To evaluate the association between quantitative and qualitative podocytes abnormalities in TEM, including features of cell death, podocyte density and clinical parameters in patients with IgAN. **METHODS:** This is a transversal study with 100 biopsy-proven IgAN patients. Clinical parameters were proteinuria, hypertension, hematuria and glomerular filtration rate (GFR). Renal samples underwent light microscopy, immunofluorescence, immunohistochemistry for WT1 and TEM. WT1-labeled cells in glomerular loops were counted as podocytes and glomerular area was measured to obtain podocyte density. TEM images were evaluated searching changes in podocytes, especially cell death features, and adjacent structures. The number of slit pores was counted and the length (µm) of the loop divided by this number, resulting in the density of slit diaphragms/µm. The perimeter of the loop covered by FPE or denuded areas were measured and divided by total perimeter, resulting in the Effacement Index (EI). Statistical analysis was performed using GraphPad Prism and a significance <0.05 was adopted. **RESULTS:** Severe proteinuria was found in 65% of patients and 18% had nephrotic levels. Podocyte density using WT1 immunolabeling was lower in cases with nephrotic proteinuria (p= 0.03; r=-0.22). Patients were separated in groups according to morphological features of podocyte death. Detached podocytes were found in all groups, but with a significantly lower proportion in those cases with morphological evidences of autophagy (p=0.03). In

most cases with detached podocytes, there were also features of podocyte death, specially autophagy and necrosis. No podocyte with apoptosis was found. Pseudocysts were not correlated with podocyte detachment (p=0.49). Cases with autophagy, had significantly lower frequency of hematuria (p=0.03). Slit diaphragm density and EI did not correlate with clinical or morphological data. In contrast, the occurrence of occluding junctions was significantly more frequent in males than females (p=0.004). **CONCLUSIONS:** Proteinuria correlated with podocyte density and not with FPE, which reinforces the theory that this change is an adaptive / protective mechanism. Detached podocytes were related to podocyte injury and not with pseudocysts, which reinforces the hypothesis that in cases of IgAN, podocyte loss is related to cell injury and not just mechanical factors. Autophagy seems to be a cellular protection mechanism, as in its presence; there was a smaller proportion of detached podocytes and fewer cases with hematuria. Thus, podocyte injury seems to be the result of a fine balance between adaptive responses and cell lesions tending to cell death. It is important to be aware of these characteristics in diagnostic routine of renal biopsies, as they may refine the assessment of severity of disease.

KEY WORDS

IgA Nephropathy, Podocytes, Cell death, Autophagy, Transmission Electron Microscopy, Proteinuria

INTRODUCTION

Podocytes have a complex structure and a special position outside of the glomerular capillary, in intimate contact with the ultrafiltrate and fixed in the glomerular basement membrane (GBM) only by its process, that is, they are poetically floating in urinary space [1]. As phylogenetically their features have been conserved along natural selection, it can be presumed that this structure is indispensable for the function, including their incapacity to replicate *in situ* [1].

This set of morphological aspects makes podocytes constantly exposed to several types of stress, with risk of detachment [1]. When damaged, they undergo structural changes as foot process effacement (FPE), pseudocyst formation, cytoplasmic accumulation of vesicles [2] among many others. Scanning and transmission electron microscopies (TEM) are still the gold standard methods to access podocyte architecture [2,3,4,5,6]. In fact, the current knowledge and mechanistic understanding of the processes of podocyte injury and detachment are largely based on TEM [1,2,7].

The relationship between nephrotic proteinuria and glomerular ultrastructural changes is not fully elucidated in IgA nephropathy (IgAN) [8]. Ancient classic concepts related IgAN pathogenesis almost exclusively to mesangium, but a growing body of evidences has proven that mesangium stress can affect podocyte behavior [9].

Podocyte detachment from GBM has already been reported in IgAN patients [10]. Mechanical factors seem to have a key role in podocyte detachment as [11] for glomerular filtration function, a transcapillary pressure is necessary, but it can become a stress on epithelial cells if it is increased above physiological levels [12]. Besides, circumscribed areas of bare glomerular basement membrane with no foot processes (FP) may give rise to uncontrolled filtrate flows starting the formation of pseudocysts [13]. The increased local filtrate flow in areas of pseudocyst is associated with a high shear stress and maybe this mechanical change could be crucial for podocyte detachment [11].

In addition, podocyte cell death by anoikis, autophagy or mitotic catastrophe (MC) are possible causes of podocyte detachments *in vivo* [14,15]. Indeed, FPE is

often associated with binucleated podocytes *in vivo*, [16,17] and this type of cell injury is frequently seen in podocyte in urine, so detachment may be caused by unknown intrinsic properties in response to injury [15].

Thus, current understanding highlights that podocyte detachment may be caused by biological cellular responses due to injuries and also by mechanical forces when filtrate across GBM tends to lift the cell body [12]. The knowledge of these detachment mechanisms is relevant as it correlates with pathological progression of disease in humans and experimental animals [18, 19], suggesting that it underlies podocytopenia and progression to glomerulosclerosis.

One way to quantify podocytopenia is by counting podocyte number *in situ* with an immunohistochemical podocyte marker [20], as WT1. This is a transcription factor found in mature podocytes that regulates podocalyxin [21] and nephrin [22] expression.

Modern understanding advocates that FPE is a final adaptative change due to mechanical challenges that leads to expansion of the GBM [23]. The first adaptation would be a replacement of slit diaphragms by a closer structure called occluding junctions [1]. Slit diaphragm is the main barrier that limits proteinuria [12]. Occluding junctions would retract cell processes and promote attachment of the cell body to GBM [1]. This response of closing the slits seems to be the most critical for podocyte preservation as it ultimately leads to FPE that implicates a better attachment to GBM, preventing podocytopenia. Otherwise, the podocyte would continue subjected to shear stresses and finally would detach [24].

Early stages of FPE are reversible but eventually reaches a point of no return from which this adaptation participates in a further protective mechanism in order to maintain the lesion in a single local of the glomerulus, generating a segmental scar known as glomerulosclerosis [13].

In addition to the evaluation of podocyte injury and FPE, morphological analysis of evidences of cell death is essential to identify which death mechanisms contribute to podocyte loss and its clinical consequences.

Morphological features of necrosis include oncosis, characterized by edema with increased cell volume, disruption of cytoplasmic membrane and loss of electron

density in the cytoplasm; the nucleus also undergoes lysis [15]. Autophagy in turn, is characterized by formation of autophagosomes, vacuoles with double-limiting membrane that sequestrate large organelles such as mitochondria and ribosomes [25,26]. Binucleated cells or podocytes with micronuclei indicate aneuploidy, implying MC as a cause for podocyte loss [15]. This loss would happen by detachment due to enlargement and complex changes in the cell structure [27], which makes it more susceptible to any mechanical stress [28]. Binucleation occurs when an injurious stimulus overcomes natural podocyte cell cycle blockage [28] and the cell starts division [29]. Due to its complex cytoskeleton, podocytes cannot complete cytokinesis and will suffer an abortive mitosis or result in binucleate cells [1]. Morphologic data suggesting apoptotic cell death would be nuclear remnant of fragmented or condensed chromatin, and are rarely presented in podocytes [1]. Although enzymatic (caspase) and biochemical assays of DNA fragmentation (TUNEL assay) are frequently used to demonstrate evidence of apoptosis, they do not definitely separate apoptosis from other types of cell death [15] and TEM remains the gold standard method to demonstrate apoptosis [1].

The aim of this study is to evaluate the association between quantitative and qualitative podocytes abnormalities, including cell death features; podocyte density and clinical parameters in patients with IgAN.

MATERIAL AND METHODS

Study design

This transversal observational study included patients who underwent renal biopsy, the material was analyzed in the Nephropathology Service of the Federal University of Triangulo Mineiro (UFTM) from 2010 to 2016 and the diagnosis were IgAN based on IgA-dominant mesangial or mesangial-capillary immune deposits through IF microscopy. A representative sample was considered with a minimum of 8 glomeruli for light microscopy and material for immunofluorescence (IF) and TEM analysis. Patients were from both genders, adolescents (person aged 10 to 19 years,

inclusive) and adults. The study was approved by Ethics Committee with the protocol number 46369815.0.0000.5154.

Two hundred seventy-two cases of IgAN were analyzed between 2010 and 2016. At first, exclusion criteria were absence of material for IF analysis or light microscopy (n = 23); incomplete clinical data (n = 38) and transplanted patients (n = 9). Then cases with inconclusive diagnosis-focal, segmental or not predominant IgA deposits in IF analysis, which were suspicious but did not allowed a definite diagnosis of IgAN (n = 52) and secondary IgAN (n = 6) were excluded. The presence of concomitant diseases such as diabetes, overlapping glomerulopathies, neoplasia, among others (n = 9); and non-representative sample, with less than eight glomeruli (n = 33) were also exclusion criteria. Two cases were excluded due to technical immunohistochemical problems. A group of 100 patients was obtained, after exclusions.

In 91 cases, it was possible to evaluate WT1 immunolabeling in podocyte nuclei.

There were 73 cases with available and adequate material for electron microscopy, but for study of cell death mechanisms, some cases were excluded due to paucity of patients: six cases with necrosis, one case with MC, two cases with autophagy+necrosis+MC and two cases with autophagy+MC, resulting in 62 cases.

For slit diaphragm morphometry, two additional cases were excluded as glomeruli were evolving for global sclerosis and in another case, there were artifacts that compromised the reliability of the morphometry. Thus, for this specific analysis, 59 cases were included.

Kidney Biopsy Evaluation

Each renal biopsy was prepared for light microscopy and IF.

Podocytes were identified using immunohistochemistry for WT-1, a podocyte differentiation marker and a transcription factor. Slides were incubated with primary monoclonal antibody, anti-WT1 (DAKO) diluted 1: 100, washed with PBS, incubated with the post-primary reagent and incubated with the polymer (Kit Novolink Polymer Detection System, BL, UK).

For TEM, cases were fixed in Karnovsky and 0.2% Ruthenium red and 0.1M sodium cacodylate buffer, and postfixed in 0.2% osmium tetroxide and Ruthenium red. After being embedded in Epon, they were sectioned with an ultramicrotome and contrasted with uranyl acetate and lead citrate. Electron microscopic examinations were performed with a transmission electron microscope (Zeiss EM 900, West Germany). Photos of glomeruli were taken by a nephropathologist who analyzed all capillary loops of all available glomeruli in each ultrathin section and photographed especially podocytes. Magnification of photos were typically 3000x, 7000x and 12000x, rarely 20000x.

Morphometry

Immunohistochemistry

All glomeruli in each case were photographed using 20x or 40x objective so that the entire circumference of the glomerulus was represented in a single image, with the microscope software AxioVision. All WT1-labeled cells in glomerular loops were counted as podocytes and the glomerular area (μ^2) was measured using ImageJ software and its respective tools point selection and free hand (ImageJ1. Version 1.52a. National Institute of Health, Rockville, MD, USA, 1997). The density of WT1-positive glomerular podocytes, that is, the number of podocytes per glomerular volume unit was calculated from a table previously validated [27], available as supplementary material in this article.

Transmission electron microscopy

TEM images were evaluated searching for changes in podocytes and adjacent structures as the interaction between GBM and FP (occluding junctions, FPE or preserved FP; pseudocysts); evidences of possible MC (lobulated nuclei, binucleation or potentially binucleated podocytes, micronucleus); evidences of possible necrosis (cytoplasmic edema, nuclear edema, oncosis, cell lysis); detachment of viable podocytes from GBM (bared GBM, podocyte detachment); changes in cellular metabolism [endosomes, cytoplasmic electrondense vesicles, lipofuscin, enlargement of endoplasmic reticulum (ER) cisterns, mitochondrial edema, myelin-like bodies]; adaptive changes in podocyte shape (microvillus transformation, cytoplasmic shedding); evidences of autophagy (autophagosomes, type 1 or type 2 autophagy); evidences of possible apoptosis (chromatin condensation; blebs); morphological evidences of functional changes in glomerular filtration barrier (hematuria, proteinuria); endothelial changes in IgAN (reduction of endothelial fenestra, enlargement of subendothelial space); early or established evidences of segmental sclerosis (segmental glomerulosclerosis, adhesion of damaged podocytes to Bowman's capsule or parietal epithelium, thick and rough GBM).

The distance between nuclei should be greater than 3 µm or the morphology should be different or should have evident pathological changes such as condensation, to characterize binucleation. Any case that does not meet these criteria was classified as a potentially binucleated podocyte, as binucleation cannot be formally excluded. In addition, podocytes with increased nuclear lobulation were classified as lobulated [30].

Slit diaphragm density

All images with available and adequate capillary loops from all available glomeruli from each case were evaluated, in 7000x or 12000x magnifications, without evaluating the same region twice. The median of images analyzed per case was 21 (3-76).

All images were analyzed using the image-analyzing program ImageJ. The length of GBM was measured by line tracing method along the outer surface of the lamina densa. Measurements were made only in capillary loops; GBMs covering the mesangial and paramesangial areas were not included. The number of slit pores (space between FP) was counted and the length (µm) of the respective loop divided this number. The result was expressed as number of slit diaphragms / µm [8].

In addition, we calculated the Effacement Index (EI), considered the ratio of the perimeter of capillary loop covered by effaced podocytes (defined by absence of FP) divided by the total perimeter of capillary loops present, as follow: EI: =PFD1+PFD2+PFD3+PFD4+⋯+ PFDn/ PT1+PT2+PT3+PT4+⋯+PTn in which PFD corresponds to the perimeter of capillary loop covered by pedicel effacement and/ or detached podocytes; PT corresponds to the total perimeter of the evaluated capillary loop; and numbers correspond to the loops presented in the sample measured. Thus, each patient has only one EI, regardless of the number of loops evaluated in the sample [31].

Definitions of clinical parameters

Hypertension was characterized by systolic blood pressure (BP) greater than 140 mmHg and diastolic BP greater than 90 mmHg. Hematuria was characterized if there were more than ten thousand red blood cells per field or presence of at least 1+ of red blood cells in urinalysis. Glomerular filtration rate (GFR) was calculated by CKD-EPI formula.

Statistical analysis

A Microsoft Excel table was prepared with clinical, laboratorial and epidemiological data from biopsy reports and morphological characteristics from renal fragments. Descriptive statistics were presented as median, 25 and 75 percentiles, mean and standard deviation. Statistical analysis was performed in the program GraphPad Prism (version 7.0). Kolmogorov-Smirnov test was used to evaluate data normality. In cases of normal distribution and similar variances, Student´s t test (t) was used for comparing two groups and ANOVA (F) parametric

test followed by Tukey post-test was used for comparing three or more groups. In cases with non-normal distribution, Mann Whitney test (U) was used for comparing two groups and Kruskal-Wallis test (H) followed by Dunn post-test was used for comparing three or more groups. In contingency tables analysis, Fisher's exact test or Chi-Square ($\chi 2$) were used. Correlation between two variables with non-normal distribution was analyzed by Spearman's test (rS). A significance of ($p < 0.05$) was adopted for all tests.

RESULTS

Clinical-epidemiological aspects

Most patients were Caucasians (67%), male (64%) and the mean age at the time of biopsy was 38.77± 12.33 years (ranging from 11 to 72 years, 25^{th}-75^{th} percentiles were 31 and 47.75 years, with six patients between 11 and 18 years old).

Most patients presented hematuria (83%). The mean proteinuria was 2.27± 2.58 g/24h (ranging from 0- 16.2 g/24h with 25^{th}-75^{th} percentiles 0.72 and 2.82 g/24h, respectively), with 65% presenting severe proteinuria (greater than 1g/ 24h) and 18% presented nephrotic levels of proteinuria. GFR had an average of 67.76± 35.21 mL/min/1.73 m^2 (ranging from 6.1 to 154.2 mL/min/1.73 m^2; 25^{th}-75^{th} percentiles were 40.43 and 92.1 mL/min/1.73 m^2, respectively). Hypertension was present in 56% of patients.

Morphological features

According to Oxford classification for IgA Nephropathy, 47% of the patients were M1; 37% E1; 75% S1; 43% T1 or T2, 12% C1 or C2.

We evaluated 5011 images obtained from 118 glomeruli in 73 cases by TEM, with an average of 69.59 images/ case. The median of podocytes analyzed per case was 19.5 (4-44). With this thorough analysis, all cases presented a wide range of podocyte morphological changes, as described in Table 1 and shown in Figure 1.

Table 1- Frequency of morphological features of podocytes in cases analyzed by TEM*

Morphological features	Frequency (n%)
Relation GBM-foot process	
Foot process effacement	62 (84.93%)
Pseudocysts	55 (75.34%)
Normal foot process	54 (73.97%)
Occluding junctions	44 (60.27%)
Evidences of possible necrosis	
Cytoplasmic edema	36 (49.31%)
Nuclear edema	28 (38.35%)
Oncosis	13 (17.80%)
Cell lysis	8 (10.95%)
Evidences of mitotic catastrophe	
Lobulated nuclei	33 (45.20%)
Potentially binucleated	16 (19.27%)
Binucleation	6 (8.21%)
Micronuclei	4 (5.47%)
Evidences of possible apoptosis	
Chromatin condensation	0
Blebs	0
Detachment of podocytes from GBM#	
Bared GBM	23 (31.50%)
Podocyte detached	15 (20.54%)
Evidences of autophagy	
Autophagosomes	55 (75.34%)
Changes in cellular metabolism	
Endosomes	58 (79.45%)
Enlargement of ER+ cisternae	42 (57.53%)
Myelin-like bodies	33 (45.20%)
Cytoplasmic electron dense vesicles	31 (42.46%)
Lipofuscin	24 (32.87%)
Mitochondrial edema	16 (21.91%)
Adaptive changes in podocyte shape	
Microvillus transformation	58 (79.45%)
Cytoplasmic shedding	22 (30.13%)
Functional changes in filtration barrier	
Proteinuria	61 (83.56%)
Hematuria	28 (38.35%)
Endothelial changes	
Reduction of endothelial fenestra	18 (24.65%)
Enlargement of subendothelial space	16 (21.91%)
Evidences of segmental sclerosis	
Adhesion of damaged podocytes to Bowman's capsule or parietal epithelium	31 (42.46%)
Segmental glomerulosclerosis or thick and rough GBM	22 (30.13%)

*TEM: transmission electron microscopy
#GMB- glomerular basement membrane
+ER- endoplasmatic reticulum

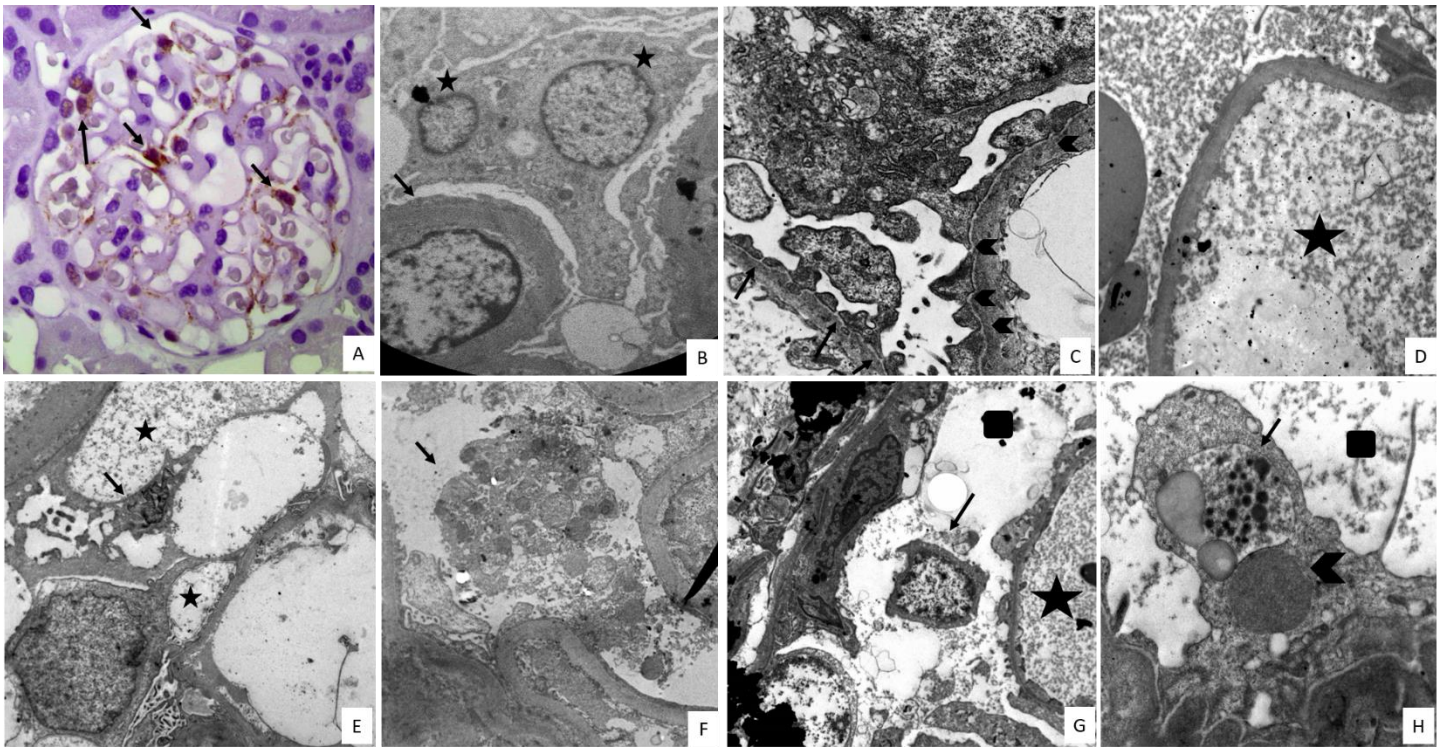

Figure 1. Podocyte morphological features. **A**- WT1 immunolabeling in glomerulus. Arrows point to podocyte nucleus evidenced by WT1. They were counted and tuft area were measured to calculate podocyte density (WT1, 40x2 obj.). **B**- Binucleated podocyte: each star is close to a nucleus with normal chromatin pattern and distancing between them about 4 micrometers. Arrow points to a glomerular capillary loop with diffuse FPE. In the bottom of the image, in the cytoplasm of this podocyte, there is a large endosome (TEM, 12,000x). **C**- In the capillary loop located in the bottom left, arrows point to preserved FP with normal slit diaphragms. In the capillary loop in the upper right, arrowheads point to FP closed together, in occluding junctions. In the podocyte cytoplasm in the upper third of the image, there are small endosomes (TEM, 12,000x). **D**- The star highlights the lumen of glomerular capillary loop. In the urinary space, there is granular material compatible with proteinuria and a red blood cell, characterizing hematuria, partially visualized in the left. Over the GBM, there are only remnants of podocyte cytoplasm, with diffuse and accentuated FPE (TEM, 7,000x). **E**- Stars highlights pseudocysts. Large pseudocysts both at the junction of the left bottom podocyte with the GBM and in the upper portion of the image. In the center, there is a very irregular, lobulated podocyte nucleus, highlighted by an arrow (TEM, 4,400x). **F**- Oncosis with associated cell lysis, highlighted by an arrow. Podocyte with ruptured cytoplasmic membrane and release of swollen organelles and cytoplasmic content in the urinary space. This is an evidence of cell death by necrosis, although the nucleus is not seen in this image. In adjacent capillary loops, there is marked FPE. (TEM, 4,400x). **G**- Detached podocyte in the urinary space near Bowman's capsule, with normal chromatin pattern, which favors this as a viable cell podocyturia. There is granular material in the urinary space compatible with proteinuria. Parietal epithelial cells are activated with nuclear and cytoplasmic edema. Occluding junctions are seen in the FP of adjacent loops (TEM, 3,000x). **H**- Podocyte with autophagy (condensation of ribosomes associated with vacuoles of various densities and sizes, surrounded by membrane), highlighted with an arrow. There is granular material in the urinary space compatible with proteinuria. This podocyte shows FPE and is lies over the already rough GBM. In its cytoplasm, there is an electron dense vesicle, highlighted with an arrowhead. There is granular material in the urinary space compatible with proteinuria, highlighted with a square (TEM, 12,000x).

At first, we evaluated WT1-immunolabeled podocytes for podocyte counting in 535 glomeruli from 91 cases. The median podocyte density was 459 (59-1317) podocytes/x10^6 μm^3. It was closely associated with the degree of proteinuria, as podocyte density was significantly lower in cases with nephrotic proteinuria (p=0.03) with a statistically significant and direct correlation (p= 0.03; r=-0.22)- Figure 2.

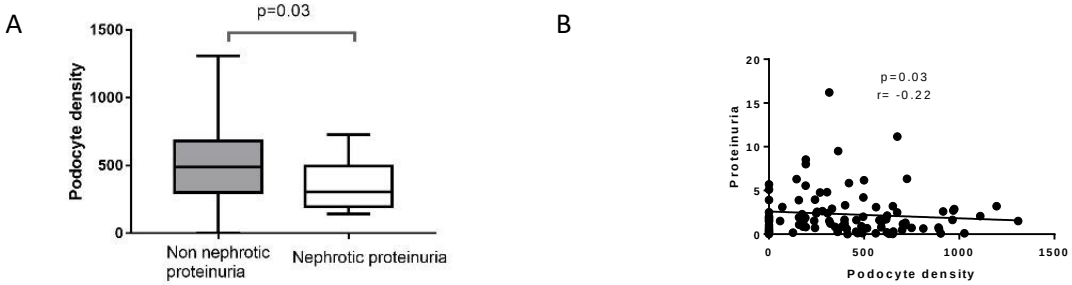

Figure 2- Podocyte density is related to proteinuria. A- Podocyte density, calculated by WT1 immunolabeling, is significantly lower in cases with nephrotic proteinuria (U, p=0.03). Data are presented as median [lower–upper quartiles]. The Mann–Whitney *U* test was used for comparison of categorical variables. B- Correlation between podocyte density and proteinuria (rS, p= 0.03; r=-0.22).

Podocyte density was not related with any other morphological feature, including Oxford classification parameters and ultrastructural changes.

Changes in foot process architecture are traditional features of podocyte lesion and can be summarized as occluding junctions and FPE. Occluding junctions were found in 44 (60.27%) cases and FPE in 62 (84.93%).

To study these features, we evaluated a median of 21 (3-76) photos of each case. The median of slit diaphragm density was 1.0 (0-1.73) pores/ µm and the median of EI was 0.21 (0-1). Both parameters did not correlate with clinical data as proteinuria, hematuria, hypertension, gender or age and neither with morphological data as Oxford parameters, podocyte density measured by WT1, pseudocysts or podocyte death features.

The occurrence of occluding junctions was significantly more frequent in males than females (Figure 3).

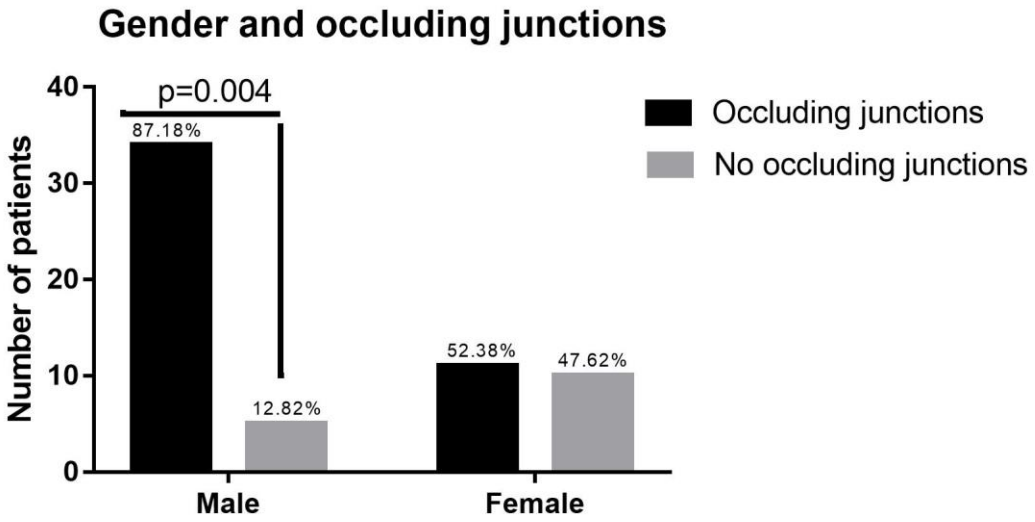

Figure 3- Occluding junctions, an abnormal connection between foot process, were significantly more frequent in males than females (Fisher´s exact test, p=0.004).

There was no significant difference between proteinuria in males and females (p=0.20).

To investigate possible mechanisms involved in podocyte depletion and death, we separated cases according to morphological features of podocyte death (Table 2).

Table 2- Frequency of morphological features of podocytes death analyzed by TEM*

Podocyte death features	n (%)
Autophagy	32 (43.83%)
No detectable death feature	18 (24.65%)
Autophagy + Necrosis	12 (16.43%)
Necrosis	6 (8.21%)
Autophagy + MC#	2 (2.73%)
Autophagy + Necrosis+ MC#	2 (2.73%)
MC#	1 (1.36%)
Total	73 (100%)

*TEM- Transmission Electron Microscopy

#MC- Mitotic Catastrophe

Necrosis, MC, autophagy with MC, and autophagy associated with necrosis and MC groups had small number of cases, so we excluded such groups for analysis. Finally, we analyzed three groups: no podocyte death features, autophagy and autophagy with necrosis.

Detached podocytes were found in all groups, in 15 (20.54%) patients. A significantly lower proportion of detached podocytes was found in cases with morphological evidences of autophagy (17.07%) compared with those cases with autophagy associated with necrosis (50%). In most cases with detached podocytes, there were features of podocyte death, both autophagy and autophagy with necrosis (Figure 4). The occurrence of pseudocysts did not correlate with podocyte detachment (p=0.49).

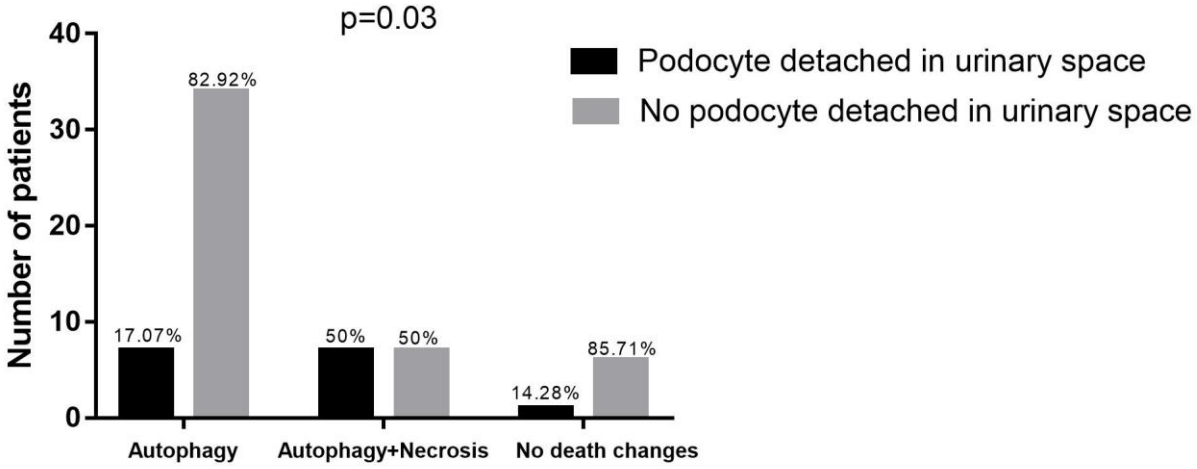

Figure 4- Cases with isolated autophagy had a significantly higher proportion of no detected podocyte detachment (χ^2, p=0.03).

The presence of autophagy is also related to hematuria as detected by TEM. Cases with autophagy, had significantly lower frequency of hematuria (p=0.03) (Figure 5).

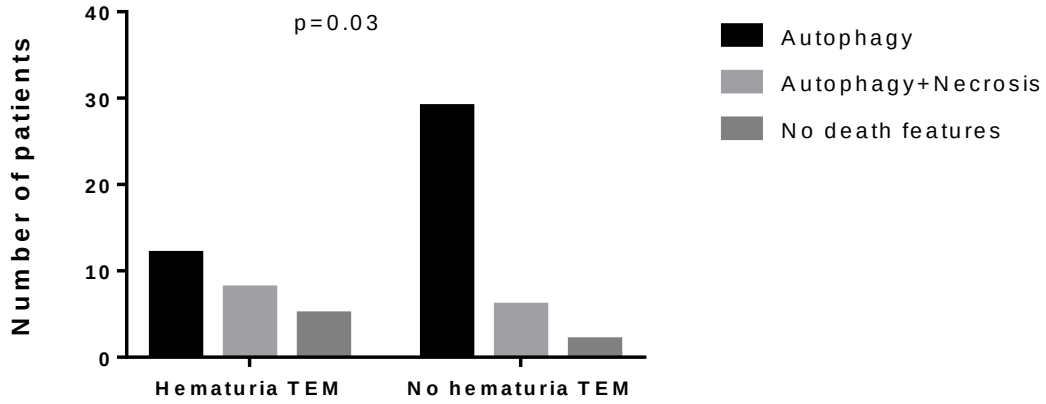

Figure 5- Cases with autophagy, had significantly lower frequency of hematuria (χ^2, p=0.03).

The number of autophagosomes in our cases was 1.25 (0-23) autophagosomes/ podocyte with evidence of autophagy. There was a tendency for a higher number of autophagosomes in cases with isolated autophagy (p= 0.08) and a tendency for a higher number of podocytes with autophagosome in patients with autophagy associated with necrosis (p=0.09).

Additionally, we searched evidences of apoptosis, but we did not find any podocyte with suggestive morphological features in our cases.

DISCUSSION

We sought to evaluate morphological features of podocyte injury and cell death in IgAN and their relation with clinical and other morphological parameters. Most of these features are not considered for ordinary diagnostic goals in glomerulopathies, including IgAN.

Podocyte lesions influence clinical presentation and prognosis [32]. Among clinical parameters, proteinuria has been especially correlated with podocyte damage or loss, evidenced by changes in expression of specific podocytes markers [33, 34, 35], necrosis followed by detachment of podocytes from GBM, [17] as well as podocytopenia [32, 36].

Our study showed an interesting correlation between nephrotic proteinuria levels and lower WT1 immunolabed podocyte density. The association between podocytopenia and proteinuria has been found in some studies [32; 36] while others found only a tendency of association between these variables [37]. Even further, there are evidences that podocyte density could predict evolution of IgAN better than proteinuria levels in the time of biopsy [37]. This is a feature not routinely evaluated in diagnostic renal biopsies, so is probably worth to better evaluate this relationship in order to implement this immunohistochemistry technique as an adjunct to prognosis.

In our study, podocyte density did not correlate with other morphological features. This is in agreement with a study which also evaluated podocyte number and did not find significant differences in the glomerular histologic changes comparing the podocytopenic with the normopodocytic group [37].

The most frequent podocyte change is FPE. A way to evaluate the extension of this process is counting the density of slit diaphragms in a length of GBM. The slit diaphragm is an electron-dense membrane that connects interdigitating FP in a space of 30 nm length, composed dominantly by nephrin, a transmembrane protein relevant for intracellular survival signaling and mechanosensation [38].

We found a median of 1.0 (0-1.73) slit diaphragm/µm and the EI was 0.21 (0-1). Although FPE is traditionally associated with proteinuria, in our cases, these indexes did

not correlate with proteinuria, in agreement with a study of human glomerulopathies, that found FPE were less severe in IgAN patients than in Minimal Change Disease and, in both diseases, there was no correlation of FPE extension with proteinuria [39]. The presence of FPE may not be sufficient to cause proteinuria, as found in autopsy of kwashiorkor patients, which FPE was found without albuminuria [40]. FPE may be a phenotypic adaptation that forms from cytoskeletal derangement leading to cell motility [12], characterizing a protective cellular adaptation, in order to tightly attach to GBM and form occluding junctions with neighboring podocytes, preventing cell detachment [24, 1]. For this reason, it may not necessarily be related with proteinuria, a clinical parameter related to poor renal prognosis.

Occluding junctions would be the first adaptation as it replaces slit diaphragms by a closer structure [1]. In our cases, this adaptation was significantly more frequent in males than females and had not been related to proteinuria levels probably because, as an adaptive process, it tends to protect against the occurrence of proteinuria.

A final route for severe injured podocyte is cell death and/or detachment. In our cases, morphological features of cell death were found as described in Table 1, regarding changes typical of necrosis, autophagy and MC. No evidences of apoptosis were found.

Podocytes are able to keep viability despite relevant change in shape, unlike other cell types. These changes have patterns that can be summarized as [1] (1) cell body hypertrophy and enlargement of the sub-podocyte space [41] resulting in pseudocysts [6]; (2) increased turnover of cell components, especially lysosomal; probably reflecting increased autophagy [42] and cytoplasmic shedding [43,44]; and (3) FPE. These changes are apparently reversible but may evolve to more severe injuries as podocyte die (especially by necrosis with cell lysis) or detach from the GBM as viable cells [1].

Classically, progressive podocyte detachment and its detection in urine have been correlated with pathological progression of disease in humans and experimental animals [18, 19, 45], suggesting that cumulative detachment underlies podocytopenia and progression to glomerulosclerosis. This relation could not be satisfactorily evaluated in our study due to its transversal design, with data only from the day of biopsy. Despite this temporal limitation, in our cases, there was no association between podocyte detachment and glomerulosclerosis or proteinuria. Similarly, a study with proteinuric glomeruli found

no epithelial cell detachment or areas of bared GBM [46]. Therefore, this relation needs to be better clarify.

Detached podocytes have been shown in a lesser extent in the urine of healthy individuals, [47] as well as in IgAN patients [10, 36] and also, in IgAN, podocyte detachment have been shown in association with necrosis [17].

In our cases, the proportion of patients with detached podocytes showing autophagy associated with necrosis (50%) is much higher than those cases with isolated autophagy (17.07%) and cases with no death changes (14.28%). In addition, cases with no podocyte detachment were more frequently associated with isolated autophagy (72.34% of cases with no podocyte detachment).

Cellular debris of dead podocytes are frequently found in advanced glomerular diseases, implying occurrence of podocyte death before detachment and, in most cases, there are signs of cell lysis suggesting necrosis pathway for death [1].

Therefore, we can infer that, in our cases, autophagy is apparently a sign of reversible podocyte lesion not necessarily associated with podocyte detachment. In our cases, features of the autophagic process were quite frequent in TEM (75.34%). Routine TEM is currently considered the only reliable method for monitoring autophagy in situ [48, 49, 50].

A balance between production and destruction of macromolecules and organelles is imperative for cell survival [51], especially in post mitotic cells as podocytes. Lysosomes are responsible for degrading macromolecules, and for the turnover of organelles by autophagy [52].

Autophagy has an apparently paradoxical role in cell death [53,54] as it is a cytoprotective process but is also associated with cell death through its relations with apoptosis and necrosis [55, 56, 57]. In animal models, autophagy has been shown to affect podocytes both helping to restore cytoplasmic podocyte integrity and, if excessive, leading to direct cell death [58], known as type II cell death or autophagic cell death (type I cell death is apoptosis and type III cell death is necrosis). [59] It is doubtful if this occurs in physiological setting [53,54].

The number of autophagosomes in our cases ranged from 0 to 23 autophagosomes/ podocyte. Podocytes have more autophagosomes than other kidney

cells, but this do not necessarily indicate increased autophagic activity or flux, as it can also result from impaired autophagosome/lysosome fusion or reduced lysosomal activity [58]. Other studies showed the number of autophagosomes per podocyte in IgAN ranging from a minimum of zero to a maximum of five, with the number of podocyte autophagosomes in IgAN being significantly lower than control group [60]. As deficiency in autophagy leads podocytes to stress, autophagy function both as a sensor of stress stimuli and as an effector, which coordinates podocyte homeostasis [58].

In line with the data described, regarding the protective role of autophagy, we found that cases with autophagy had significantly lower frequency of hematuria detected by TEM. The most frequent cause of glomerular hematuria is IgAN [62], being a classical symptom of this disease, a marker of disease activity and probable implicated in progression of the disease [63]**.**

Pathogenic mechanisms of glomerular hematuria remain unclear [64] and is also debatable if podocyte injury leads to hematuria; if hematuria enhance podocyte injury or both. It is well stablished that GBM components are mainly synthesized by podocytes [17] and there is a theoretical possibility that injured podocytes impair renewed biosynthesis of GBM proteins [65] in a possible mechanism that favors hematuria.

On the other hand, the passage of red blood cells through capillary wall can damage podocytes, especially in macroscopic hematuria in IgAN [63] and, as well as tubular epithelial cells, podocytes may be a cellular target of hemoglobin-mediated oxidative damage, leading to podocyte detachment [66]. Another way of podocyte injury secondary to hematuria is due to microvesicles containing microRNA that are released by erythrocytes during their passage throughout the glomerular filtration barrier. These microvesicles have a role in oxidative stress regulation and intercellular communication by regulating gene expression [67]. A third way of podocyte injury in hematuria setting is the higher deposition of immune complexes in mesangium during the episodes of macroscopic hematuria, which induces cell proliferation and secretion of several inflammatory mediators which can be released to the urinary space and induce podocytes and proximal tubular epithelial cells injury [68,69].

We speculate that autophagy exerts protective action on podocytes reducing its damage and the chance of developing hematuria. Our results taken together with

literature data, indicate that autophagic activity in podocytes is a protective factor against kidney injury [61]. More studies with molecular approaches in this setting are needed to better evaluate the meaning of accumulation of morphological different autophagossomes in podocytes in IgAN. There are many studies ongoing with the aim to better clarify autophagy role in podocyte fate.

Another important consideration regarding our study is that we did not find any feature of podocyte apoptosis despite the careful search in 5011 images of 118 glomeruli. Morphologic data of apoptotic podocytes are rarely found and either a well-known group that systematically search this field never found a stigma of apoptosis in podocytes, even after analysis of 40,000 images [1]. They advise that even in heavily injured podocytes, nuclear chromatin is well preserved [1], as we found in the majority of our cells.

Therefore, we demonstrated that podocyte changes are related to clinical parameters, such as nephrotic proteinuria, which correlated with quantitative reduction in podocytes; and hematuria, less frequent in cases with autophagy in podocytes. In addition, FPE seems to be an adaptive change to avoid podocyte detachment and would not generate, by itself, proteinuria. We demonstrated evidences of podocyte necrosis and MC in IgAN, as well as a significant proportion of autophagy, however, as reported in the literature, there was no evidence of apoptosis. Autophagy seems to protect against podocyte detachment which is not related to pseudocysts, an evidence that podocytopenia is rather a consequence of podocyte damage than of mechanical changes.

CONCLUSIONS

The aim of this study was to evaluate, in patients with IgAN, the association between quantitative and qualitative podocytes abnormalities and clinical parameters. Interestingly, our results indicate that patients with lower podocyte density have higher levels of proteinuria and podocyte detachment is more frequent in cases of necrosis. In contrast, autophagy seems to be a cytoprotective mechanism against podocyte detachment and also against hematuria. Therefore, many ultrastructural changes can impact clinical parameters, not just the most described and studied of them, foot process effacement. The ultrastructural analysis must go far beyond foot process morphology.

We highlight the importance of systematized TEM analysis in cases of IgAN and the relevance of being aware of these characteristics in the diagnostic routine of renal biopsies as they may add to the assessment of severity of disease in patients with IgAN.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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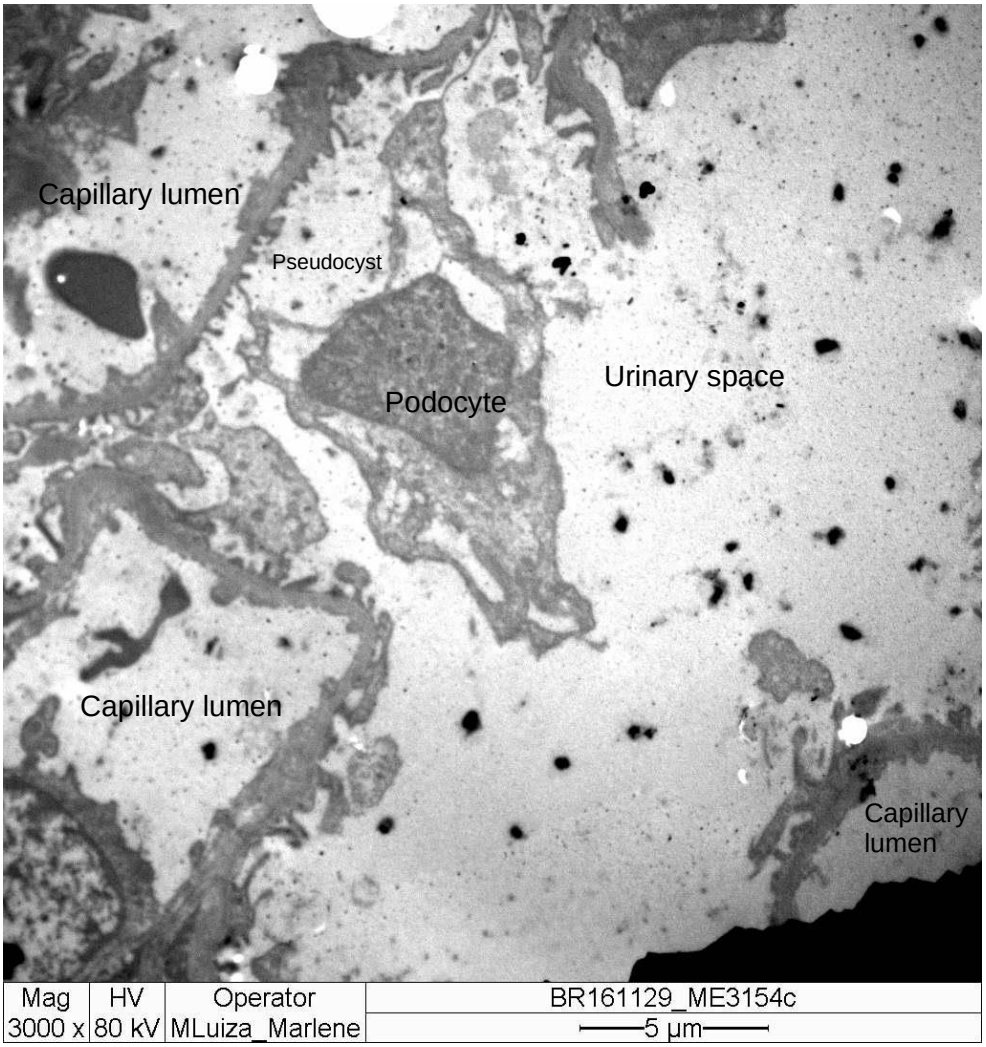
SUPPLEMENTARY MATERIAL

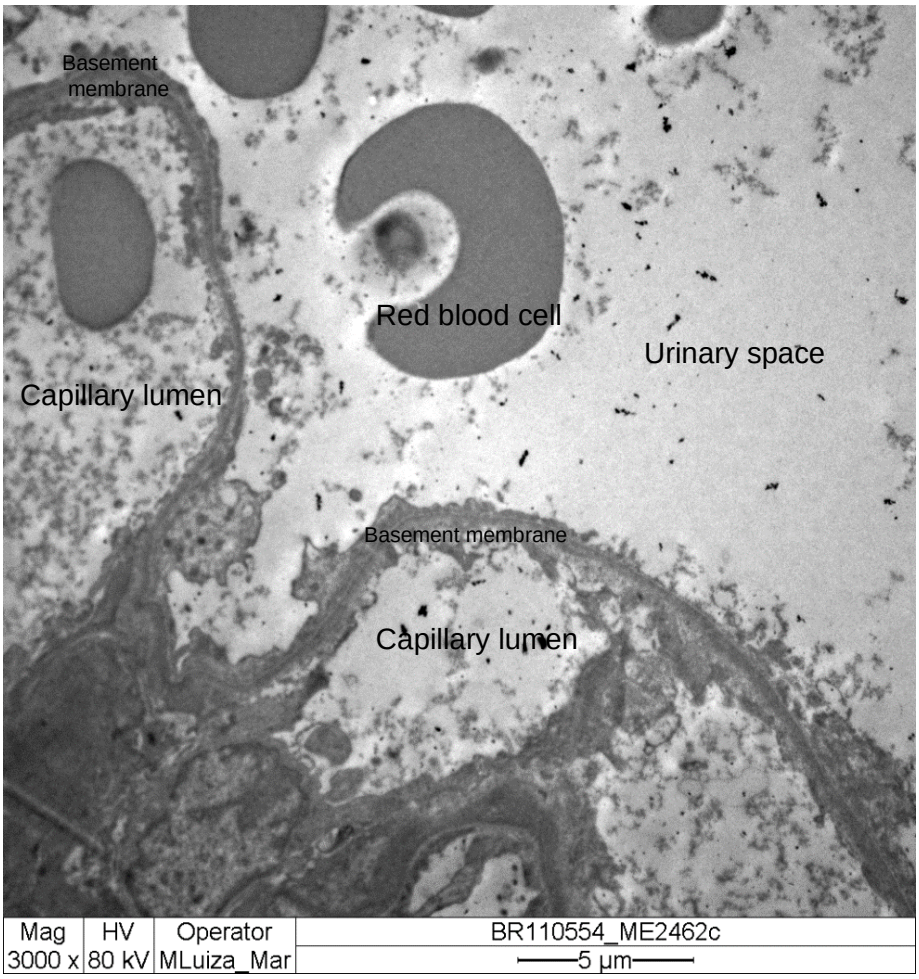
1- Table available as supplementary material in the article Venkatareddy M., et al. Estimating podocyte number and density using a single histologic section. J. Am Soc Neprhol, 25, p.1118-1129, 2014, filled with our data regarding podocyte density using WT1-immunolabeled podocytes.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Tabela - Densidade de podócitos											
2												
3		Section						Corrected				
4		thickness	Apparent	Shape	True	Correctio	Podocyte	podocyte	Total Tuft	Total Tuft	Podocyte	GV/P
5	#	(um)	(um)	k	(um)	CF	No	Nt	(um^3)	(um^3)	(pods/x10^6 um3)	(um^3)
6	BR100316	2	6,5	0,72	8,40	0,19	17	3,27	27475,0	54950	59	16808
7	BR100474	2	6,5	0,72	8,40	0,19	41	7,88	25133,0	50266	157	6375
8	BR100739	2	6,5	0,72	8,40	0,19	20	3,85	27554,0	55108	70	14327
9	BR100839	2	6,5	0,72	8,40	0,19	90	17,31	14053,0	28106	616	1624
10	BR100842	2	6,5	0,72	8,40	0,19	45	8,65	17845,0	35690	242	4124
11	BR101052	2	6,5	0,72	8,40	0,19	46	8,85	13704,0	27408	323	3098
12	BR101133	2	6,5	0,72	8,40	0,19	41	7,88	20301,0	40602	194	5149
13	BR101167	2	6,5	0,72	8,40	0,19	26	5,00	12915,0	25830	194	5166
14	BR110107	2	6,5	0,72	8,40	0,19	25	4,81	14227,0	28454	169	5918
15	BR110212	2	6,5	0,72	8,40	0,19	108	20,77	18500,0	37000	561	1781
16	BR110234	2	6,5	0,72	8,40	0,19	51	9,81	20588,0	41176	238	4198
17	BR110246	2	6,5	0,72	8,40	0,19	60	11,54	10237,0	20474	564	1774
18	BR110249	2	6,5	0,72	8,40	0,19	49	9,42	11421,0	22842	413	2424
19	BR110252	2	6,5	0,72	8,40	0,19	81	15,58	21400,0	42800	364	2748
20	BR110285	2	6,5	0,72	8,40	0,19	68	13,08	15998,0	31996	409	2447
21	BR110301	2	6,5	0,72	8,40	0,19	61	11,73	18497,0	36994	317	3153
22	BR110335	2	6,5	0,72	8,40	0,19	93	17,89	14159,0	28318	632	1583
23	BR110346	2	6,5	0,72	8,40	0,19	54	10,39	11139,0	22278	466	2145
24	BR110361	2	6,5	0,72	8,40	0,19	25	4,81	19098,0	38196	126	7944
25	BR110407	2	6,5	0,72	8,40	0,19	73	14,04	11874,0	23748	591	1692
26	BR110427	2	6,5	0,72	8,40	0,19	74	14,23	20461,0	40922	348	2875
27	BR110433	2	6,5	0,72	8,40	0,19	73	14,04	11897,0	23794	590	1695
28	BR110550	2	6,5	0,72	8,40	0,19	67	12,89	33417,0	66834	193	5187
29	BR110552	2	6,5	0,72	8,40	0,19	53	10,19	29154,0	58308	175	5721
30	BR110654	2	6,5	0,72	8,40	0,19	80	15,39	12413,0	24826	620	1614
31	BR110674	2	6,5	0,72	8,40	0,19	97	18,65	23507,0	47014	397	2520
32	BR110719	2	6,5	0,72	8,40	0,19	49	9,42	19472,0	38944	242	4133
33	BR110725	2	6,5	0,72	8,40	0,19	177	34,04	18591,0	37182	915	1092
34	BR110744	2	6,5	0,72	8,40	0,19	45	8,65	17090,0	34180	253	3950
35	BR110777	2	6,5	0,72	8,40	0,19	61	11,73	18739,0	37478	313	3195
36	BR110777	2	6,5	0,72	8,40	0,19	89	17,12	27814,0	55628	308	3250
37	BR110807	2	6,5	0,72	8,40	0,19	60	11,54	29776,0	59552	194	5161
38	BR110901	2	6,5	0,72	8,40	0,19	50	9,62	9693,0	19386	496	2016
39	BR111122	2	6,5	0,72	8,40	0,19	88	16,92	12988,0	25976	652	1535
40	BR111134	2	6,5	0,72	8,40	0,19	43	8,27	26060,0	52120	159	6303
41	BR111210	2	6,5	0,72	8,40	0,19	55	10,58	29143,0	58286	181	5510
42	BR111214	2	6,5	0,72	8,40	0,19	41	7,88	12893,0	25786	306	3270
43	BR120404	2	6,5	0,72	8,40	0,19	64	12,31	17024,0	34048	361	2766
44	BR120601	2	6,5	0,72	8,40	0,19	53	10,19	11130,0	22260	458	2184
45	BR120703	2	6,5	0,72	8,40	0,19	30	5,77	15115,0	30230	191	5240
46	BR121005	2	6,5	0,72	8,40	0,19	59	11,35	17144,0	34288	331	3022
47	BR130102	2	6,5	0,72	8,40	0,19	62	11,92	10055,0	20110	593	1687
48	BR130118	2	6,5	0,72	8,40	0,19	74	14,23	7883,0	15766	903	1108
49	BR130401	2	6,5	0,72	8,40	0,19	67	12,89	23995,0	47990	268	3724
50	BR130404	2	6,5	0,72	8,40	0,19	81	15,58	16940,0	33880	460	2175
51	BR130507	2	6,5	0,72	8,40	0,19	52	10,00	7690,0	15380	650	1538
52	BR130525	2	6,5	0,72	8,40	0,19	34	6,54	8506,0	17012	384	2602
53	BR130630	2	6,5	0,72	8,40	0,19	72	13,85	17431,0	34862	397	2518
54	BR130801	2	6,5	0,72	8,40	0,19	89	17,12	12680,0	25360	675	1482
55	BR130803	2	6,5	0,72	8,40	0,19	149	28,66	16029,0	32058	894	1119
56	BR131204	2	6,5	0,72	8,40	0,19	131	25,19	17562,0	35124	717	1394
57	BR140101	2	6,5	0,72	8,40	0,19	116	22,31	11566,0	23132	964	1037
58	BR140208	2	6,5	0,72	8,40	0,19	101	19,42	9975,0	19950	974	1027
59	BR140529	2	6,5	0,72	8,40	0,19	84	16,15	16160,0	32320	500	2001
60	BR140601	2	6,5	0,72	8,40	0,19	144	27,69	12467,0	24934	1,111	900
61	BR140903	2	6,5	0,72	8,40	0,19	103	19,81	14150,0	28300	700	1429
62	BR141106	2	6,5	0,72	8,40	0,19	73	14,04	12101,0	24202	580	1724
63	BR150118	2	6,5	0,72	8,40	0,19	70	13,46	10298,0	20596	654	1530
64	BR150561	2	6,5	0,72	8,40	0,19	143	27,50	10498,0	20996	1,310	763
65	BR150708	2	6,5	0,72	8,40	0,19	80	15,39	10606,0	21212	725	1379
66	BR150734	2	6,5	0,72	8,40	0,19	102	19,62	13931,0	27862	704	1420
67	BR150814	2	6,5	0,72	8,40	0,19	147	28,27	15854,0	31708	892	1122
68	BR150825	2	6,5	0,72	8,40	0,19	160	30,77	27340,0	54680	563	1777
69	BR151028	2	6,5	0,72	8,40	0,19	119	22,89	14106,0	28212	811	1233
70	BR151112	2	6,5	0,72	8,40	0,19	23	4,42	2950,0	5900	750	1334
71	BR160322	2	6,5	0,72	8,40	0,19	36	6,92	23787,0	47574	146	6872
72	BR160335	2	6,5	0,72	8,40	0,19	46	8,85	9097,0	18194	486	2057
73	BR160424	2	6,5	0,72	8,40	0,19	36	6,92	13938,0	27876	248	4026
74	BR160522	2	6,5	0,72	8,40	0,19	24	4,62	12938,0	25876	178	5606
75	BR160531	2	6,5	0,72	8,40	0,19	40	7,69	9608,0	19216	400	2498
76	BR160614	2	6,5	0,72	8,40	0,19	33	6,35	20052,0	40104	158	6319
77	BR160638	2	6,5	0,72	8,40	0,19	293	56,35	23554,0	47108	1,196	836
78	BR160720	2	6,5	0,72	8,40	0,19	43	8,27	11829,0	23658	350	2861
79	BR160809	2	6,5	0,72	8,40	0,19	56	10,77	10974,0	21948	491	2038
80	BR160862	2	6,5	0,72	8,40	0,19	79	15,19	14742,0	29484	515	1941
81	BR160916	2	6,5	0,72	8,40	0,19	68	13,08	23248,0	46496	281	3555
82	BR160958	2	6,5	0,72	8,40	0,19	94	18,08	12587,0	25174	718	1393
83	BR160961	2	6,5	0,72	8,40	0,19	135	25,96	9855,0	19710	1,317	759
84	BR161007	2	6,5	0,72	8,40	0,19	84	16,15	25445,0	50890	317	3150
85	BR161069	2	6,5	0,72	8,40	0,19	105	20,19	25428,0	50856	397	2518
86	BR161127	2	6,5	0,72	8,40	0,19	114	21,92	10675,0	21350	1,027	974
87	BR161143	2	6,5	0,72	8,40	0,19	93	17,89	14404,0	28808	621	1611
88	BR161218	2	6,5	0,72	8,40	0,19	63	12,12	14418,0	28836	420	2380
89	BR161221	2	6,5	0,72	8,40	0,19	51	9,81	9906,0	19812	495	2020
90	BR161227	2	6,5	0,72	8,40	0,19	82	15,77	8137,0	16274	969	1032
91	BR161258	2	6,5	0,72	8,40	0,19	71	13,65	10134,0	20268	674	1484

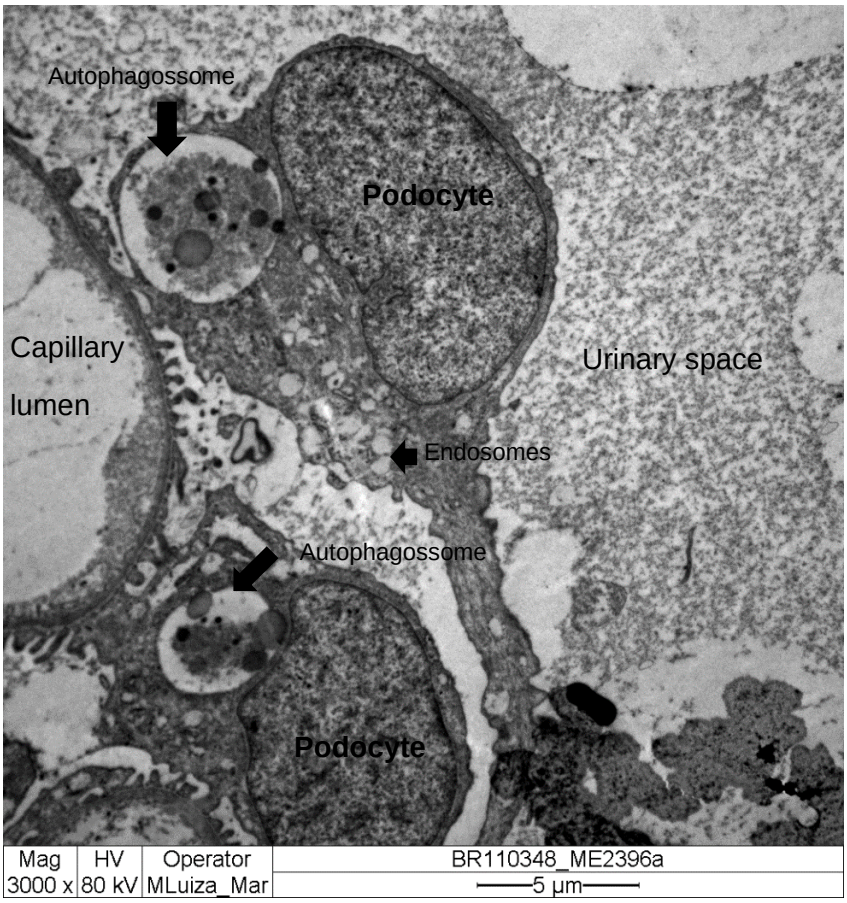
2- Additional images from our cases:



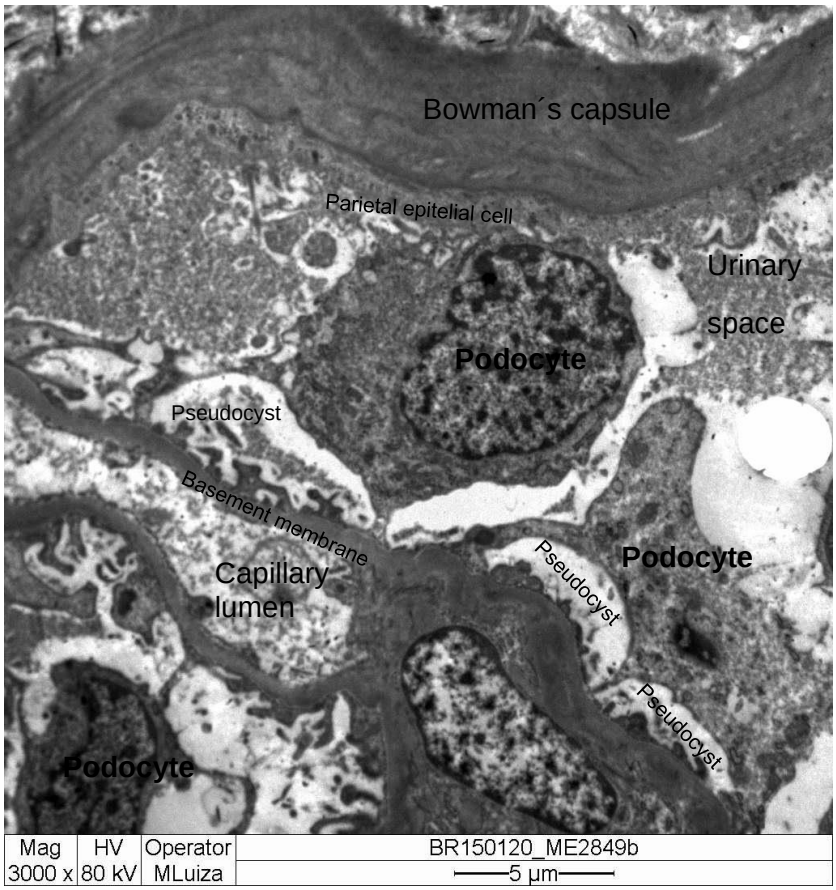
A- Podocyte with multiple pseudocysts, change in shape probably due to increased shear stress, which apparently increases the possibility of loss of adhesion to the GBM.



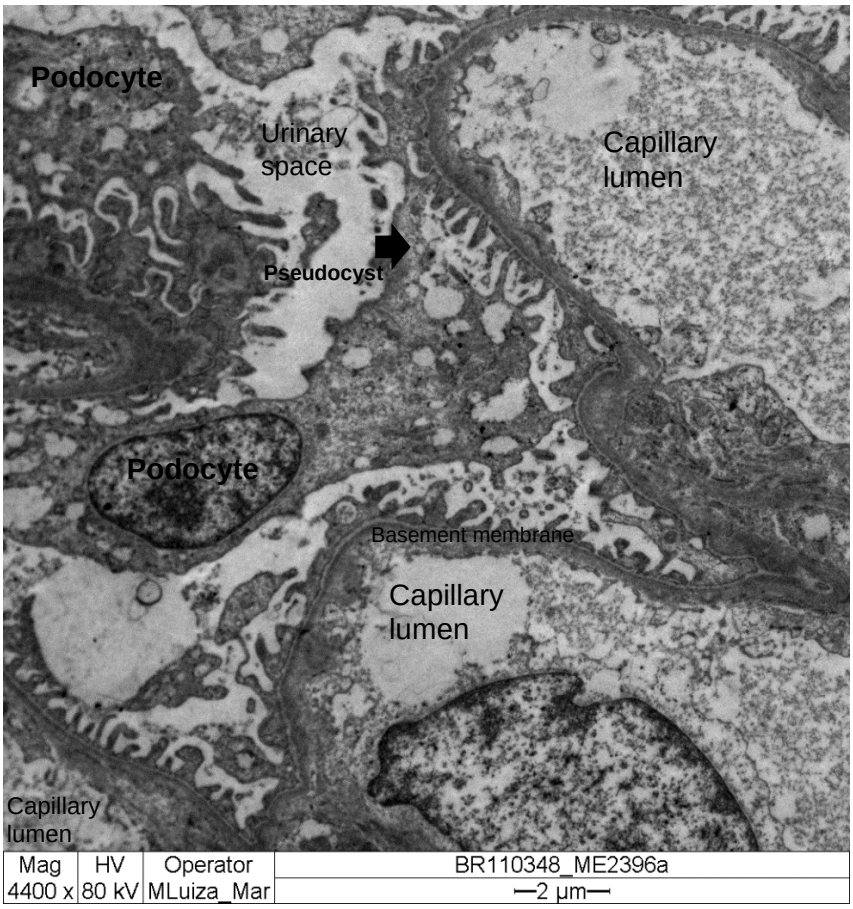
B- Areas of bare GBM, only sparse remnants of podocytes and red blood cells in the urinary space (hematuria).



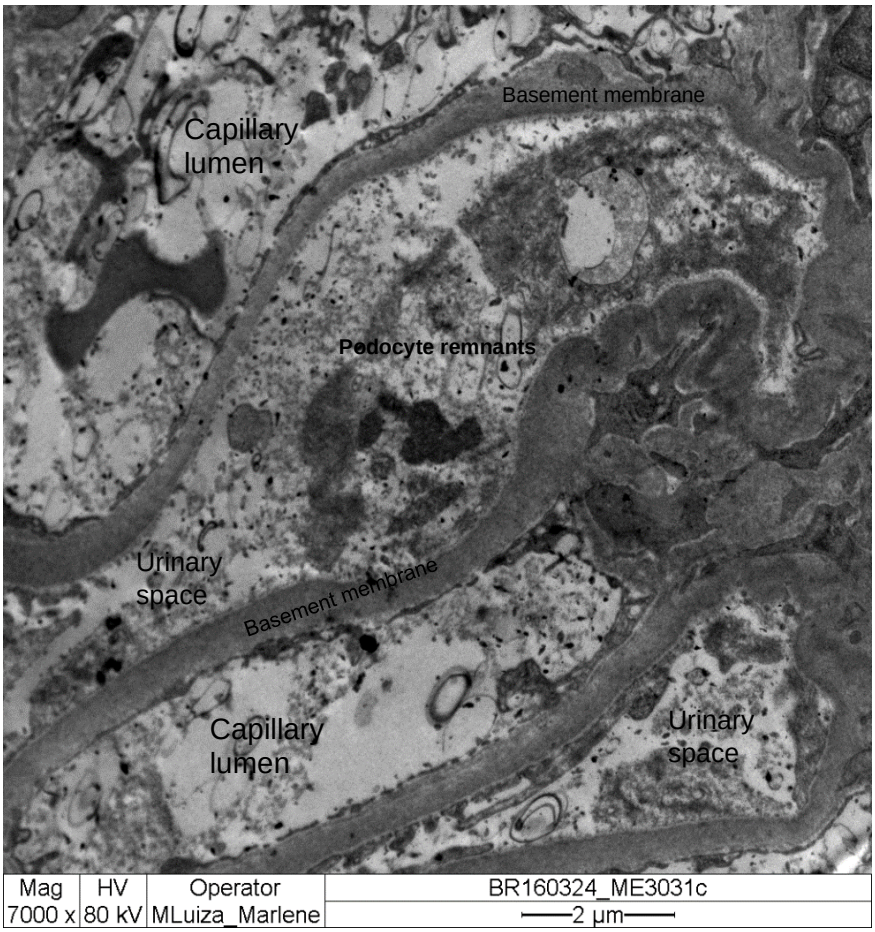
C- There are two podocytes in the image, with their respective nuclei with usual aspect. In both, there are endosomes and autophagossomes in the cytoplasm. There is granular material in the urinary space compatible with proteinuria.



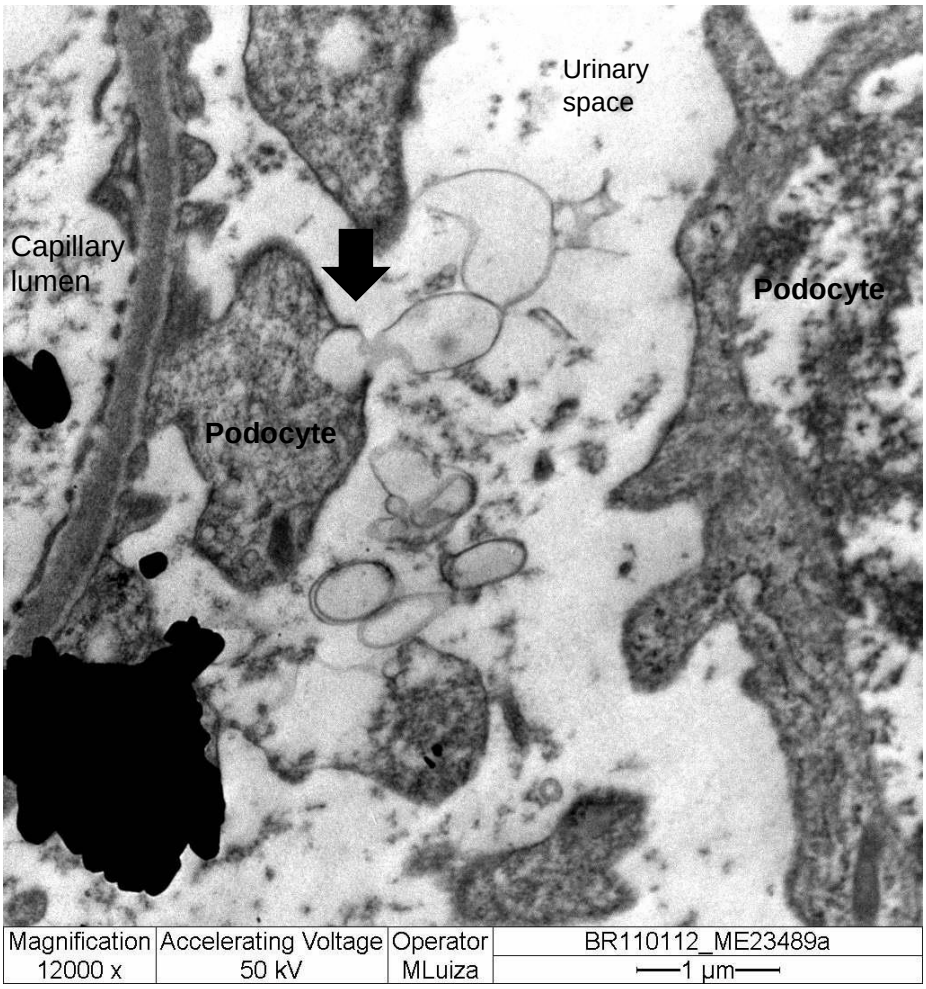
D- Podocytes with pseudocysts. One is also attached to the parietal epithelial cell.



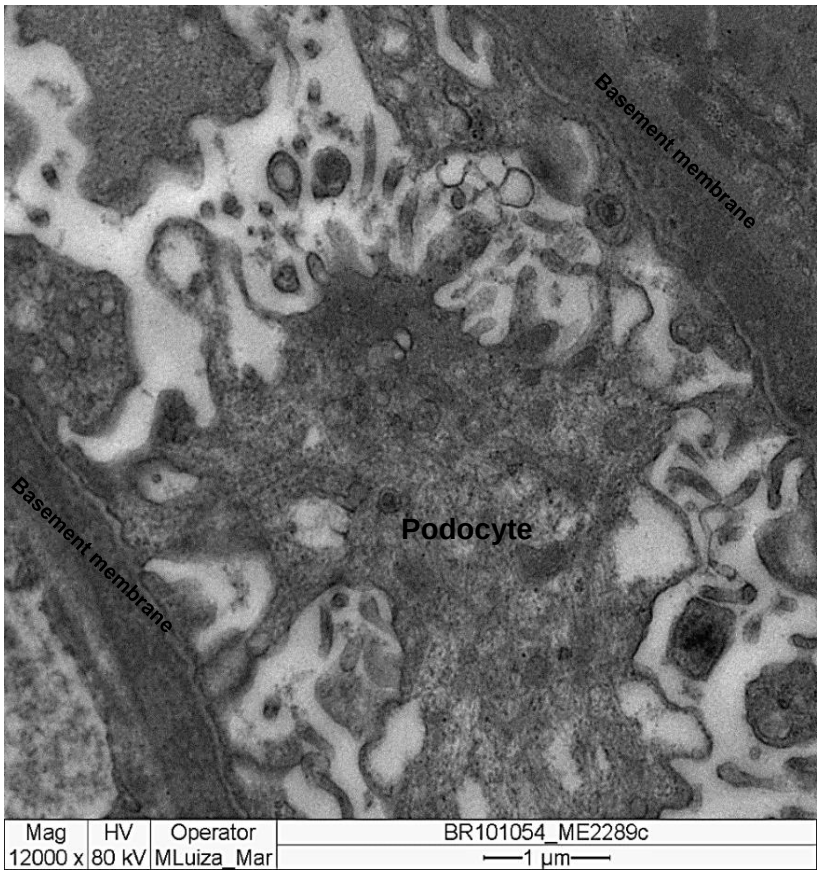
E- Podocyte with pseudocyst at its junction with the GBM. It is remarkable the possible communication between these cavities.



F- Extensive area of bare GBM with podocyte remnants in the urinary space, a morphological evidence of cell death by necrosis, characterized by oncosis and plasmatic membrane lysis.



G- Preserved foot process and a segment of podocyte cytoplasm exteriorizing endosome content into the urinary space (arrow).



H- Podocyte cell body with shedding. In the adjacent foot process, there is a double membrane round structure compatible with autophagosome.

6 COMENTÁRIOS / CONSIDERAÇÕES FINAIS

Acreditamos que no futuro serão descobertos métodos não invasivos para o diagnóstico da NIgA, especialmente biomarcadores séricos e/ou urinários. Neste contexto, será essencial predizer os parâmetros da classificação de Oxford a partir dos dados clínico-laboratoriais. Assim, mesmo sem a biópsia renal, será possível estabelecer o prognóstico de cada paciente e a melhor estratégia terapêutica. Demonstramos neste estudo que todos os parâmetros da classificação de Oxford possuem correspondência com algum dado clínico-laboratorial. Isto viabiliza novas abordagens e protocolos no futuro.

Nossos resultados indicam que lesões de esclerose segmentar não relacionadas especificamente a podocitopatias também podem influenciar parâmetros clínicos que afetam a evolução da doença renal, como a proteinúria. Assim, favorecemos a incorporação do método de subclassificação do parâmetro S na rotina de avaliação da biópsia renal, a fim de contribuir para maior esclarecimento da fisiopatologia. O futuro determinará um consenso sobre o papel de cada subtipo na evolução dos pacientes e se há sentido em manter esse método.

A variedade de subtipos de glomerulosclerose segmentar que podem ser encontradas em cada glomérulo e em cada biópsia renal, ainda é um desafio no estabelecimento do grau de influência de cada subtipo nas manifestações clínicas. Os estudos disponíveis são raros, exigindo avaliação adicional em diferentes populações para confirmar e reforçar nossos resultados.

Com relação às lesões podocitárias, demonstramos que há relação com parâmetros clínicos, uma vez que redução quantitativa nos podócitos mensurada pela imunomarcação pelo WT1 está correlacionada à proteinúria nefrótica e que nos casos com presença de autofagia em podócitos, há significativamente menor frequência de hematúria.

Nossos dados favorecem a hipótese de que o apagamento dos pedicelos seja uma alteração adaptativa para evitar o destacamento dos podócitos e que, essa alteração não seria capaz, por si só, de gerar proteinúria.

Os podócitos danificados, apresentam evidências de morte celular como necrose e catástrofe mitótica. Há ainda expressiva proporção de podócitos com autofagia na NIgA. Em relação à apoptose, confirmamos dados previamente encontrados na literatura sobre a não detecção desse tipo de morte celular em podócitos.

Na avaliação dos podócitos que se desprenderam da MBG, foi encontrado que os casos com apenas autofagia tinham menor proporção de podócitos soltos e a maior proporção destes foi encontrada em casos com autofagia e necrose associadas. Em contrapartida, os casos sem destacamento de podócitos estavam mais frequentemente associados a autofagia isolada. Portanto, diante da controvérsia existente acerca do papel da autofagia na podocitopenia, nossos dados sugerem que a autofagia seja um mecanismo citoprotetor. No entanto, mais estudos são necessários para estabelecer o papel da autofagia no destino dos podócitos. Além disso, os casos com presença de podócitos destacados não se relacionaram a pseudocistos, uma evidência de que, em nossos casos de NIgA, a podocitopenia relaciona-se mais com lesão podocitária do que com alteração mecânica.

A utilização da técnica de MET na rotina diagnóstica das biópsias renais contribui para a avaliação da gravidade da doença em pacientes com NIgA, além de suas outras funções diagnósticas já estabelecidas. Com o avançar de novos estudos na área das lesões podocitárias nessa doença, novas correlações entre lesões serão feitas, o que possibilita descoberta de novas vias fisiopatológicas e, no futuro, intervenções terapêuticas específicas para evitar e minimizar os danos podocitários, a fim de proporcionar melhor prognóstico aos pacientes com essa doença.

7 CONCLUSÕES

O objetivo deste estudo foi avaliar, em pacientes com NIgA, a associação entre parâmetros morfológicos e clínicos. Interessantemente, nossos resultados indicam que cada parâmetro da Classificação de Oxford pode ser predito por um ou mais parâmetros clínicos. Especialmente o parâmetro S necessita ser mais estudado, pois diversas variações morfológicas parecem impactar de forma diferente na proteinúria. Pacientes com menor densidade de podócitos apresentam níveis mais elevados de proteinúria e o destacamento de podócitos é mais frequente nos casos de necrose. Por outro lado, a autofagia parece ser um mecanismo citoprotetor contra o destacamento de podócitos e também contra a hematúria. Portanto, muitas alterações ultraestruturais podem impactar os parâmetros clínicos, e não apenas a mais descrita e estudada, o apagamento dos pedicelos. A análise ultraestrutural deve ir muito além da morfologia dos pedicelos.

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