Clinical Kidney Journal

ckj

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Leading European Nephrology

Clinical Kidney Journal, 2015, vol. 8, no. 4, 433–439

doi: 10.1093/ckj/sfv049
Advance Access Publication Date: 9 July 2015
Original Article

ORIGINAL ARTICLE

Glomerular IgG subclasses in idiopathic and malignancy-associated membranous nephropathy

Jennie Lönnbro-Widgren[1], Kerstin Ebefors[2], Johan Mölne[3], Jenny Nyström[2], and Börje Haraldsson[1]

[1]Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [2]Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, and [3]Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Correspondence to: Jennie Lönnbro-Widgren; E-mail: jennie.lonnbro.widgren@gu.se

Abstract

Background: In idiopathic membranous nephropathy (MN), antibodies directed towards the glomerular phospholipase A_2 receptor (PLA_2R) have mainly been reported to be of IgG4 subclass. However, the role of the different IgG subclasses in the pathogenesis of MN, both in idiopathic MN and in secondary cases, is still unclear. In this retrospective study, we test the hypothesis that the absence of glomerular IgG4 and PLA_2R in patients with MN indicates malignant disease.

Methods: The distribution pattern of glomerular IgG subclasses and PLA_2R was studied in 69 patients with idiopathic MN and 16 patients with malignancy-associated MN who were followed up for a mean of 83 months.

Results: A significant correlation between the absence of IgG4 and PLA_2R and malignancy-associated MN was found. Thus, IgG4 was positive in 45 of 69 patients (65%) with idiopathic MN but only in 5 of 16 patients (31%) with malignancy-associated MN. The other IgG subclasses did not differ statistically between the groups, IgG2-positivity being present in more than 94% of patients in both groups. Thirty-five of 63 patients (56%) with idiopathic MN and 3 of 16 (19%) patients with malignancy-associated MN had glomerular deposits of PLA_2R.

Conclusions: We have found that the absence of glomerular IgG4 and PLA_2R is common in patients with malignancy-associated MN. In our material, IgG2 could not be used as a marker of underlying malignant disease. Finally, neither IgG1 nor IgG3 seems to be involved in the pathogenesis of MN.

Key words: cancer, glomerulonephritis, kidney, membranous nephropathy, proteinuria

Introduction

Membranous nephropathy (MN) is one of the most common causes of adult-onset nephrotic syndrome [1]. In developed countries, the majority of the cases of MN are idiopathic and ~25% of the cases are secondary to underlying disease such as malignancy, hepatitis B or C, autoimmune disease, or due to toxicity of drugs [2]. Previous studies report that 5–20% of all cases of MN are malignancy associated [3]. In ~ 45% of these cases, the renal diagnosis antedates diagnose of malignancy, in 40% there is a simultaneous presentation of malignancy and nephrotic syndrome, and in the remaining 10% of the cases the renal diagnosis

Received: September 25, 2014. **Accepted:** June 3, 2015

appears after diagnosis of malignancy. Thus, both diseases often present within 1 year of each other [3]. Discrimination between idiopathic MN and malignancy-associated MN is of great clinical importance as treatment of secondary forms of MN is directed towards the underlying disease, while immunosuppressive medication is recommended in idiopathic MN [4]. Since clinical presentation and routine laboratory examinations in idiopathic MN and malignancy-associated MN do not differ, investigation to exclude malignancy has to be performed [5]. The question is whether all patients with MN should be screened, or how to identify patients at risk. Recently the M-type phospholipase A_2 receptor, PLA_2R, was identified as a major antigen involved in the development of idiopathic MN [6]. Indeed, 70–80% of patients with idiopathic MN seem to have serum anti-PLA_2R antibodies. There could be several reasons why the sensitivity is <100%, such as influence of immunosuppressive drugs, involvement of other antigens than PLA_2R in the pathogenesis of idiopathic MN, misclassification of patients with idiopathic MN, or absence of immunologic disease at the time of blood analysis [6–8]. Initially, anti-PLA_2R antibodies were not found in patients with secondary MN, but now they have been detected in patients with hepatitis B, malignancy and sarcoidosis-associated MN [9, 10]. Expression of PLA_2R in glomerular deposits has been proposed to correlate with the presence of anti-PLA_2R antibodies in serum in patients with idiopathic MN [7]. It has also been suggested that the sensitivity might be even higher for the deposits than for the presence of serum PLA_2R antibodies [11]. The IgG antibodies that bind to PLA_2R have been reported to be predominantly of IgG4 subclass, but the involvement of the different IgG subclasses in the pathogenesis of MN is still not fully elucidated [12]. A recent report suggests an IgG subclass switch in the antibody response, from IgG1 to IgG4 in the later stages of idiopathic MN [13]. Previous studies on the glomerular distribution of IgG subclasses present partly contradicting results [14, 15], although the absence of IgG4 may indicate malignancy-associated MN [15]. We analysed consecutive patients with MN that had been carefully monitored over a period of 1–12 years. The follow-up period allowed us to distinguish between patients with idiopathic MN and malignancy-associated MN with reasonable accuracy. Patients with other forms of secondary MN, or unclear cases, were excluded, resulting in two well-defined patient groups. The pathologist examining the renal biopsies had no knowledge of which form of MN the patient had. Hereby, it was possible to test the hypothesis that patients with malignancy-associated MN could be identified based on the lack of glomerular staining for IgG4 and PLA_2R.

Materials and methods

Recruitment of patients

Patients were identified on the basis of the renal biopsy files at the Department of Pathology at Sahlgrenska University Hospital. All adult patients (>15 years old) that were diagnosed with MN between January 2000 and April 2012 were considered for inclusion, a total of 210 patients. Thirty-four patients were excluded since they had moved to other parts of Sweden, 11 patients had missing medical records and 10 patients declined to participate in the study. Among the remaining 155 patients, 44 had systemic lupus erythematosus, 10 patients had other forms of secondary MN and 73 patients had idiopathic MN. Twenty-eight patients had a prior history of malignant disease or a malignancy at the time of or after the appearance of kidney disease. This study was approved by the regional ethical review board in Gothenburg, approval number 432-09. Written informed consent was obtained from patients before collecting clinical data and performing biopsy examinations. The youngest patient was 15 years old at the time of diagnosis, but all patients were >18 years old at the time of inclusion in the study. This study adheres to the Declaration of Helsinki.

Diagnosis of MN

The definition of idiopathic MN was (i) renal biopsy MN, (ii) absence of other forms of MN such as systemic lupus erythematosus, hepatitis B and C, and toxic drug reaction, and (iii) absence of malignancy during the follow-up period that was at least 12 months (82 ± 5 months). The definition of malignancy-associated MN was (i) the existence of malignancy discovered at the time of, or after, the biopsy, as well as in treated cases; or (ii) reduction or disappearance of proteinuria after therapy for malignancy and (iii) no evidence of other secondary forms of MN. In 3 of the 28 patients with malignancy, the time between MN and cancer was 138, 168 and 180 months and the nephrotic syndrome did not relapse when cancer was later diagnosed. The probability of developing cancer at a high age was assumed more likely than a connection between malignancy and MN in these three cases. In three additional cases, the nephrotic syndrome was in complete remission despite the fact that the malignant disease was uncured and the patients had metastatic disease. In five cases, there was more than 3 years between the occurrence of malignancy and kidney disease, and a connection between the two entities was difficult to determine. In our aim to include clearly defined patients with idiopathic MN and malignancy-associated MN, we considered these 11 patients not to be typical for neither idiopathic nor malignancy-associated MN and therefore excluded them from the study.

Histological examination of immune deposits

One renal pathologist (J.M.), blinded to the clinical and laboratory data, reviewed all renal biopsies from the included patients. All biopsy specimens were examined using light microscopy, immunohistochemistry and electron microscopy. The kidney tissue was adequately preserved and available for staining for IgG immune deposits except in five cases (four idiopathic MN and one malignancy-associated MN).

Standard light microscopy examination generally demonstrated a membranous pattern and excluded other glomerulonephritis including lupus. Immune deposits (IgG, IgA, IgM, light chains, C1q, C3c and C5b-9, all Dako, Copenhagen, Denmark) were examined using a standardized immunoperoxidase method. The EnVision™ Flex high pH (Link) detection kit (Dako K8000) was used. This is an indirect immunohistochemical technique using unlabelled primary antibodies. The procedure is standardized with a total processing time of 2.5 h. The most important steps are as follows. Consecutive series of paraffin sections are produced at a 4-µm constant thickness setting, floated on a 37°C water bath, and collected on serially numbered polylysin-coated glass slides (Dako). Sections are de-paraffinized in xylene–ethanol at room temperature and rehydrated in phosphate-buffered saline (PBS) before antigen retrieval using protease XXIV (Sigma-Aldrich, St Louis, MO, USA) for 30 min (IgG4). Endogenous peroxidase activity was blocked by immersion in peroxidase-blocking solution (Dako K8000) for 5 min at room temperature and immunostaining performed in a computer-assisted Autostainer Plus processor (Dako). Incubation time for primary antibodies was 30 min at room temperature, terminated

Table 1. Characteristics of the patient population with malignancy-associated MN

Cases	Sex	Age	Malignancy	Time from onset of proteinuria to biopsy (months)	Time from biopsy to identification of malignancy (months)	Treatment of tumour	Remission		Glomerular		Follow-up time after biopsy (months)	Outcome
							Tumour	Proteinuria	IgG4	PLA2R		
1	M	61	Prostate cancer	6	31	S	Yes	PR	Neg	Neg	148	Alive
2	M	56	Lung cancer	8	11	Cs + R	No	No	Neg	Neg	23	Dead
3	F	70	Uterus cancer	2	9	No	No	No	Neg	Neg	10	Dead
4	M	76	Lymphoma	1	6	Cs + Ch	No	PR	Neg	Neg	30	Dead
5	M	83	Prostate cancer	1	0	H	No	PR	Neg	Neg	8	Dead
6	M	65	Prostate cancer	4	0	S	Yes	CR	Neg	Neg	72	Alive
7	F	68	Buccal cancer	1	12	S + R	Yes	CR	Neg	Neg	62	Alive
8	M	58	Lymphoma	1	0	Cs + Ch + BMtx	Yes	CR	Neg	Pos	60	Alive
9	M	78	Prostate cancer	9	1	H	No	No	Pos	Neg	2	Dead
10	F	49	Leukaemia	3	0	Cs + Ch + BMtx	No	CR	Neg	Neg	36	Dead
11	M	60	Prostate cancer	0,5	0	H + R	No	No	Pos	Pos	33	Alive
12	F	66	Lung cancer	1	18	No	No	No	Pos	Neg	27	Dead
13	F	65	Breast cancer	7	17	S + H	Yes	CR	Pos	Pos	33	Alive
14	M	79	Prostate cancer	2	1	No	No	No	Neg	Neg	8	Dead
15	M	73	Lung cancer	2	4	S	Yes	CR	Neg	Neg	18	Alive
16	F	80	Colon cancer	5	0	S	Yes	CR	Pos	Neg	16	Alive

CR, complete remission of proteinuria (<300 mg/24 h); PR, partial remission of proteinuria (<3.5 g/24 h and 50% reduction in proteinuria); S, surgery; R, radiation; H, hormonal therapy; Cs, chemotherapy including steroids; Ch, chemotherapy including alkylating agents; BMtx, bone marrow transplantation; Ig, immunoglobulin; PLA_2R, phospholipase A_2 receptor.

by repeated washings, followed by incubation with a dextran polymer coated with secondary antibodies and horseradish peroxidase for another 30 min. Slides were transferred to fresh hydrogen peroxide plus 3,3′-diaminobenzidine tetrahydrochloride (DAB) solutions for 4 min. Finally, slides were stained with Mayer's haematoxylin and permanently mounted under cover slips. Omitting or replacing the primary antibodies produced negative controls. IgG subclass antibody expression was studied using the same immunohistochemical protocol. IgG1 (clone9052-01, Southern Biotech, Birmingham, AL, USA) was used at a final concentration of 1:100, IgG2 (clone 9080-01, Southern Biotech) at 1:100, IgG3 at 1:500, IgG3 (clone 9210, Southern Biotech) at 1:100 and IgG4 (clone MRQ-55, Cell Marque, Rocklin, CA, USA) at 1:100. Optimal primary antibody dilutions were obtained using serial dilutions of each antibody on human tonsils and five cases of MN.

Histological examination of PLA_2R

Consecutive series of paraffin sections were produced at a 4-µm constant thickness setting, floated on a 37°C water bath and collected on serially numbered polylysin-coated glass slides (Dako). Sections were de-paraffinized in xylene–ethanol at room temperature with an endogenous peroxidase-blocking step. The sections were rehydrated in PBS, and heat-induced epitope retrieval using citrate buffer pH 6.2 was performed, followed by a blocking step and incubation with a polyclonal rabbit anti-PLA_2R (Atlas Antibodies, Sweden) at a dilution of 1:8000 overnight at 4°C. POLAP (Zytomed, Germany) was used as the detection system. Labelled sections were analysed by three independent scientists in a blinded fashion by using a scoring method, where 0 = negative and 1 = positive staining for PLA_2R.

Statistical analysis

Chi-square test (Fisher's exact test), Spearman's rank-sum coefficient of correlation and ANOVA was used for statistical analysis and $P \leq 0.05$ was considered as being significant. Descriptive statistics are presented as the means (±SEM).

Results

Background of patients with malignancy-associated MN

The total prevalence of cancer among patients with MN in our material was 8.1% (17 out of 210) based on the extensive clinical records at hand. That number is fairly accurate, but we do acknowledge that some of the 210 patients were lost to follow-up, or had missing files, which excluded them from the study. The prevalence of 8.1% in the MN population is 10 times higher than in the general population according to the Swedish cancer register in 2010. Table 1 (and Supplementary Figure S1) gives an overview of the characteristics of the patient population with malignancy. In half of the cases, the malignant disease was already known at the time of biopsy, or discovered within a month from biopsy. In the rest of the cases, the malignant disease was diagnosed within 2 years from kidney biopsy, except for one patient with prostate cancer (31 months after kidney biopsy). In this case, serum prostate-specific antigen was elevated at least 6 months before prostate biopsy was performed and the prostate cancer diagnosis was confirmed. In total, three patients did not receive treatment for cancer, due to metastatic disease, the proteinuria persisted and they died due to the malignant disease. Thirteen patients received treatment for the malignant disease with complete resection and cure of tumour; remission of proteinuria was seen in seven of these cases (six complete remission/one partial remission). In the remaining six cases, the malignancy was not cured and five of these patients died due to the malignant disease. Proteinuria persisted in three of these cases, while a partial remission of proteinuria was noted in two cases. One patient received heavy treatment for leukaemia, including bone marrow transplantation, and achieved complete remission of both leukaemia and nephrotic syndrome. This patient later had a recurrence of leukaemia and died due to complications of the malignancy. It is unclear whether proteinuria reappeared or not because of lack of urinary test for protein at that point.

Baseline characteristics

Table 2 gives an overview of the clinical and laboratory data of all the patients at the time of renal biopsy. The patients with malignancy-associated MN were significantly older (67 ± 2 years) than those with idiopathic disease (52 ± 2 years), $P < 0.001$. Other baseline data did not differ statistically between the groups.

Histological findings

The MN cases displayed everything from faint irregularities in the glomerular capillaries to a distinct membranous pattern, but few other histological changes in the glomeruli (no proliferation, no crescents). Diagnosis was confirmed using immunoperoxidase and electron microscopy, and other forms of glomerulonephritis, including lupus, were excluded. Figure 1A–D shows immunoperoxidase detection of IgG subclasses exemplified in one typical patient with MN.

Comparison of glomerular IgG subclasses between patients with idiopathic MN and malignancy-associated MN

IgG4 subclass deposits were found in the renal biopsy in 45 of 69 patients with idiopathic MN and in 5 of 16 patients with malignancy, a statistically significant difference ($P < 0.05$). The positive predictive value for IgG4 as an indicator of idiopathic MN was 90% (95% CI 78.19–96.67). For more details, see Table 3. There was no difference in staining pattern for IgG1, IgG2 or IgG3 between the two groups. To see whether we could find a sharper tool in distinguishing between idiopathic and malignancy-associated MN, we created a recognition category score based on the four distinct

Table 2. Baseline characteristics of all patients

	Idiopathic MN ($n = 69$)	Malignancy-associated MN ($n = 16$)	Significance, P-value
Sex (male/female)	45/24	10/6	NS
Smoking (yes/no), missing data 3 patients	40/26	9/7	NS
Age (years)	52 ± 16	68 ± 10	<0.001
Serum-albumin (g/L)	24 ± 8	21 ± 7	NS
Urine-albumin (g/day)	5.4 ± 3	5.5 ± 3	NS
Urine-protein (g/day)	5.9 ± 3	6.0 ± 3	NS
eGFR (mL/min/m^2)	82 ± 32	76 ± 24	NS
Time from symptom to biopsy, months (range)	15 ± 6 (5–360)	3 ± 1 (0.5–9)	NS
Length of follow-up, months (range)	82 ± 5 (12–164)	37 ± 9 (2–164)	<0.05

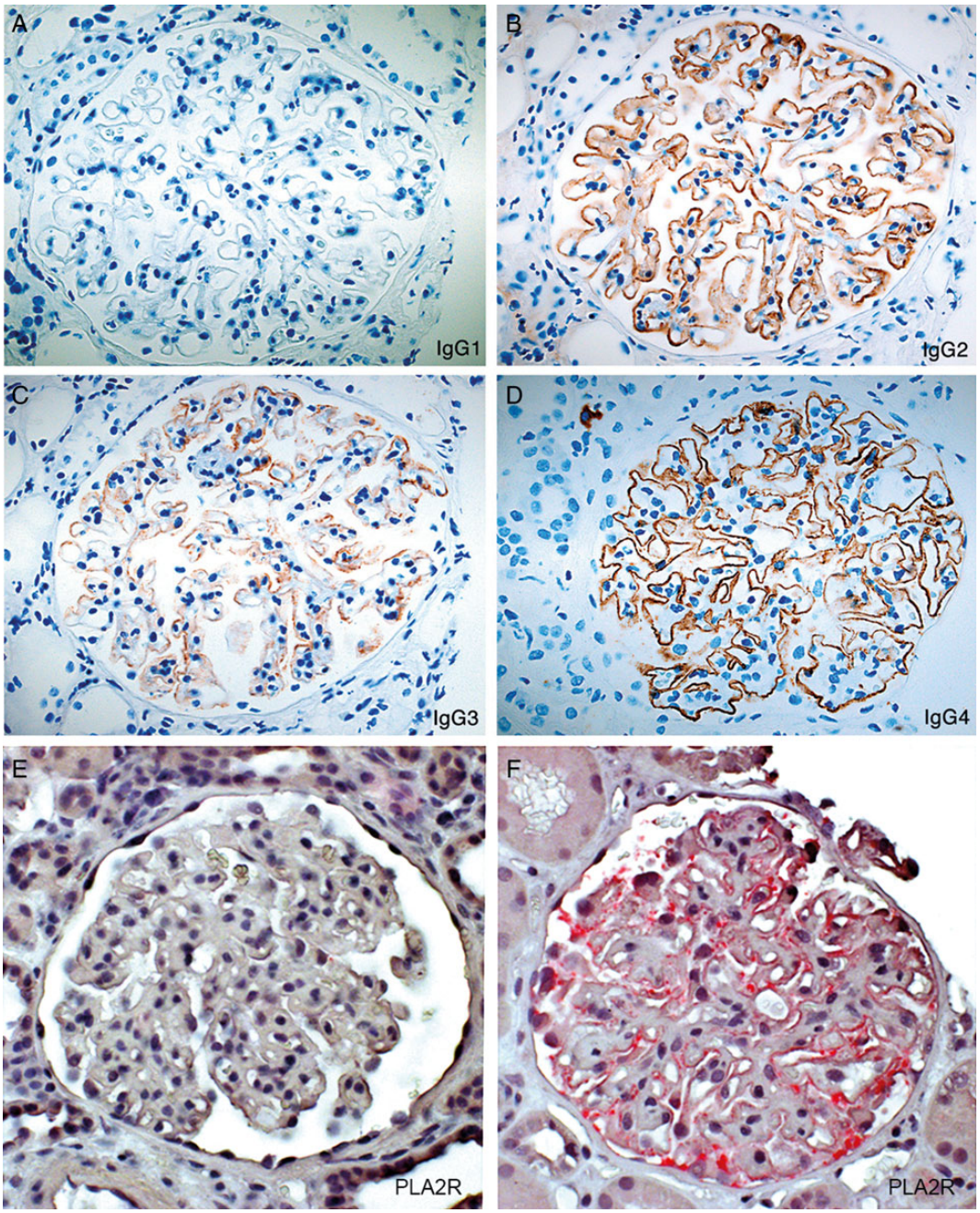

Fig. 1. Immunoperoxidase detection of IgG subclasses exemplified in one typical patient with MN (**A–D**) and POLAP detection of phospholipase receptor 2 (PLA_2R) exemplified in two representative cases of idiopathic MN (**E–F**.) (A) (IgG1) Completely negative (B) (IgG2) staining in a membranous granular pattern; (C) (IgG3) slightly uneven membranous granular pattern and (D) (IgG4) strong IgG staining in an almost linear membranous pattern. (E) Case lacking positive staining for PLA_2R and (F) case positive for PLA_2R. Magnification ×40.

categories of staining pattern that are possible, Supplementary Figure S2. In the malignant group, 63% of the patients were positive for IgG2 and negative for IgG4 (category 2) compared with 25% of the idiopathic patients. Patients with idiopathic MN were in 55% of the cases positive for IgG2 and IgG4 (category 3) compared with 31% in the malignant group. Supplementary Figure S2 illustrates the marked difference in the distribution pattern between patients with idiopathic MN and malignancy-associated MN. However, neither IgG2 nor IgG4 can be used to identify patients with malignancy with any reasonable precision.

PLA_2R staining

Glomerular PLA_2R was detected in 35 of 63 patients with idiopathic MN and in 3 of 16 patients with malignancy-associated MN, a statistically significant difference ($P < 0.05$) (Table 3). In six patients with idiopathic MN, there was not enough material left to perform staining for PLA_2R. Figure 1E and F shows the detection of PLA_2R in two representative cases of MN. 86% (30 of 35) of the patients with positive staining for glomerular PLA_2R were also positive for IgG4 subclass. However, in a large percentage (29 of 35 patients), IgG2 was also detectable. No patient was positive

Table 3. Result of staining for glomerular IgG subclasses and PLA_2R

	Idiopathic MN ($n = 69$)			Malignancy-associated MN ($n = 16$)			
	Positive	Negative	% Positive	Positive	Negative	% Positive	Difference between groups, P-value
IgG4	45	24	65	5	11	31	<0.05
IgG3	15	54	22	3	13	19	NS
IgG2	56	13	81	15	1	94	NS
IgG1	1	68	1	1	15	6	NS
PLA_2R	35	28	56	3	13	19	<0.05

Ig, immunoglobulin; I-MN, idiopathic MN; M-MN, malignancy-associated MN.

Table 4. IgG subclasses in patients with positive staining for glomerular PLA_2R

Glomerular IgG subclasses	Number of patients (% of PLA_2R positive)
IgG4	30 (86)
IgG3	13 (37)
IgG2	29 (83)
IgG1	0

for IgG1, Table 4. In the malignant group, PLA_2R was positive in three patients; two of these were also positive for IgG4. There was a significant positive correlation in the idiopathic group between the presence of IgG4 and PLA_2R, but not in the malignant group ($P < 0.05$).

Discussion

In our material, the prevalence of malignancy in patients with MN was close to 10%, which is within the wide range published in previous studies [3]. The most frequent cancer in our cohort was located in the prostate (six) and in the lung (three). It is noteworthy that there was no case of skin cancer despite the fact that skin cancer is quite common in Sweden. Our data support previous findings of cancer prevalence in MN [5], as well as the fact that the existing few case reports of correlation between skin cancer and MN include melanoma and Kaposi's sarcoma [16, 17].

The trigger of MN in patients with malignancy is unclear. Different mechanisms have been postulated [2], such as formation of *in situ* and/or circulating immune complexes, tumour antigens, or extrinsic factors such as viral infection. There might be different pathogenic mechanisms involved depending on the type of malignancy and the patient's immune system. However, it is likely that the malignancy-associated MN could also be induced by reactions to secreted PLA_2 proteins. Thus, extensive studies of PLA_2 have revealed that the human genome contains nine secretory PLA_2 genes [18]. Group IIA secretory PLA_2 seems to accumulate during inflammatory conditions such as arthritis. The enzyme has also been found to have a direct antibacterial activity against many Gram-positive bacteria. Group IIA and IB secretory PLA_2 are also proposed to play a role in the development of cancer, although the exact mechanism on cell proliferation is unknown. It seems that the inflammatory effect of secretory PLA_2 does not always require lipolytic enzymatic activity but can be secondary to direct binding to membrane receptors on the target cells. One could speculate that certain cancer cells release secretory PLA_2 that affects the kidneys and leads to the development of MN through an immune response. However, in the case of malignancy-associated MN, the immune response less often seems to involve antibodies of the IgG4 subclass.

We found a significant correlation between the absence of glomerular IgG4 and PLA_2R and malignancy-associated MN, a result that is consistent with previous reports [7, 15]. Furthermore, 45 of 69 (65%) patients with idiopathic MN were positive for IgG4. Analysis of IgG1–3 was performed and IgG1 and IgG3 were present in a low number of cases while IgG2 was found in a high number of cases. However, in our material, a positive staining for IgG2 could not be used as an indicator of underlying malignancy.

Of 63 (56%) patients with idiopathic MN, 35 had PLA_2R in glomerular deposits. This is lower than previous reports [7, 11], which could possibly be due to aged biopsy materials in this retrospective study. Three patients in the malignant group had glomerular PLA_2R, and it cannot be ruled out that the presence of MN and malignancy in these cases was coincidental.

The dominance of IgG2 and IgG4 antibodies in MN fits well with the notion that these two subclasses are less prone to complement activation than IgG1 and IgG3 [19, 20]. Thus, the patients with MN have little inflammation such as infiltrating inflammatory cells or crescents. Recently, it has been proposed that there is a subclass switch from IgG1 to IgG4 during the progression of idiopathic MN [13]—a phenomenon for which we could not find any evidence. IgG4 antibodies possess an ability of exchanging Fab arms, a mechanism that provides the base for their anti-inflammatory activity with poor ability to activate complement through the classical pathway and a low affinity of Fc receptors [21]. The effect of IgG4 on PLA_2R, as well as the normal function of PLA_2R, is still not fully understood [2]. In addition, it is still not clear how the deposition of immune complexes induces the glomerular damage and podocyte loss. One hypothesis is that the podocytes are exposed to increased oxidative stress, and the antioxidative defence system indeed has been shown to be downregulated in MN [22].

Since malignant disease can appear years after diagnosis of MN, early differentiation between idiopathic MN and malignancy-associated MN is crucial to avoid a delay in finding and treating malignancy. In our study, baseline laboratory data did not differ between the groups, while older age indicated a risk for malignancy-associated MN. Also, the absence of glomerular IgG4 and PLA_2R strongly indicates underlying malignant disease.

Naturally, there are some limitations in our study. Firstly, it is a retrospective study, and therefore we had no possibility of measuring anti-PLA_2R antibodies in serum at the time of biopsy. Secondly, although we have included a larger number of patients with malignancy-associated MN than most previous studies, the number of cases is still small, and we have reduced statistical power. Nevertheless, we conclude that there is not yet any specific diagnostic tool to identify patients with malignancies among those who display a nephrotic syndrome with a morphological

Clinical Kidney Journal
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pattern of MN. The absence of IgG4 antibodies and glomerular deposits of PLA_2R should raise suspicion in particular in patients of higher age.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

Acknowledgements

The study was supported by the Swedish Medical Research (grants 9898 and 14764), the National Association for Kidney Diseases, the John and Brit Wennerström Research Foundation, IngaBritt and Arne Lundberg Research Foundation and Sahlgrenska University Hospital (grant LUA75450). We thank Dr G Lambeau for providing staining the staining protocol for glomerular PLA_2R.

Conflict of interest statement

None declared.

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