Complement System and C4d expression in cases of Membranous nephropathy

Sistema complemento e expressão de C4d em casos de glomerulonefropatia membranosa

ABSTRACT

Introduction: Membranous nephropathy (MN) is one of the major causes of nephrotic syndrome. The complement system plays a key role in the pathophysiology of MN. Objectives: To identify the complement pathway possibly activated in MN cases and correlate the presence of C4d with more severe clinical and histological markers. Methods: Sixty nine cases from renal biopsy with membranous nephropathy were investigated. The presence of C1q was analyzed by direct immunofluorescence; and expression of C4d by immunohistochemistry. Clinical and epidemiological data were obtained upon biopsy request. Results: The presence of focal segmental glomerulosclerosis, global glomerulosclerosis, vascular lesions and tubulointerstitial fibrosis were collected by anatomopathological report. C4d(+) was found in 58 (84%), and C1q(+) was found in 12 (17%) of the cases. Twelve patients had C4d(+)/C1q(+), 46 had C4d(+)/C1q(-), and 11 patients had C4d(-)/C1q(-), probably indicating the activation of the classical, lectin and alternative pathways, respectively. Conclusion: C4d was associated with increased interstitial fibrosis, but not with clinical markers of poor prognosis. Through the deposition of C4d and C1q we demonstrated that all complement pathways may be involved in MN, highlighting the lectin pathway. The presence of C4d has been associated with severe tubulointerstitial lesions, but not with clinical markers, or can be taken as a universal marker of all cases of MN.

Key words: complement activating enzymes; complement system proteins; glomerulonephritis, membranous.

RESUMO

Introdução: A Glomerulopatia membranosa (GM) é uma das principais causas da síndrome nefrótica. O sistema do complemento desempenha um papel chave na fisiopatologia do GM. Objetivos: Identificar a via do complemento possivelmente ativada nos casos de GM e correlacionar a presença de C4d com marcadores clínicos e histológicos mais graves. Métodos: Foram investigados 69 casos de biópsia renal com GM. A presença de C1q foi analisada por imunofluorescência direta e a expressão de C4d por imunohistoquímica. Dados clínicos e epidemiológicos foram obtidos mediante solicitação de biópsia renal. Resultados: A presença de glomerulosclerose segmentar focal, glomeruloesclerose global, lesões vasculares e fibrose tubulointersticial foi coletada por relato anatomopatológico. C4d(+) foi encontrado em 58 (84%), e C1q(+) foi encontrado em 12 (17%) casos. Doze pacientes tinham C4d (+)/C1q (+), 46 tinham C4d (+)/C1q (-) e 11 pacientes tinham C4d (-)/C1q (-), indicando provavelmente a ativação da via clássica, da lectina e da alternativa, respectivamente. Conclusão: O C4d foi associado ao aumento da fibrose intersticial, mas não com marcador clínico de mau prognóstico. Através da deposição de C4d e C1q, demonstrou-se que todas as vias do complemento podem estar envolvidas em GM, destacando a via da lectina. A presença de C4d tem sido associada a lesões tubulointersticiais graves, mas não com marcadores clínicos, ou pode ser tomada como um marcador universal de todos os casos de GM.

Palavras-chave: enzimas ativadoras do complemento; glomerulonefrite membranosa; proteínas do sistema complemento.
**INTRODUCTION**

Membranous nephropathy (MN) is one of the major causes of nephrotic syndrome in adults. It has a variable clinical course, and one third of patients progress to end-stage renal disease.\(^1\)

Older age, renal failure at diagnosis, intensity of proteinuria, glomerulosclerosis, vascular lesions and tubulointerstitial fibrosis are associated with poor prognosis.\(^2\) Thus, knowledge of clinical and histological markers of severity and prognosis is essential for an optimal therapeutic approach.\(^3\)

MN is due to the *in situ* deposition of antibodies against antigens podocyte and studies suggest that M-type phospholipase A2 receptor is one of the major antigens involved, implicated in approximately 70% of cases of primary MN.\(^4\) These immune complexes activate the complement system (CS), and are associated with podocyte injury, proteinuria and consequent renal failure.\(^4\)

The CS may be activated by three pathways: (1) the classical pathway, by binding immunoglobulins to the pathogen and C1q activation; (2) the lectin pathway, in which plasma proteins of the collectin family (mannose-binding proteins - MBLs - and ficolins) binds to immunoglobulins; and (3) the alternative pathway, which is constantly activated at low levels, resulting in spontaneous hydrolysis of the C3 component.\(^5\) Activation of any of these three pathways leads to the formation of the membrane attack complex (C5b-9), which promotes target cell membrane damage.\(^6\)

The complement system is critical for the onset of several glomerulopathies.\(^6\) In MN, cytotoxicity mediated by the complement system has been implicated as a major factor due to the effector function of C5b-9, which is found in the kidney and urine of patients with MN.\(^7\) In primary MN, IgG4 is the main immunoglobulin deposited in the kidney and the classical theory suggests that does not bind C1q and hence does not lead to activation of the classical pathway, but in the lectin pathway.\(^8\) Furthermore it has been demonstrated direct binding of MBL to the IgG4-PLA2R.\(^9\) However, there are case reports and experimental studies in which the expression of other IgG subclasses, and the presence of components of other complement pathways were observed in MN;\(^10-12\) thus, there is still no consensus on which complement pathway is more activated.\(^7\)

C4d is a degradation product of C4 during activation of the classical and lectin pathways of the CS, and because of its strong covalent bonds to the tissue, it has been used as a marker of antibody-mediated response and of the complement system activation.\(^13\) The classical clinical application of C4d is in the diagnosis of antibody-mediated rejection,\(^13\) but it has been used in glomerulopathies as a marker of complement activation and worse prognosis.\(^14\) Although these studies show high positivity for C4d in MN,\(^15,16\) there are no studies evaluating its correlation with clinical and histological markers.

Given the importance of the participation of the complete system in MN, this study aimed to evaluate MN cases regarding C4d expression, thus identifying the complement pathway possibly activated, and correlating the presence of C4d with more severe clinical and histological markers.

**MATERIALS AND METHODS**

This study was approved by the Triângulo Mineiro Federal University Research Ethics Committee, approval number 999. Cases in which individuals were diagnosed with MN were selected from Renal Biopsy Files of the Nephropathology/General Pathology Service of UFTM from 1996 to 2012. The diagnoses had all been made by the same nephropathologist, and were confirmed by light microscopy and immunofluorescence analysis, as well as electronic analysis.

Probable cases of secondary MN were excluded, as well as cases with incomplete clinical data, in which there was deposit of all immunoglobulins, mesangial deposits and cases with no histological representation for light microscopy or immunofluorescence. In total, 69 cases were studied.

Clinical and epidemiological data such as gender, age, ethnicity, presence or absence of systemic hypertension (SH), presence or absence of renal insufficiency (RI), presence or absence of nephrotic proteinuria (NP), and average proteinuria (g/24h) were obtained upon biopsy request.

SH was defined when blood pressure values > 140 x 90mmHg and/or when the patient used antihypertensive drugs. RI was defined as creatinine clearance < 60 ml/min, which was calculated using the CKD-EPI equation. NP was considered when ≥ 3.5g/24h.

Anatomopathological report allowed for the following histological data to be collected: presence or
absence of focal segmental glomerulosclerosis (FSGS), presence or absence of global glomerulosclerosis (GS) > 10%, vascular lesions (arteriolar hyalinosis and intimal fibroelastic thickening), and tubulointerstitial fibrosis (IF/TA); the latter two were classified as absent (0), mild (1), moderate (2) or severe (3). The stage of MN was also graded according to the criteria of Ehrenreich and Churg.

The presence of C1q was analyzed by direct immunofluorescence, through polyclonal rabbit anti-human C1q Complement antibody response (1:5, Dako®, Inc., Glostrup, Denmark)

Expression of C4d was performed by immunohistochemistry using the immunoperoxidase technique. Then the sections were deparaffinized and hydrated, and antigen retrieval was performed in a Pascal pressure chamber (Dako®, Denmark) at 121°C and 18 psi. After incubation with anti-C4d primary antibody (1:600, ALPCO®, Salem, NH, USA), the sections were developed using Novolink Max Polymer Kit (Leica Microsystems, Inc., Wetzlar, Germany) as a secondary antibody, and diaminobenzidine (Dako®, Denmark).

Immunostaining for C4d was considered positive in cases with more than 50% of immunostained glomeruli.

For statistical analysis, data were entered into a Microsoft Excel spreadsheet and later analyzed using GraphPad Prism software version 5.0 (GraphPad Software, Inc; La Jolla, CA, USA). The variables were tested for normal distribution using the Kolmogorov-Smirnov test, and homogeneity analysis was also performed. ANOVA test was used for comparisons between three or more groups of parametric variables, Kruskal-Wallis H test was used for comparison between three or more groups of non-parametric variables, Student’s t-test was used for comparison between two groups of parametric variables, Mann-Whitney U test was used for comparison between two groups of non-parametric variables, and the Chi-square ($X^2$) and Fisher’s exact test were used to compare proportions, i.e. qualitative variables. Differences were considered statistically significant when “p” was less than 5% ($p < 0.05$).

RESULTS
The average age was 46.91 years (13-80 years). Of these cases, 44 patients (64%) were over 40 years. Forty-four (63.8%) patients were male and 52 (75.3%) patients were Caucasian. As for clinical findings, 11 patients (15.9%) had kidney failure at the time of biopsy. SH was found in 39 patients (53.6%) and NP was found in 42 patients (60.1%). The median value of proteinuria was 5.04 ± 3.7g/24h.

Histopathological data regarding the stages of MN, FSGS, GS, vascular lesions, IF/TA, deposition of C1q and C4d are detailed in Table 1.

C4d was positive in 58 (84%) cases (Figure 1). Of these, 12 cases (17%) also exhibited deposition of C1q (no deposition of immunoglobulins or mesangial deposits), indicating probable cases of activation of the classical complement pathway. In 46 cases (67%), only C4d was positive indicating activation of the lectin pathway of the CS.

In 11 cases (16%) both C4d and C1q were negative, indicating a probable ongoing activation of the alternative pathway. Thus, the lectin pathway (C1q-C4d+) was more activated than the classical pathway (C1q+ C4d+) and the alternative pathway (C1q- C4d-) in the study sample.

The C4d(+) cases showed no higher prevalence of kidney failure ($p = 0.364$), systemic hypertension ($p = 0.743$), nephrotic proteinuria ($p = 1$) or higher mean values of proteinuria ($U = 270; p = 0.431$) in comparison with the C4d(-) cases.

When analyzing the presence of C4d with histological markers, higher mean GS ($U = 42, p = 0.202$), presence of FSGS ($p = 0.49$) or more severe vascular injury ($X^2 = 0.92; p = 0.82$) were not observed in the C4d(+) cases in comparison with the C4d(-) cases. As for tubulointerstitial injury, C4d positive cases had greater degrees of IF/TA (moderate or severe) compared to C4d negative cases ($p < 0.001$) (Figure 2).

DISCUSSION
MN is one of the main glomerulopathies, and it has been subject of several studies aimed at better understanding its pathophysiology since the 1950s so that better treatment options are developed.17

Although many studies have focused on discovering the target antigen, nowadays attention has turned to the effector mechanisms of podocyte injury, particularly the activation of the complement system and the role of different cytokines.

In this sample, older Caucasian male individuals predominated (64% older were than 40 years), which is in keeping with other Studies.18 We found similar levels of hypertension and renal failure, and slightly lower levels of nephrotic syndrome when compared
Table 1. Histopathological data and deposition of C4d and C1q in patients with membranous nephropathy

<table>
<thead>
<tr>
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<th>n (%)</th>
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<tbody>
<tr>
<td>Membranous nephropathy (Stage)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>25 (36)</td>
</tr>
<tr>
<td>2</td>
<td>37 (54)</td>
</tr>
<tr>
<td>3</td>
<td>7 (10)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Focal Segmental Glomerulosclerosis</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>49 (71)</td>
</tr>
<tr>
<td>Yes</td>
<td>23 (33)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glomerular Sclerosis &gt; 10%</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>46 (67)</td>
</tr>
<tr>
<td>Yes</td>
<td>10 (14)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vascular Injury (Classification)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>14 (20.2)</td>
</tr>
<tr>
<td>Mild</td>
<td>42 (60.8)</td>
</tr>
<tr>
<td>Moderate</td>
<td>12 (17.3)</td>
</tr>
<tr>
<td>Severe</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Absent</td>
<td>11 (16)</td>
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<td></td>
<td></td>
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<tr>
<td>Interstitial Fibrosis and Tubular Atrophy (Classification)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>58 (84)</td>
</tr>
<tr>
<td>Moderate</td>
<td>57 (83)</td>
</tr>
<tr>
<td>Severe</td>
<td>12 (17)</td>
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</tbody>
</table>

Figure 1. Immunostaining of C4d in the kidney of patients with membranous nephropathy: (A) Immunostaining of C4d in the positive control with lupus nephritis (400x); (B) Immunostaining of C4d in a case with positive C4d (800x).

to that reported in the Literature. Also, there was a higher distribution of cases in stages 1 and 2, which is the same observed by a Spanish group of researchers, but different from other studies in which stages 2 and 3 prevailed.

Regarding the histological lesions, focal segmental glomerulosclerosis (20 - 29%) and global glomerulosclerosis > 10% (23 - 33%) were found to be less prevalent, as well as moderate or severe tubulointerstitial lesions (13 - 19%). Higher degrees of moderate or severe vascular lesions (35 - 51%) were observed in this sample.

Other studies analyzing morphological changes in MN patients showed different findings. Chen et al., studied 129 patients with MN and found that 10% of the cases had FSGS, 75% had moderate or severe atherosclerosis, and 33% had moderate-severe tubulointerstitial fibrosis. On the other hand, in a study by Troyanov et al., consisting of 389 cases of MN, there was an association of vascular lesions (in 25%
Figure 2. Intensity of interstitial fibrosis/tubular atrophy (IF/TA) according to C4d deposition in the kidneys of patients with membranous nephropathy: IF/TA was moderate or severe in positive C4d, when compared to the negative C4d patients (p < 0.001).

of the cases, moderate or severe) and interstitial fibrosis (in 18%, moderate to severe).

It is known that the complement system plays a key role in the pathophysiology of MN, however the evidences regarding which specific complement pathway predominates in MN is still inconclusive. C4d was positive in 58 (84%) cases in this study. However, there were more positive cases, between 92 and 100%, in other studies that also investigated the occurrence of C4d in MN. In a study by Song et al., deposition of C4 was not observed in cases of primary MN, unlike the cases of MN secondary to systemic lupus erythematosus. Different methodologies can explain such discrepancies. The number of cases evaluated was also different; there was a higher number of cases in this study than in the aforementioned studies.

Not only C4d but also C1q was found to be positive in 12 cases (17%), which may indicate a potential participation of the classical pathway. A case of recurrent MN has been recently reported after renal transplantation, in which deposits of IgG3 and C1q were observed, indicating a possible involvement of the classical pathway in glomerular injury.

In a study by Segawa et al., the pattern of segmental MN was found in 6/16 cases, and it was associated with deposition of C1q and IgG3 as well. Obana et al., also found the presence of C1q in cases of segmental MN. In another study, IgG1 subclass predominated over IgG4 in stages I and II of MN, indicating that the classical pathway might be present in early MN lesions.

There were no deposits of C4d or C1q in 11 patients (16%). In these cases, we concluded that activation of the alternative complement pathway may be in progress. Segawa et al. found that some patients were also positive for factor B, a marker of the alternative pathway and this finding was also observed when immunostaining for all immunoglobulins and complement factors was performed in MN cases in another study.

Recently, Bally et al. reported a case of primary MN in a patient with deficiency of MBL, intense IgG4 deposits, PLA2R and factor B and weak deposits C1q and C4d, indicating the alternative pathway activation of the complement in this case.

In experimental models of MN, glomerular injury was found to be likely due to absence, dysfunction or inhibition of the counterregulatory proteins of the alternative complement pathway. Thus, it is possible that IgG triggers these injuries by activating the classical complement pathway, leading to the activation of the alternative pathway and especially the lectin pathway later.

Therefore, as seen in this study, the lectin pathway is believed to be predominant in MN cases although there may be cases in which activation of the other pathways is triggered as well.

What can explain the non-uniformity of the complement system activation pathways is the fact that there may be other antigens (like thrombospondin, bovine serum albumin, superoxide dismutase 2 and aldose reductase) than only PLA2R in MN, whose mechanisms of renal injury they are not fully known.

In this sample, C4d was not associated with clinical markers of severity or worse prognosis. Histological analysis allowed for association of the presence of C4d with increased tubulointerstitial fibrosis. There are no reports in the literature linking C4d to clinical and/or histological markers in MN, but that is observed in other glomerulopathies. In IgA nephropathy, the presence of C4d was associated with worse prognosis.

Conclusion

We concluded that C4d expression is important in cases of MN, thus indicating the participation of the CS in the pathophysiology of glomerular lesions. Although the lectin pathway appears to be the most prevalent, participation of the classical and alternative pathways was found to be possible. The presence of C4d has been associated with more severe tubulointerstitial lesions. Further studies on the complement...
system in MN are necessary to provide a better understanding of its pathophysiology and an optimal clinical approach.

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References


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