

# C4d immunohistochemical staining is a sensitive method to confirm immunoreactant deposition in formalin-fixed paraffin-embedded tissue in membranous glomerulonephritis

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**Summary.** Although the diagnosis of membranous glomerulonephritis (MGN) may be suspected on routine histology of formalin-fixed paraffin-embedded tissue, fresh-frozen tissue must be used to show the immunologic nature of the process by direct immunofluorescence (IF). The efficiency of IF or immunoperoxidase (IP) detection of IgG and C3 using paraffin sections is controversial. This study was designed to evaluate whether glomerular C4d deposition using an IP method in formalin-fixed paraffin-embedded tissue may be a useful marker for MGN. We showed characteristic glomerular, granular basement membrane deposition of C4d in 31 (100%) cases of idiopathic MGN and in 5 cases (100%) of pure class V membranous lupus nephritis, in which we had a positive diagnosis of the lesions for conventional IF study. Control cases were negative. Nineteen cases of different glomerulopathies, including IgA nephropathy, primary type I membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis and minimal change disease showed diverse reproducible patterns of C4d deposition, without intrinsic background. Our results indicate that staining of formalin-fixed paraffin-embedded tissue for C4d can be used for confirmation of granular basement membrane immunoreactant deposition in cases of MGN. This proved to be a reliable method that could potentially obviate the need for rebiopsy in cases with absence of glomeruli in renal frozen sections or when other adjunct IF or IP methods on paraffin sections are negative. C4d immunostaining, using an IP method, deserves a place as an adjunct

method in the biopsy diagnosis of MGN.

**Key words:** C4d, Glomerulonephritis, Immunoperoxidase methodology, Immunofluorescence method, Membranous glomerulonephritis, Paraffin embedded section, Polyclonal antibody

## Introduction

Membranous glomerulonephritis (MGN) is a major cause of nephrotic syndrome in adults. It is characterized by the presence of immunoglobulins and complement deposits along the subepithelial side of the glomerular basement membrane as a form of chronic antigen-antibody-mediated disease. MGN may occur in association with known etiologic agents or known disorders (secondary MGN) or can be idiopathic (primary MGN). In about 85% of patients the disease is idiopathic.

Early in the disease, the glomeruli may appear normal by light microscopy. Thus, pathological diagnosis demands fresh-frozen tissue to show the granular deposits of IgG and C3 by immunofluorescence (IF) microscopy. In addition, glomerular deposition of C4d investigated by IF microscopy has been reported as a sensitive indicator of MGN (Kusunoki et al., 1989).

However, in some cases the biopsy can contain scant number of glomeruli, and the tissue selected for IF microscopy may show absence of those and be useless for diagnosis. Results of immunofluorescence on paraffin-embedded sections is controversial and it is still difficult to obtain reproducible results (Wagrowska-Danilewicz and Danilewicz, 2009). The efficiency of immunoperoxidase (IP) detection of IgG and C3 using

paraffin sections is controversial too.

In this report we demonstrate that formalin-fixed paraffin-embedded tissue can be stained for the immunoreactant C4d, using an IP method, to show the characteristic deposition in MGN, obviating the need for repeat biopsy. This method has been revealed as a useful and reproducible tool in the diagnosis of the disease.

### Material and methods

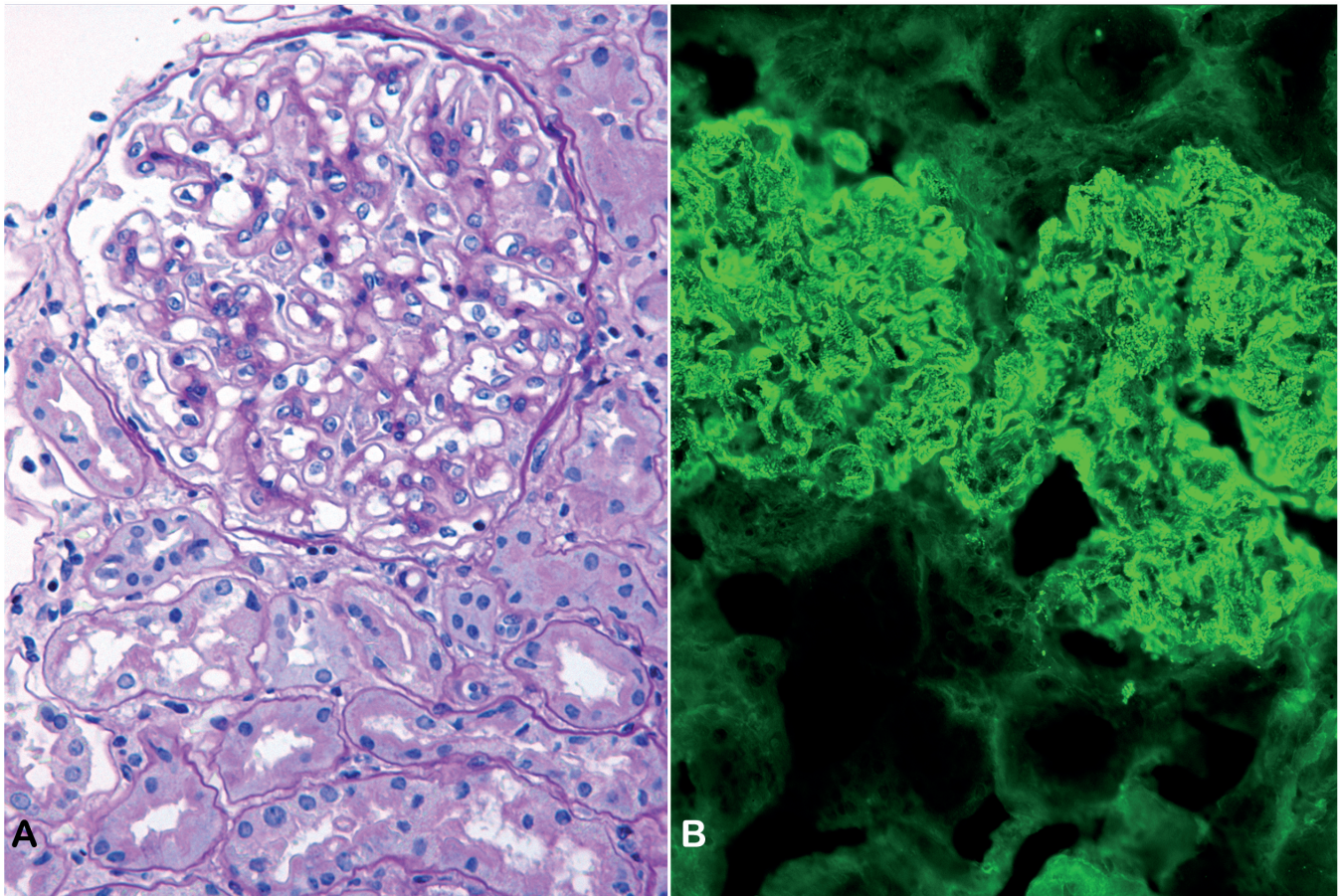
Thirty one patients diagnosed as having idiopathic MGN between January 2004 and September 2010 were the subjects of this study. The indication of renal biopsy was for evaluation of nephrotic syndrome. The mean age of the patients was  $57.9 \pm 15.01$  years (range 23-79 years, median 61 years). Twenty three patients were male and eight were female. The relation male:female was 2.9:1.

Biopsy cores were sectioned under a dissecting microscope into two parts: one was introduced into buffered neutral formalin for light microscopic examination, and the remainder was embedded in optimal cutting temperature (OCT) compound (Miles

Laboratories, Elkhart, IN, USA) for IF staining. In addition, in four selected cases another part of the core was inserted into 2.5% glutaraldehyde in 0.2M Sorenson's sodium phosphate buffer for electron microscopic examination. Formalin-fixed tissue sections were routinely stained with hematoxylin and eosin, periodic acid Schiff, Masson's trichome, and methenamine silver. Fresh frozen tissue was stained with FITC-conjugated polyclonal antibodies directed against human IgG, IgA, IgM, C3, C4, C1q, fibrinogen, and albumin (Dako, Glostrup, Denmark).

The deposition of C4d was detected in formalin-fixed paraffin-embedded tissue. IP method for C4d antibody (polyclonal, prediluted, Cell Marque Corporation, Roklin, CA, USA, Ventana dispenser catalog number 760-4436) was performed in an automated platform (BenchMark Ultra autostainer, Ventana Medical Systems Inc., Tucson, AZ, USA) which includes tissue CC1 standard pre-treatment.

For the evaluation of C4d deposition, morphologically normal adult renal tissue, formalin-fixed and paraffin embedded, obtained from unaffected



**Fig. 1.** Membranous glomerulonephritis, stage I. **A.** The glomerulus is normocellular, with basement membranes of normal thickness and texture (PAS stain, original magnification  $\times 200$ ). **B.** Immunofluorescence reveals a thin rim of finely granular, global deposits outlining the walls of the glomerular capillary loops (antibody specific for IgG, original magnification  $\times 100$ ).



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parts of ten renal core biopsies performed for localized renal cell carcinoma were used as controls.

In addition, sections of twenty four glomerulopathies were included in the study: there were five cases of pure class V membranous lupus nephritis (MLN), six cases of IgA glomerulonephritis (IgAGN), four cases of primary type I membranoproliferative glomerulonephritis (MPGN), five cases of focal segmental glomerulosclerosis (FSGS) and four cases of minimal change disease (MCD).

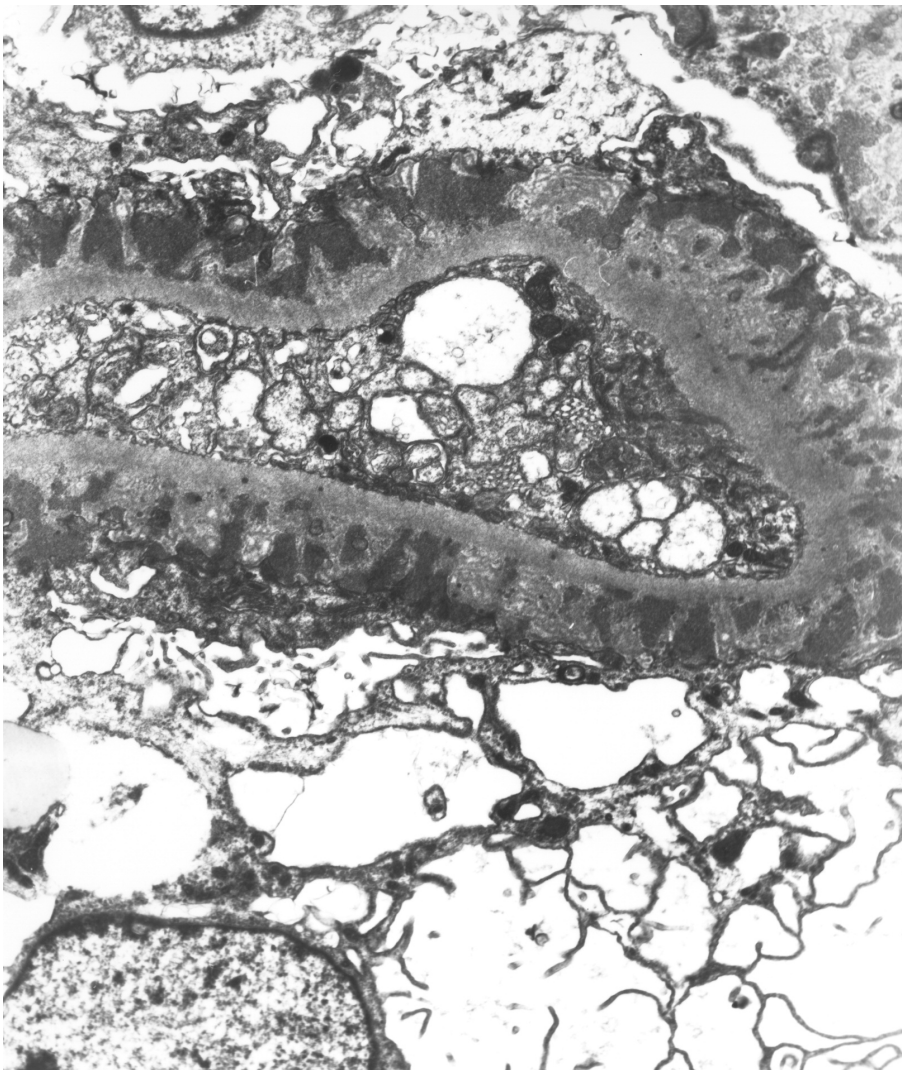
**Results**

The pathologic diagnosis was based on the microscopic appearance of the glomeruli. They were normocellular with totally normal light microscopy aspect (Fig. 1A) or showed slight or marked global thickening of capillary walls. In all cases they showed

diffuse, global, granular intense staining of capillary walls for IgG (Fig. 1B) and C3. Additional deposits with lower intensity of IgM, 3 cases, IgA, 2 cases, and C1q, 1 case, were observed in the capillary loops. The electron microscope revealed the presence in all four cases of electron-dense deposits along the subepithelial side of the basement membrane with adjacent projections of glomerular basement membrane material in one case. The foot processes were obliterated (Fig. 2).

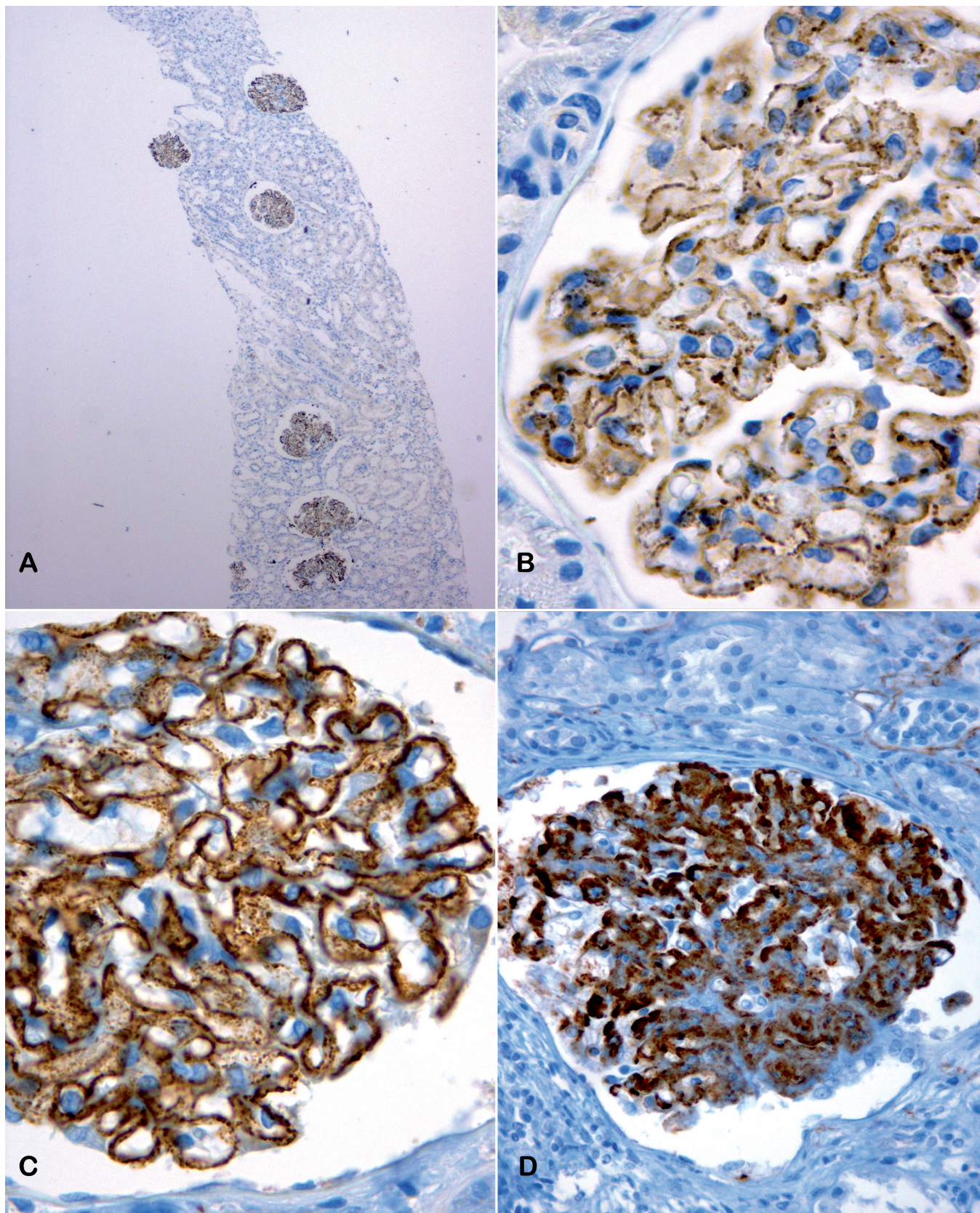
According to the classification of Germuth and Rodriguez (1973) glomerular lesions were stage I in 22 cases (71.0%), stage II in 8 cases (25.8%), and stage III in one case (3.2%).

Diffuse, global, fine, intense, granular deposits of C4d were identified along the glomerular capillary loops in 31 cases (100%) (Fig. 3A). The deposits were finely granular outlining the walls of glomerular capillaries in stage I (Fig. 3B). These deposits were heavier in stages



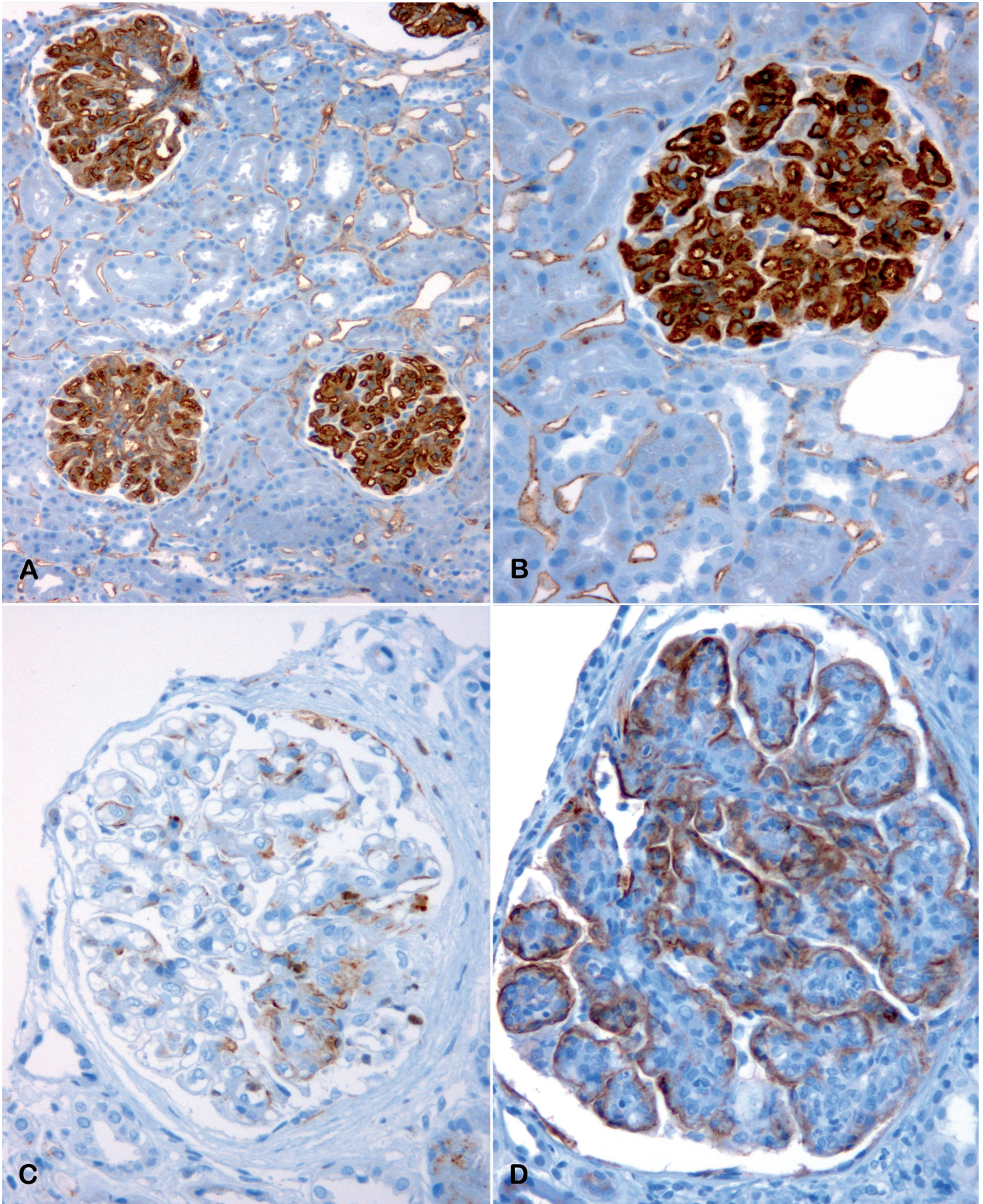
**Fig. 2.** Membranous glomerulonephritis, stage III. Abundant electron dense deposits are noted in the outer portion of the thickened glomerular basement membrane. There are projections of the basement between the deposits. Foot process effacement can be seen (electron microscopy, original magnification x 4,500).





**Fig. 3.** Membranous glomerulonephritis. **A.** Panoramic view of glomeruli showing basement membranous deposition of C4d. (polyclonal antibody for C4d, original magnification x 16). **B.** Stage I of the disease. Thin rim of C4d<sup>+</sup> finely granular deposits outlining the walls of the loops (original magnification x 400). **C.** Stage II of the disease. Thicker layer of C4d<sup>+</sup> granular deposits (original magnification x 400). **D.** Stage III of the disease. Dense masses of C4d<sup>+</sup> deposits in thickened capillary loops (original magnification x 200).





**Fig. 4.** Representative immunoperoxidase study in different glomerulonephritis. **A.** Panoramic view of class V membranous lupus nephritis showing global, striking, granular membranous glomerular deposits of C4d. These deposits are also visible along of peritubular capillaries (original magnification x 100). **B.** Detail of class V membranous lupus nephritis showing C4d membranous deposition and a background of mesangial deposits. The peritubular capillaries show strong, granular and diffuse deposition of C4d (original magnification x 200). **C.** Faintly detectable mesangial, subendothelial and capsular C4d deposit in IgA nephropathy (original magnification x 200). **D.** Granular deposition of C4d along the periphery of the glomerular capillary loops in membranoproliferative glomerulonephritis (original magnification x 200).



II (Fig. 3C) and III (Fig. 3D). C4d deposition within the mesangial area was not seen.

Controls showed no evidence of false positivity.

The five cases of pure class V MLN showed delicate or striking, global, granular deposits of C4d along the glomerular basement membrane with a background of occasional mesangial deposits (Fig. 4A). These membranous deposits were similar to those found in idiopathic MGN. Besides, in one case diffuse, granular C4d deposits in peritubular interstitial capillaries (PTCs) were present. These capillaries showed no neutrophil or mononuclear cell margination or fibrin thrombi (Fig. 4B).

Four cases of IgAGN showed a scant, irregular, mesangial, subendotelial and capsular C4d deposit (Fig. 4C). Primary type I MPGN cases showed marked peripheral, capillary wall granular staining for C4d (fringe-like pattern). Mesangial deposits were absent (Fig. 4D).

FSGS showed scant glomerular deposition of C4d in the synechial attachment to Bowman's capsule and in occasional mesangial zones.

C4d deposition was not seen in cases of MCD.

## Discussion

Direct IF on frozen tissue is the most widely used technique for diagnosis of renal antigen-antibody-mediated disease. This method is simple, fast and sensitive. However, not uncommonly, glomeruli are not present in the portion of tissue submitted for IF. In these cases formalin-fixed, paraffin-embedded tissue sections prepared for light microscopy can be used for IF or IP methods following antigen retrieval. The data on the value of IF in paraffin-embedded sections in the assessment of human renal biopsies are controversial, and the results are not reproducible (Wagrowska-Danilewicz and Danilewicz, 2009). Using this method, diagnostic findings were obtained in only 50% of cases of idiopathic MGN (Nasr et al., 2006). On the other hand, the efficiency of IP detection of IgG and C3 using paraffin sections is controversial too. Many renal pathologists have not been satisfied with the results of IgG and C3 staining in paraffin embedded renal biopsy specimens. Thus, although the IgG deposits were revealed in about 80% of cases (Mölne et al., 2005; Chowdhury et al., 2005), C3 deposits were detected in only 50% of cases of MGN (Chowdhury et al., 2005).

C4d is a stable degradation product of complement factor C4, which is produced via two pathways in the complement cascade system, the classical and the mannose-binding lectin (MBL) pathways (Murata and Baldwin, 2009). The MBL pathway is antibody-independent. On the other hand, the classical complement cascade is initiated by changes in immunoglobulin molecules after binding to specific antigens and is followed by the cleavage of C4 domains into C4a and C4b. In presence of C4b binding protein, C4b is cleaved by C3bINA into C4c and C4d. The latter

covalently binds to endothelial surfaces and basement membranes through a thiolester group.

The presence of C4d in the kidney is generally detected for the diagnosis of antibody-mediated rejection in renal transplants. The deposit is usually recognized in PTCs on frozen sections by IF staining with anti-C4d monoclonal or polyclonal antibodies (Nadasdy et al., 2005). However, in these cases intrinsic C4d deposition in normal glomeruli has been noted (Regele et al., 2002; Suzuki et al., 2007). Furthermore, in the normal kidney using IP staining in frozen sections with monoclonal antibody, C4d can be detected in the glomerular mesangium and in the walls of some renal arterioles (Zwirner et al., 1989). The pattern of positive glomerular staining is granular and segmental in the mesangium. The PTCs and the tubules are negative (Zwirner et al., 1989).

Suzuki et al. (2007) demonstrated that the intrinsic staining in the normal glomerulus is undetectable using an anti-C4d polyclonal antibody by IP methodology in paraffin-embedded tissue. Therefore, immunohistochemical glomerular staining for C4d using the polyclonal antibody which is suitable for paraffin embedded tissue indicates pathogenic complement activation. These authors included in their study a small series of MGN and demonstrated a granular pattern of C4d deposition along the glomerular basement membrane. Thus, in 11 cases C4d deposition pattern of IP staining was negative in one case (9.1%), focal segmental in two cases (18.2%) and diffuse global in 8 cases (72.7%) (Suzuki et al., 2007). The C4d negative case was a MGN, stage I.

The advantage of IP demonstration of C4d in glomerular disease prompted us to study the deposition of this factor, especially in early idiopathic MGN. The morphology of the diffuse, small, granular, membranous deposits of C4d in the capillary loops of our cases is similar to that observed using the usual IF staining for IgG and C3 on frozen sections. On the other hand, our control kidneys showed absence of staining.

Our results indicate that the deposition of C4d revealing a granular and diffuse pattern along the glomerular capillary loops, demonstrated by IP, is a distinctive characteristic of MGN. With the IP technique the antigen is visualized in the light microscope and therefore can be correlated to glomerular structures. This sensitive method can be used with confidence in the diagnosis of this process.

C4d glomerular deposition in pure class V MLN has been morphologically indistinguishable from idiopathic MGN. A background of scant mesangial deposits was observed occasionally. The finding of granular PTC C4d deposition, as observed in one of our five cases of lupus nephritis, is uncommon. This deposition has been described (Lerut et al., 2005) and observed in 6.8% of lupus nephritis patients, mostly with diffuse proliferative, class IV glomerulonephritis (Li et al., 2007). The pattern of PTC C4d deposition in lupus nephritis (granular) is distinct from that observed in



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antibody-mediated rejection (linear) (Li et al., 2007).

The presence of C4d antigen with the same location as IgG deposits in the glomerulus suggests that complement activation via the classical pathway usually occurs in idiopathic MGN. Glomerular C4d deposits most likely represent stable C4d-Ig complexes, as well as covalently bound C4d fragments to glomerular membrane in close vicinity to the immune deposits (Kusunoki et al., 1989; Zwirner et al., 1989). Thus, C4d membranous deposition is indicative of continuous complement activation induced by deposition of immune complexes.

C4d deposition in PTCs in lupus nephritis might be the consequence of immune complex formation (Li et al., 2007).

The nineteen cases of different glomerulopathies included in this study to control disease specificity such as IgAGN, primary type 1 MPGN, FSGS and MCD showed distinct reproducible patterns of C4d deposition, without intrinsic background in paraffin sections.

In conclusion, these results indicate that formalin-fixed paraffin-embedded tissue can be stained for the immunoreactant C4d to show characteristic deposition in MGN, potentially obviating the need to repeat biopsy in cases with absence of glomeruli in the frozen tissue and in the tissue selected for electron microscopy, or when other adjunct methods for diagnosis in paraffin sections are negative. C4d staining in formalin-fixed and paraffin-embedded renal biopsy tissue using an IP method is effective as an adjunct for diagnosis of MGN.

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