Cancer Risk after Cyclophosphamide Treatment in Idiopathic Membranous Nephropathy

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Abstract

Background and objectives Cyclophosphamide treatment improves renal survival in patients with idiopathic membranous nephropathy. However, use of cyclophosphamide is associated with cancer. The incidence of malignancies in patients with idiopathic membranous nephropathy was evaluated, and the cancer risk associated with cyclophosphamide use was estimated.

Design, setting, participants, & measurements Patients who attended the clinic were included prospectively from 1995 on. A crude incidence ratio for the occurrence of malignancy was calculated. Incidence ratios were subsequently standardized to potential confounders. Latency between cyclophosphamide therapy and the occurrence of cancer was estimated by stratifying for time since the start of treatment. Finally, Poisson regression was used to obtain a multiple adjusted incidence ratio and investigate the dose–response relationship between cyclophosphamide and cancer.

Results Data were available for 272 patients; the mean age was 51 years, and 70% of the patients were men. Median follow-up was 6.0 years (interquartile range=3.6–9.5), and 127 patients were treated with cyclophosphamide. Cancer incidence was 21.2 per 1000 person-years in treated patients compared with 4.6 per 1000 person-years in patients who did not receive cyclophosphamide, resulting in crude and adjusted incidence ratios of 4.6 (95% confidence interval, 1.5 to 18.8) and 3.2 (95% confidence interval, 1.0 to 9.5), respectively.

Conclusion Cyclophosphamide therapy in idiopathic membranous nephropathy gives a threefold increase in cancer risk. For the average patient, this finding translates into an increase in annual risk from approximately 0.3% to 1.0%. The increased risk of malignancy must be balanced against the improved renal survival.

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Introduction

Idiopathic membranous nephropathy (iMN) is a common cause of nephrotic syndrome in adults. Current guidelines advise treatment with steroids and alkylating agents in patients who are at high risk for ESRD or have severe, persistent nephrosis (1). However, many physicians and patients are reluctant to use cyclophosphamide because of the increased risk of cancer after cyclophosphamide therapy in patients with granulomatosis with polyangiitis (formerly Wegener's granulomatosis), rheumatoid arthritis, and non-Hodgkin's lymphoma (2–4). Ascertaining the association between cyclophosphamide therapy and malignancy in iMN is challenging because of the concomitant immunosuppressive therapy, the relative rarity of malignancies, and the fact that membranous nephropathy may occur secondary to cancer (5,6). As a result, data on cancer risk in cyclophosphamide-treated iMN patients are sparse. Obviously, such information is important when balancing risks and benefits of immunotherapy in iMN. Therefore, we studied the incidence of cancer in a cohort of iMN patients and investigated the role of cyclophosphamide as a risk factor.

Materials and Methods

A detailed description of patients and methods is given in Supplemental Appendix.

We included adult patients with biopsy-proven iMN who visited our outpatient clinic between 1995 and 2009. Patients were referred by either a family doctor in our catchment area or an allied center. Secondary causes were ruled out per standard policy (7). Written informed consent was obtained. The study was conducted in accordance with the Declaration of Helsinki and approved by our hospital's review board.

Most patients were treated according to our restrictive regimen, which is detailed elsewhere (7). In summary, patients underwent a standardized, timed urine measurement (8) and received supportive treatment. Immunosuppressive therapy was advised when patients reached a serum creatinine concentration above 1.5 mg/dl or suffered severe or life-threatening symptoms of nephrotic syndrome. Oral cyclophosphamide and pulse intravenous methylprednisolone combined with high-dose oral prednisone was the preferred treatment. Occasionally, alternative immunosuppressive drugs were prescribed (9,10).

The outcome for the present study was incident malignancy recorded from medical records (including the date of diagnosis). Mortality and the date of the final consultation were recorded as well.

We prespecified potential confounders as age at the time of biopsy, sex, ever smoking, having a first degree relative with a history of malignancy, CKD stage, and nephrotic syndrome. Immunosuppressive therapy was considered a possible confounder if it was initiated before cyclophosphamide therapy. Immunosuppressive therapy after cyclophosphamide could have acted as an intermediary, and therefore, adjustment could result in underestimation of possible malignancy risk. Sex, date of birth, and height were recorded during the urinary analysis, whereas biopsy and follow-up laboratory data were obtained from medical records. We recorded family history, smoking, cyclophosphamide exposure (including total cumulative dose), and use of other immunosuppressive drugs over the entire follow-up duration.

Baseline data are presented as mean±SD, median and interquartile range (IQR), or frequency and proportion. A chi-squared test was used to evaluate differences in frequencies. The difference in means for normally distributed variables was compared using a *t* test. A Wilcoxon rank-sum test was used to compare medians for skewed variables.

Person-time was calculated from the start of therapy to the occurrence of malignancy or the last consultation date in the cyclophosphamide-exposed group. For unexposed patients, person-time was calculated from biopsy to malignancy or the last consultation. Ideally, one would start measuring person-time for controls at the moment that they would have started treatment. To mimic this moment of exposure, the median time between biopsy and initiation of therapy in the cyclophosphamide group was estimated and deducted from the person-time for each control. Control patients who had negative person-times as a result were excluded from the analyses.

Subsequently, the cumulative incidence of malignancy was calculated assuming that death was competing with malignancy risk. Incidence rates were calculated and used to estimate the incidence ratio (IR) of malignancy after cyclophosphamide exposure. To estimate the latency between cyclophosphamide exposure and cancer, IRs were calculated by 2-year strata of person-time. Standardized

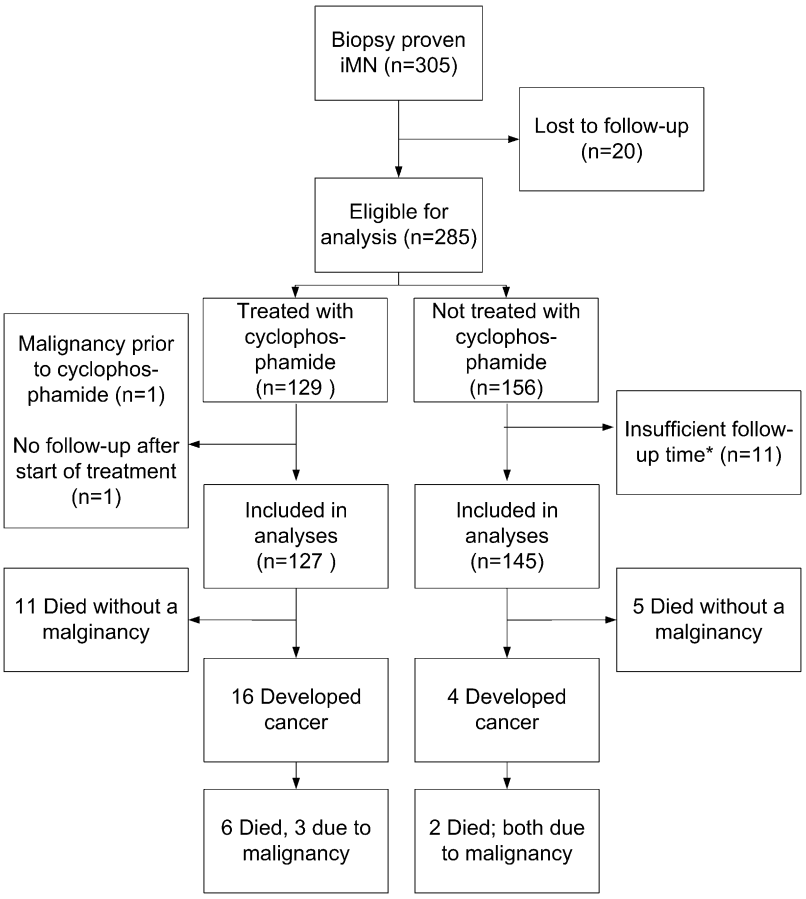

Figure 1. | **Flowchart for inclusion in the cohort.** *The median time between biopsy and start of cyclophosphamide exposure was deducted from the exposure time of controls to adjust for time not at risk. As a result, 11 patients had negative exposure times and therefore, were excluded from the analyses (Materials and Methods). Two cyclophosphamide-treated patients were excluded: one patient was excluded because of a lung carcinoma *in situ* before treatment, and the other patient was lost to follow-up after the initiation of therapy. iMN, idiopathic membranous nephropathy.

IRs were calculated by weighting for the distribution of the confounders in the cyclophosphamide-treated group (11). Multiple imputation by chained equations was used to impute missing data on smoking status, family history, and cumulative cyclophosphamide dose (12). Poisson regression was used to obtain a multiple adjusted IR for the association between cyclophosphamide and malignancy, taking the imputations into account. In addition, the dose–response relation between cumulative cyclophosphamide exposure and the occurrence of malignancy was investigated by creating 20-g categories of cumulative exposure and including these categories in an adjusted Poisson regression. For all analyses, 95% confidence intervals (95% CIs) around the IRs were calculated.

Membranous nephropathy can be incorrectly classified as idiopathic when it occurs secondary to an undetected malignancy. These patients are unlikely to respond to supportive therapy and therefore, more likely to receive cyclophosphamide, which would inflate the association between cyclophosphamide and cancer. To address this issue, we analyzed the serum samples of all patients with a malignancy for the presence of antibodies against the phospholipase A2 receptor (PLA2R) in serum. Samples were obtained at the time of urine analysis, stored at −80°C, and analyzed using an immunofluorescence test (Euroimmun AG, Lübeck, Germany).

In sensitivity analyses, the multivariate analysis was repeated including only patients with complete data. Then, we excluded patients who had received immunosuppressive drugs other than cyclophosphamide. Finally, cancer incidence was standardized by age and sex to the general population using incidence estimates obtained by The Netherlands Cancer Registry over the past decade (13).

Results

Between 1995 and 2009, 305 patients with iMN visited our center. Twenty patients were lost to follow-up. Eleven patients with negative person-times after correction for time not at risk and two cyclophosphamide-treated patients were excluded (Figure 1). The present study includes 272 patients (Table 1). Most patients were men, and the

Table 1. Population characteristics at baseline

Variables	Cyclophosphamide-Treated Patients (n=127)	Patients Not Treated with Cyclophosphamide (n=145)	P Value
At baseline (the time of biopsy)			
Men (n)	101 (80%)	89 (61%)	0.001
Body mass index (kg/m^2)	26.6±3.6	27.1±5.0	0.40
Age (yr)	53±13	49±15	0.05
Year of biopsy	2001±7	2002±6	0.10
Follow-up duration (yr)	7.0 (4.0–11.4)	5.4 (3.0–8.4)	0.26
Positive family history for malignancy[a]	12/82 (15%)	12/98 (12%)	0.64
Current/former smoker[a]	56/94 (60%)	55/107 (51%)	0.24
eGFR-MDRD4 (ml/min per 1.73 m^2)	60±24	75±22	<0.001
Serum creatinine (mg/dl)	1.2 (1.0–1.6)	1.0 (0.8–1.1)	<0.001
Serum albumin (g/dl)	2.2±0.68	2.7±0.63	<0.001
Nephrotic syndrome at presentation	121 (95%)	119 (82%)	0.001
Protein to creatinine ratio (g/g)	8.9 (5.7–11.1)	4.6 (3.0–7.6)	<0.001
ACEi/ARB use	112 (88%)	134 (92%)	0.24
Statin use	81 (64%)	87 (60%)	0.52
Other BP-lowering medication	39 (31%)	22 (15%)	0.002
Therapy			
Interval from baseline to start of therapy (mo)	12 (5–26)		
Serum creatinine at start of therapy (mg/dl)	1.6 (1.2–2.0)		
Prior immunosuppressive therapy	27 (21%)	22 (15%)	0.19
Cumulative cyclophosphamide dose (g)	37 (21–46)	n/a	
Outcomes			
Death	17 (13%)	7 (5%)	0.01
Malignancies	16 (13%)	4 (3%)	0.002

Data are presented as mean±SD, median (interquartile range), or percentage where appropriate. eGFR-MDRD4, eGFR calculated with the abbreviated Modification of Diet in Renal Disease equation for mass spectrometry-standardized creatinine; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; n/a, not applicable.
[a]The denominator differs from the total number of patients because of missing data for this variable.

mean age was 51±14 years. Most patients presented with nephrotic syndrome (88%) and well preserved kidney function (mean eGFR=68±24 ml/min per 1.73 m^2). During follow-up, 127 patients (47%) received cyclophosphamide, 123 patients (45%) did not receive any immunosuppression, and 22 patients did receive immunosuppressive therapy but were never treated with cyclophosphamide. Cyclophosphamide-treated patients were more likely to be men, be older, and have more severe proteinuria and a lower eGFR at the initial visit compared with untreated patients. The median time between biopsy and start of cyclophosphamide treatment was 12 months (IQR=5–26).

During follow-up (median=6.0 years, IQR=3.6–9.5 years), 20 patients (7%) developed a malignancy, and 24 patients (9%) died, 5 of whom died because of a malignancy. The cumulative incidence of cancer was 11% (95% CI, 7% to 18%) after 10 years (Figure 2). Malignancy incidence was 21.2 per 1000 person-years in the cyclophosphamide group compared with 4.6 per 1000 person-years in the controls, resulting in an unadjusted IR of 4.6 (95% CI, 1.5 to 18.8). There was no clear relation between IR and the time of follow-up (Figure 3). Table 2 shows a decrease in the IR of malignancy after cyclophosphamide exposure when standardized to age, sex, prior immunosuppressive therapy, smoking, and positive family history (Table 2).

Missing values for smoking (*n*=71, 26%), family history of malignancy (*n*=92, 34%), and cumulative cyclophosphamide dose (*n*=15, 6%) were estimated and imputed. After the imputations, the proportion of smokers decreased to 54%, whereas the proportion of patients with a family history of cancer remained 13%. A multiple-adjusted IR of 3.1 (95% CI, 0.9 to 9.9) for cyclophosphamide exposure was calculated by entering all possible confounders in a Poisson regression. However, the association between cyclophosphamide treatment and cancer in the Poisson regression was not substantially influenced by smoking status, prior immunosuppressive therapy, family history of malignancy, CKD stage, or presence of the nephrotic syndrome. The most parsimonious model included only age and sex as confounding factors and gave an IR for cyclophosphamide exposure of 3.2 (95% CI, 1.0 to 9.5).

Figure 4 shows the adjusted relation between cumulative cyclophosphamide dose in 20-g categories and malignancy. The respective median cumulative doses per category were 0, 12, 36, 46, and 71 g. Compared with the untreated patients, the age- and sex-adjusted IRs by increasing dose category were 4.5 (95% CI, 1.1 to 18.4), 2.6 (95% CI, 0.7 to 9.4), 3.9 (95% CI, 1.0 to 14.7), and 1.7 (95% CI, 0.2 to 15.4), respectively.

Detailed information on the patients who developed a malignancy is given in Table 3. Three cyclophosphamide-treated and two untreated patients died because of a malignancy. Baseline anti-PLA2R was positive in 13 patients (65%), negative in 5 patients (25%), and unknown in 2 patients (10%). There was no association between time to the occurrence of a malignancy and anti-PLA2R serostatus.

In a sensitivity analysis limited to 201 patients with complete data for smoking and family history of malignancy, the adjusted IR was slightly higher at 3.3 (95% CI, 0.9 to 12.0). In a second sensitivity analysis, we excluded all patients treated with other immunosuppressive drugs; therefore, 78 cyclophosphamide-exposed and 123 unexposed patients remained. The resulting adjusted IR was 5.1 (95% CI, 1.2 to 21.5). When we standardized by age and sex to the general population, an IR of 1.7 (95% CI, 0.9 to 2.6) was observed. Remarkably, the IR for malignancy in the unexposed patients was 0.5 (95% CI, 0.01 to 0.9). Details of the sensitivity analyses are presented in Supplemental Appendix.

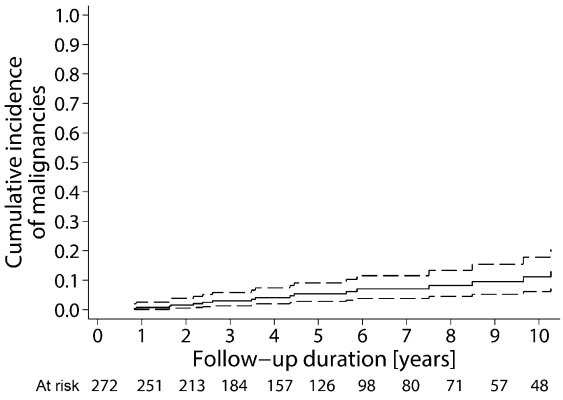

Figure 2. | Cumulative incidence of malignancy for the total cohort of iMN patients. The solid line is the point estimate, and the dashed lines are the 95% confidence interval for the cumulative incidence. Death was considered competing for malignancy risk.

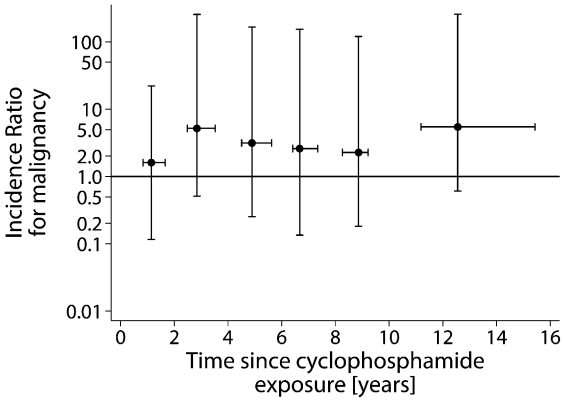

Figure 3. | Unadjusted incidence ratio of malignancy in iMN patients by time since cyclophosphamide exposure in 2-year strata. Vertical lines represent the 95% confidence intervals around the estimated incidence ratio. The horizontal lines signify interquartile range of follow-up time within each 2-year stratum.

Discussion

The present study shows that the incidence of malignancy in cyclophosphamide-treated iMN patients was approximately three times higher compared with patients not exposed to cyclophosphamide. For a 55-year-old patient, this result translates into an increase in annual cancer risk from approximately 0.3% to 1.0% (13). Previous studies have shown that treatment with alkylating agents reduced the

Table 2. Standardized incidence ratios for malignancy after cyclophosphamide exposure by potentially confounding variables

Risk Factor	Incidence Ratio	95% CI
Unadjusted	4.6	1.5 to 18.8
Univariate-adjusted		
Age	3.3	1.0 to 10.6
Men	3.3	1.1 to 10.0
Smoking	5.8	1.6 to 20.8
Prior therapy	4.2	1.3 to 13.4
Family history of malignancy	7.1	1.6 to 32.0
CKD stage	5.0	1.5 to 18.8
Nephrotic syndrome	4.3	1.3 to 14.1
Age- and sex-adjusted	3.2	1.0 to 9.5

Age was categorized as <45, 45–54, 55–64, 65–74, and ≥75 years. The unadjusted row shows the unadjusted incidence ratio. The univariate-adjusted rows are incidence ratios for cyclophosphamide exposure adjusted for the individual confounder. Ever smokers were compared with patients who never smoked. Prior therapy was defined as immunosuppressive drug therapy other than prednisone before cyclophosphamide therapy was initiated. A multiple adjusted incidence ratio of 3.1 (95% confidence interval [95% CI], 0.9 to 9.9) was obtained with a Poisson regression after multiple imputation of missing values for smoking and family history of malignancy. The most parsimonious model was adjusted only for sex and age (the latter as a continuous variable). The model showed a moderate fit (R^2=0.15).

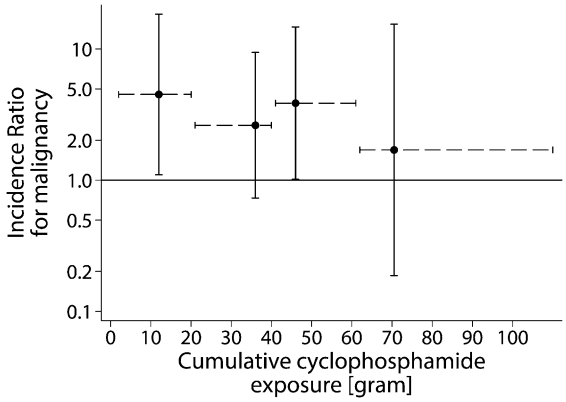

Figure 4. | The dose–response relation between cumulative cyclophosphamide exposure in categories (not treated, 1–20, 21–40, 41–60, and >60 g) and the adjusted incidence ratio of malignancy in iMN patients. The untreated group has been chosen as the reference, and thus, the incidence ratio for malignancy is one in that group. The vertical lines represent the 95% confidence intervals around the incidence ratios. The dashed horizontal lines represent the range within the categories for the cumulative cyclophosphamide dose.

incidence of ESRD 3- to 5-fold (14–17). At first glance, this decreased renal risk is matched by an increased risk of cancer. However, relative association measures may be misleading. The 10-year ESRD risk of 30%–40% in untreated patients is best compared with the 10-year malignancy risk of 7%–18% in treated patients. Moreover, only 5 of 20 patients with cancer died as a consequence, and 9% of all patients died during follow-up. By comparison, mortality risk is 50% after only 5 years in patients with ESRD (Dutch Dialysis and Transplantation Registry; www.renine.nl, personal communication). Moreover, dialysis as well as kidney transplant patients are more likely to die from cardiovascular or infectious causes than malignancy. Admittedly, different types of cancer tend to occur in dialysis and transplant patients (18). These malignancies may have a different prognosis from the ones observed after cyclophosphamide therapy. Nonetheless, the risks associated with ESRD may still outweigh those risks of the cyclophosphamide treatment in patients with progressive iMN. Finally, others have shown that very low serum albumin levels as a result of severe nephrosis can result in life-threatening thromboembolic complications (19). In these patients, attenuating the immediate complication risk outweighs the long-term malignancy risk.

A strength of the present study is the prospective inception of the cohort, reducing the likelihood of selection bias. Also, patients in our cohort have been followed long enough for cancer to occur. In addition, possible confounding risk factors were taken into account. Moreover, because patients were treated according to a uniform strategy closely adhering to recent guidelines (20), the data presented here can be generalized to current clinical practice. In addition, sensitivity analyses were performed in which patients treated with other immunosuppressive agents were excluded. In all analyses, similar associations between cyclophosphamide use and cancer were observed.

The IR reported here was similar to the IRs in other patient populations (2–4,21). Surprisingly, malignancy risk was only 1.7 times higher in cyclophosphamide-treated patients compared with age- and sex-matched persons in the general population. The incidence for unexposed patients was lower than in the general population. A likely explanation for this finding is that our patients were screened for cancer at baseline. In addition, residual confounding (*e.g.*, because of smoking) may be present compared with the general population, whereas confounding was adjusted for in the Poisson regression. Therefore, the results from the regression analysis are the most valid.

Remarkably, only 2 of 20 observed malignancies were bladder cancers (22), and no skin cancers were observed. Lefaucheur *et al.* (5) studied malignancy in iMN patients and also did not report skin cancer. However, increased risk of skin cancer was observed in patients with rheumatoid arthritis and ANCA-associated vasculitis (2,21).

Table 3. Characteristics of the patients who developed a malignancy during follow-up

Sex	Cyclophosphamide Treatment	Age at Biopsy (yr)	Type of Malignancy	Died during Follow-Up	Cause of Death	Time between Biopsy and Malignancy (yr)	Time between Treatment and Malignancy (yr)	Time between Biopsy and Death or Final Follow-Up (yr)	Serum Anti-PLA2R Antibodies at Review
Men	No	70	Lung	No		1.9		1.9	n/a
Men	No	37	Lung	Yes	Malignancy	6.9		7.2	−
Men	No	64	Chronic lymphocytic leukemia	No		3.4		6.9	+
Men	No	74	Lymphoma n.o.s.	Yes	Malignancy	1.8		1.8	+
Men	Yes	45	Prostate	No		18.9	10.3	18.9	+
Men	Yes	33	Leukemia n.o.s.	Yes	Malignancy	20.6	16.2	20.6	−
Men	Yes	44	Colon	No		12.5	10.9	15.2	+
Men	Yes	70	Prostate	No		12.7	11.8	14.8	n/a
Women	Yes	43	Chronic myelofibrosis	No		4.5	3.5	10.9	+
Men	Yes	57	Colon	No		10.7	9.6	11.5	−
Men	Yes	66	Prostate	Yes	Unknown	10.1	4.5	14.9	+
Men	Yes	54	Bladder	Yes	Malignancy	8.1	1.6	9.1	+
Men	Yes	48	Chronic lymphocytic leukemia	Yes	Cardiovascular	33.9	8.5	33.9	+
Men	Yes	70	Non-Hodgkin's lymphoma	Yes	Unknown	7.6	7.5	11.1	−
Women	Yes	62	Lung	Yes	Malignancy	3.6	3.6	4.6	+
Men	Yes	72	Larynx	No		4.2	2.6	7.8	+
Men	Yes	65	Colon	No		5.8	5.6	7.2	+
Men	Yes	59	Renal cell carcinoma	No		8.0	4.4	8.0	+
Women	Yes	66	Acute myeloid leukemia	No		2.5	2.2	3.0	+
Men	Yes	79	Bladder	No		1.7	1.7	4.1	−

In two patients, serum samples were no longer available; therefore, anti-PLA2R serostatus could not be ascertained. PLA2R, phospholipase A2 receptor; n.o.s., not otherwise specified.

However, those patients were treated with multiple drug regimens for prolonged periods. Perhaps they are more prone to virally induced cancers. Finally, we cannot exclude that small skin cancers were underreported in our study. Conversely, hematologic malignancies were relatively common in our cohort. Therefore, we feel that physicians should maintain awareness for signs of malignancy, including (but not limited to) bladder cancer, in cyclophosphamide-treated patients.

We observed some early-onset malignancies. We hypothesized that these malignancies may be caused by the inclusion of patients with secondary membranous nephropathy, in whom an early diagnosis of cancer was missed. However, this hypothesis is unlikely, because the frequency of anti-PLA2R seropositivity in patients with a malignancy was approximately equal to the reported prevalence of anti-PLA2R antibodies in iMN patients (23). In addition, there was no apparent association between the presence of anti-PLA2R autoantibodies and the time to malignancy. Several works have reported the onset of bladder cancer within 1 year after the start of cyclophosphamide therapy (24–26). Obviously, reported early cases may reflect incidental findings. Note that our limited data do not allow for additional inferences on the association between anti-PLA2R antibodies and malignancy risk.

A clear dose–response relationship between cyclophosphamide exposure and malignancy risk has been reported in non-Hodgkin's lymphoma (4). In these patients, risk increased 2.4- and 14.5-fold when the cumulative dose was less than 20 and over 50 g, respectively. Reported cumulative doses in rheumatoid arthritis and vasculitis were higher still, averaging over 80 g in patients who developed cancer (27). By comparison, the mean cumulative dose in our patients was 37 g, and only 20% of the patients received more than 50 g. We did not observe a clear dose–response relation, possibly because of the uniform treatment that patients received.

When weighing the risks and benefits of cyclophosphamide treatment, other complications should be considered. About one in three patients suffers a serious adverse event (20), mostly leucopenia, infections, and thrombotic complications, shortly after the start of therapy. Particularly worrisome for patients of a reproductive age is the risk of infertility. In these patients, the duration of cyclophosphamide therapy is limited to 3 months, which results in a relatively safe cumulative dose of less than 10 g (27). Other drugs have been suggested as first-line treatment, such as mycophenolate mofetil, cyclosporin A, tacrolimus, and rituximab (28,29). Although these drugs induce remission of proteinuria, they have not been unequivocally shown to be as effective as cyclophosphamide in a direct comparison of long-term outcome. Moreover, mycophenolate mofetil and calcineurin inhibitors have been associated with malignancy in the transplantation setting, possibly through viral mediators (29). For rituximab, however, data on long-term risks are limited. Thus, more data are needed on the efficacy and safety of other immunosuppressive drugs before replacing cyclophosphamide in the treatment of iMN.

When interpreting the data, a few issues have to be taken into account. Although screening for malignancy in patients with membranous nephropathy is standard clinical practice, we cannot exclude that malignancies at the time of diagnosis may have been missed. Conversely, there was no standard screening for cancer during follow-up. Because of its known carcinogenic effects, physicians may have been more proactive in screening patients treated with cyclophosphamide (2–4), which may have inflated IRs. Although missing values for potential confounders were dealt with in accordance with best practice, incomplete reporting may have led to misclassification of confounder status. Additionally, we cannot exclude residual confounding caused by unknown or unrecorded confounders (*e.g.*, alcohol use or environmental exposures). Twenty patients were lost to follow-up shortly after urine analysis, which may have resulted in selection bias of unknown direction. Finally, there were some empty cells in the latency analysis. To deal with this issue in a conservative manner, a single event was added to both the cyclophosphamide and unexposed groups.

We showed that cyclophosphamide therapy gives a threefold increase in the risk of cancer within 10–15 years after the start of treatment for iMN. For a 55-year-old patient, this result translates into an increase in annual risk from approximately 0.3% to 1.0%. The data presented here help weigh the benefits against the risks of cyclophosphamide therapy in iMN, thus enabling physicians and patients to make an informed decision on treatment modality.

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Disclosures

None.

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Cancer Risk after Cyclophosphamide Treatment in Idiopathic Membranous Nephropathy: Supplementary appendices

Full description of Patients and Methods

We included adult patients with biopsy proven idiopathic membranous nephropathy who visited our outpatient clinic between 1995 and 2009. Patients were either referred by a family doctor in our catchment area or by an allied centre. Secondary causes were ruled out per standard policy.[1] Specifically, diagnostic procedures included chest X-rays, serology (ANA) to exclude systemic disease, and tests for hepatitis B and C. A mammography was performed in women over the age of 50 years and prostate specific antigen was obtained in men over 50 years of age. Additional investigations were undertaken if clinical suspicion for malignancy was raised by history, physical examination, other diagnostic test or, specifically, the presence of iron deficiency anemia. Written informed consent was obtained, and the study was conducted in accordance with the declaration of Helsinki and approved by the Radboud University Medical Centre medical ethics committee.

Most patients were treated according to our restrictive immunosuppressive regimen, detailed elsewhere.[1] In summary, patients underwent a standardized, timed urine measurement.[2] Subsequently, all patients received supportive treatment. This consisted of blood pressure control and proteinuria reduction with ACEi and/or ARBs, and further blood pressure lowering drugs to achieve target levels below 130/80 mmHg. Additionally, statins were given to treat hypercholesterolemia, and anti-coagulant therapy was considered in patients with severe hypoalbuminemia (<2.0 g/dl). In patients who reached a serum creatinine concentration above 1.5 mg/dL, immunosuppressive therapy was advised. Severe or life threatening symptoms of nephrotic syndrome were considered an indication to start treatment with immunosuppressive agents as well. Oral cyclophosphamide (1.5 mg/kg daily for twelve months) and pulse intravenous methylprednisolone (1 gram on days one to three, 61 to 63 and 121 to 123) in combination with high dose oral prednisone (0.5 mg/kg every other day for five months before tapering) was the preferred treatment. Occasionally, alternative immunosuppressive drugs were prescribed either as part of a clinical trial or when cyclophosphamide was contraindicated.[3,4] In patients of reproductive age, the duration of cyclophosphamide therapy was reduced to three months, followed by nine months of azathioprine or mycophenolate mofetil. The resulting cumulative cyclophosphamide dose was less than 10 grams, which is considered safe to preserve fertility. From 1999 onward, trimethoprim-sulfamethoxazole was added to the regimen to prevent *pneumocystis jiroveci* pneumonia.

The outcome for the present study was incident malignancy, recorded from medical records and including the date of diagnosis. Mortality and the date of the final consultation were recorded as well

We pre-specified potential confounders, being age at time of biopsy, gender, ever smoking, having a first degree relative with a history of malignancy, chronic kidney disease stage (CKD, including substages 2a/b and 3a/b) and presence of nephrotic syndrome at the time of biopsy. Immunosuppressive therapy was considered a possible confounder if it was initiated prior to cyclophosphamide therapy. Immunosuppressive therapy *after* cyclophosphamide could have acted as an intermediary, and thus adjustment could result in underestimation of possible malignancy risk. Gender, date of birth and height were recorded during urinary analysis at our center, whereas biopsy and follow-up laboratory data were obtained from medical records by two of the authors (PvD and JvdB). We recorded family history, smoking history, cyclophosphamide exposure (including total cumulative dose) and the use of other immunosuppressive drugs over the entire follow-up duration, including immunosuppressive drugs after cyclophosphamide treatment. Specifically we registered chlorambucil, azathioprine, mycophenolate mofetil, ciclosporin A, tacrolimus, methotrexate, prednisone and/or methylprednisone and experimental drugs (e.g. synthetic adrenocorticotropic hormone or rituximab), were obtained from medical records as well.

Statistical methods

Baseline data are presented as mean ± standard deviation (SD), median and inter quartile range (IQR) or frequencies and proportions. X^2 test was used to evaluate differences in frequencies. The difference in means for normally distributed variables was compared using t-test. Wilcoxon's rank sum test was used to compare medians for the skewed variables.

Person time was calculated as the time from start of therapy until the occurrence of malignancy or the end of follow-up in the cyclophosphamide exposed group. Ideally, one would like to start measuring person time for controls at the moment that they would have started treatment. To mimic this moment of exposure, the median time between biopsy and initiation of therapy in the cyclophosphamide group was estimated and deducted from the time between biopsy and malignancy or end of follow-up in the control group. Consequently, time during which the exposed group was not actually at risk for malignancy due to cyclophosphamide exposure was not falsely included in the control group. If controls had negative person time as a result, they were excluded from the analyses.

Subsequently, the cumulative incidence of malignancy was calculated, assuming mortality prior to the occurrence of cancer was competing with malignancy risk. Unadjusted incidence rates were calculated and used to estimate the incidence ratio (IR) of malignancy after cyclophosphamide exposure. In order to estimate latency between cyclophosphamide exposure to outcome, the incidence ratio of malignancy was calculated by two year strata of person time. If empty cells were encountered when calculating the within stratum incidence ratios, a single event was added to both the cyclophosphamide and unexposed group. Potential confounding was investigated by stratifying according to age, gender, ever-smoking, prior immunosuppressive therapy , family history of malignancy, CKD stage and presence of the nephrotic syndrome. Age was categorized as under 44, 45 to 54, 55 to 64, 65 to74 and over 75 years. Standardized incidence ratios (SIR) were calculated by weighting for the distribution of the potential confounders in the cyclophosphamide treated group,

as described by Rothman, Greenland and Lash.[5] Note that no events were added to empty cells when SIRs were calculated.

Multiple imputation by chained equations was used to impute missing data on smoking status, family history and cumulative cyclophosphamide dose. Data on malignancies, age, gender and prior immunosuppressive therapy were complete and used in the imputation model, as these variables were also considered for the final analysis model. In addition, baseline serum creatinine, serum albumin, mean arterial pressure, body mass index and baseline presence of nephrotic syndrome were included. The natural logarithm of continuous variables was taken in order to stabilize variance. Forty imputations were created using logistic regression for smoking status and family history, and linear regression for cumulative dose. The imputed data was checked visually using scatter plots. Poisson regression was used to obtain a multiple adjusted IR for the association between cyclophosphamide and malignancies, whilst taking the imputations into account. In addition, the dose-response relation between cumulative cyclophosphamide exposure and the occurrence of malignancy was investigated by creating 20 gram categories of cumulative exposure and including these in an adjusted Poisson regression. For all of the analyses above, 95% confidence intervals around the incidence ratios have been calculated.

Membranous nephropathy can occur secondary to cancer and can be incorrectly classified as idiopathic when that cancer is not yet detected. These patients are unlikely to respond to conventional therapy, and thus more likely to receive cyclophosphamide. As a result the association between cyclophosphamide and cancer (especially early cancers) may be inflated. Therefore, we checked for serum antiphospholipase A2 receptor antibodies (anti-PLA2R) at the time of referral, assuming that, if patients with early malignancies were predominantly seronegative for anti-PLA2R, the association between early cancers and cyclophosphamide would be the result of previously undiagnosed cancers. The serum samples were obtained at the time of urine analysis and stored at -80°C. Anti-PLA2R status was measured using a commercially available indirect immunofluorescence test (Euroimmun AG, Lübeck).

In sensitivity analyses, a Poisson regression was performed using only patients with complete data for smoking and family history. Secondly, analyses were repeated excluding patients who had received immunosuppressive drugs other than cyclophosphamide. Finally, age and gender cancer specific incidence in the present cohort was standardized to the general population using incidence estimates obtained by the Netherlands Cancer Registry over the past decade.[6] To do so, person time was stratified according to age and gender. The expected number of cases within each stratum was calculated. Subsequently, a standardized incidence ratio was obtained by dividing the observed by the expected number of cases.

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Supplementary tables

Supplementary Table 1. Immunosuppressive therapy with drugs other than cyclophosphamide by cyclophosphamide exposure.

	Cyclophosphamide exposed (n=127)		**Not exposed to cyclophosphamide (n=145)**	
Initial treatment* (before CP)	Chlorambucil	5	Chlorambucil	1
	MMF	11	MMF	9
	ACTH	4	ACTH	7
	High dose corticosteroids (Coggins)	7	High dose corticosteroids (Coggins)	4
			Aza	1
Total initial treatment		27 (21%)		22 (15%)
Additional treatment* (after CP)	MMF	13		
	ACTH	2		
	Aza	7		
	CsA	2		
	ACTH & Tacrolimus	3		
	ACTH & MMF	1		
	CsA & Aza	1		
	MMF & Aza	2		
	MMF, Tacrolimus & Aza	1		
Total secondary treatment		32 (25%)		

*MMF: mycophenolate mofetil, ACTH: synthetic Adrenocorticotropic Hormone, Aza: azathioprine, CsA: cyclosporin A.*Ten patients have received other immunosuppressive therapy both prior to and after cyclophosphamide therapy and have been counted double in this table.*

- The median duration of cyclophosphamide therapy was 12 (IQR 12 to 16) months in cyclophosphamide treated patients. Patients treated with other immunosuppresion, and not cyclophosphamide, were treated for a median of 9 (IQR 5 to 12) months. Patients who received both CP and other immunosupression were treated with cyclophosphamide for 12 (IQR 6 to 15) months and other drugs for 12 (IQR 8 to 22) months, resulting in a total treatment duration of 27 (IQR 17 to 59) months.
- Coggins' regimen consists of high dose oral prednisone for 8 weeks.
- Chlorambucil was given as part of the Ponticelli regime (0.2 mg/kg daily orally).
- MMF was given as part of a clinical trial, if patients had a pregnancy wish or if they experienced severe side effects of cyclophosphamide. The regular MMF dose was 500mg to 1000mg twice daily orally for 9 to 12 months.
- ACTH was administered for nine months during a clinical trial (clinicaltrials.gov: NCT00694863). Patients received 1 mg by intramuscular injections in increasing dose with a maximum of 1 mg twice weekly.
- Azathioprine was given in case of side effects of cyclophosphamide. Dose was usually 100mg per day. It was decreased to 50 mg per day over time in most patients and stopped after a year of treatment.

- Cyclosporin A was occasionally given if patients refused alkylating agents, dosage varied between 100 and 300mg daily. Tacrolimus was given instead of cyclosporin in more recent years.

Sensitivity analyses

Patients who received immunosuppressive drugs other than cyclophosphamide were excluded. Of the cyclophosphamide treated patients, 17 received other immunosuppression prior to cyclophosphamide, 22 received other immunosuppression afterwards, and 10 patients received other immunosuppression both before and after cyclophosphamide treatment. In total, 201 patients were included, 78 were treated with cyclophosphamide, 123 patients were not treated with immunosuppression at all. The population characteristics are presented in the table below. We then performed univariate, standardized and multiple adjusted analyses, the latter using Poisson regression. The adjusted analyses were preformed after multiple imputation of missing values for family history and smoking. Table 3 shows the incidence ratio ratios for cancer after cyclophosphamide exposure: unadjusted, standardized to potential confounders and after multiple adjustment.

Supplementary Table 2: Baseline population characteristic after exclusion of patients treated with other immunosuppressive drugs.

Variables	Cyclophosphamide treated (n=78)	Not treated with cyclophosphamide (n=123)	P
At baseline (the time of biopsy)			
males (n, %)	61 (78%)	77 (63%)	0.02
BMI (kg / m^2)	26.9 ± 3.6	27.2 ± 5.2	0.58
age (years)	56.1 ± 11.1	49.2 ± 14.7	<0.001
year of biopsy	2001 ± 5.1	2002 ± 6.5	0.81
follow-up duration (years)	6.0 (3.7 - 10.7)	5.5 (2.9 - 8.4)	0.73
positive family history for malignancy*	9/52 (17%)	10/84 (12%)	0.38
current/former smoker*	37/61 (61%)	49/89 (55%)	0.46
eGFR-MDRD4 (ml/min/1.73m^2)	62 ± 21	77 ± 20	<0.001
serum creatinine (mg/dl)	1.2 (1.0 – 1.5)	0.9 (0.8 – 1.1)	<0.001
serum albumin (g/dl)	2.0 (1.7 – 2.5)	2.8 (2.4 – 3.1)	<0.001
nephrotic syndrome at presentation	75 (96%)	100 (81%)	0.002
protein : creatinine ratio (g / g)	8.8 (5.9 – 11.1)	4.5 (2.7 – 6.6)	<0.001
ACEi/ARB use	68 (87%)	113 (92%)	0.28
statin use	49 (63%)	73 (59%)	0.62
other BP lowering medication	24 (31%)	17 (14%)	0.004
Therapy			
interval from baseline to start of therapy (months)	9 (4 - 16)	n/a	.
serum creatinine at start of therapy (mg/dl)	1.4 (1.1 – 1.9)	n/a	.
cumulative cyclophosphamide dose (g)	37 (27 - 46)	n/a	.
Outcomes			
death	11 (14%)	6 (5%)	0.02
malignancies	11 (14%)	3 (2%)	0.002

*The denominator differs from the total number of patients due to missing data for this variable.
Data are presented as mean ± standard deviation, median (inter quartile range) or percentages

where appropriate. BMI: Body mass index; eGFR-MDRD4: estimated glomerular filtration rate, calculated with the abbreviated Modification of Diet in Renal Disease equation for mass spectrometry standardized creatinine; ACEi: angiotensin converting-enzyme inhibitor; ARB: angiotensin II receptor blocker; BP: blood pressure.

Supplementary Table 3: Incidence ratios for malignancy after cyclophosphamide exposure by possible confounders. Analysis limited to patients without immunosuppression other than cyclophosphamide.

Risk Factor	Incidence Ratio	95% Confidence Interval
Unadjusted	6.3	1.7 - 35.1
Univariate adjusted:		
Age	3.8	0.8 - 17.1
Male gender	4.6	1.3 - 16.7
Smoking	5.3	1.4 - 19.9
Family history of malignancy	7.4	1.6 - 35.0
CKD stage	10.3	2.8 - 38.0
Nephrotic syndrome	6.2	1.4 - 27.3
Multiple adjusted*	5.1	1.2 - 21.5

*After multiple imputation, complete case analysis: (n=150; IRR=3.4 [95%CI: 0.8 - 14.9]). The analysis was adjusted for age, gender and ever smoking in both the imputed and complete case analyses.

In conclusion, when restricting the analyses to persons who have not received any other immunosuppressive drugs, the risk associated with cyclophosphamide exposure is five times greater compared to patients receiving supportive care only. Obviously, confidence intervals are wider compared to previously conducted analyses, as fewer patients have been included.

In the final analysis, age and gender cancer specific incidence in the present cohort was standardized to the general population. Person time was stratified according to age and gender. The expected number of cases within each stratum was calculated. Subsequently, a standardized incidence ratio was obtained by dividing the observed by the expected number of cases.

Supplementary Table 4. Incidence ratio of malignancy for the total cohort, cyclophosphamide exposed and unexposed patients standardized to the general Dutch population stratified by gender and age.

			Total cohort			Cyclophosphamide exposed			Unexposed		
Gender	Age	Average Population Incidence (per 100,000 persons per year)	Person time (years)	observed cases	expected cases	Person time (years)	observed cases	expected cases	Person time (years)	observed cases	expected cases
Females	15-19	31.3	6.4		0.00				6.4		0.00
	20-24	57.4	14.9						14.9		
	25-29	104.7	27.8		0.03	2.1		0.00	25.7		0.03
	30-34	169.0	35.8		0.06				35.8		0.06
	35-39	280.0	42.3		0.12	2.7		0.01	39.6		0.11
	40-44	453.0	51		0.23	11.3		0.05	39.7		0.18
	45-49	640.6	58.5	1	0.37	15.4	1	0.10	43.1		0.28
	50-54	801.9	49.3		0.40	11.9		0.10	37.4		0.30
	55-59	1035.7	65.3		0.68	18.4		0.19	46.9		0.49
	60-64	1286.8	58.6		0.75	30.4		0.39	28.2		0.36
	65-69	1535.6	55.5	2	0.85	32.3	2	0.50	23.2		0.36
	70-74	1663.1	28.9		0.48	16.5		0.27	12.4		0.21
	75-79	1904.7	11.6		0.22	5		0.10	6.6		0.13
	80-85	1942.4	5.5		0.11	3.3		0.06	2.2		0.04
Males	15-19	37.5	13.7		0.01				13.7		0.01
	20-24	53.7	19.7		0.01	2.8		0.00	16.9		0.01
	25-29	68.4	34.1		0.02	18.4		0.01	15.7		0.01
	30-34	87.3	56.2		0.05	30.1		0.03	26.1		0.02
	35-39	126.2	83.6		0.11	40.3		0.05	43.3		0.05
	40-44	217.4	110.3	1	0.24	46.4		0.10	63.9	1	0.14
	45-49	410.1	139.4		0.57	84.6		0.35	54.8		0.22
	50-54	762.2	158.6	1	1.21	110.4	1	0.84	48.2		0.37
	55-59	1307.2	141.4	1	1.85	83.1	1	1.09	58.3		0.76
	60-64	2015.1	133.8	2	2.70	64.1	2	1.29	69.7		1.40
	65-69	2744.5	131.9	3	3.62	69.7	2	1.91	62.2	1	1.71
	70-74	3392.7	59.8	2	2.03	35.4	1	1.20	24.4	1	0.83
	75-79	3660.0	26.6	4	0.97	16.4	3	0.60	10.2	1	0.37
	80-85	3555.4	9.0	3	0.32	9.0	3	0.32			
				20	17.7		16	9.2		4	8.4
SIR				1.13			1.73			0.47	
lower				0.64			0.88			0.01	
upper				1.63			2.58			0.94	

SIR: Standardized Incidence Ratio. Lower and upper represent the lower and upper limit of the 95% confidence interval, respectively. Person time distributed over the strata according to ageing. For example if a 42 year old male had a 12 year follow-up, 3 person years would contribute to person time in the age stratum 40 to 44, 5 years to the stratum 45 to 49 and the final 4 years to the stratum 50 to 54.