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Diabetic nephropathy : from histological findings to clinical features

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Citation
Klessens, C. Q. F. (2017, November 22). *Diabetic nephropathy : from histological findings to clinical features*. Retrieved from https://hdl.handle.net/1887/55808

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Note: To cite this publication please use the final published version (if applicable).

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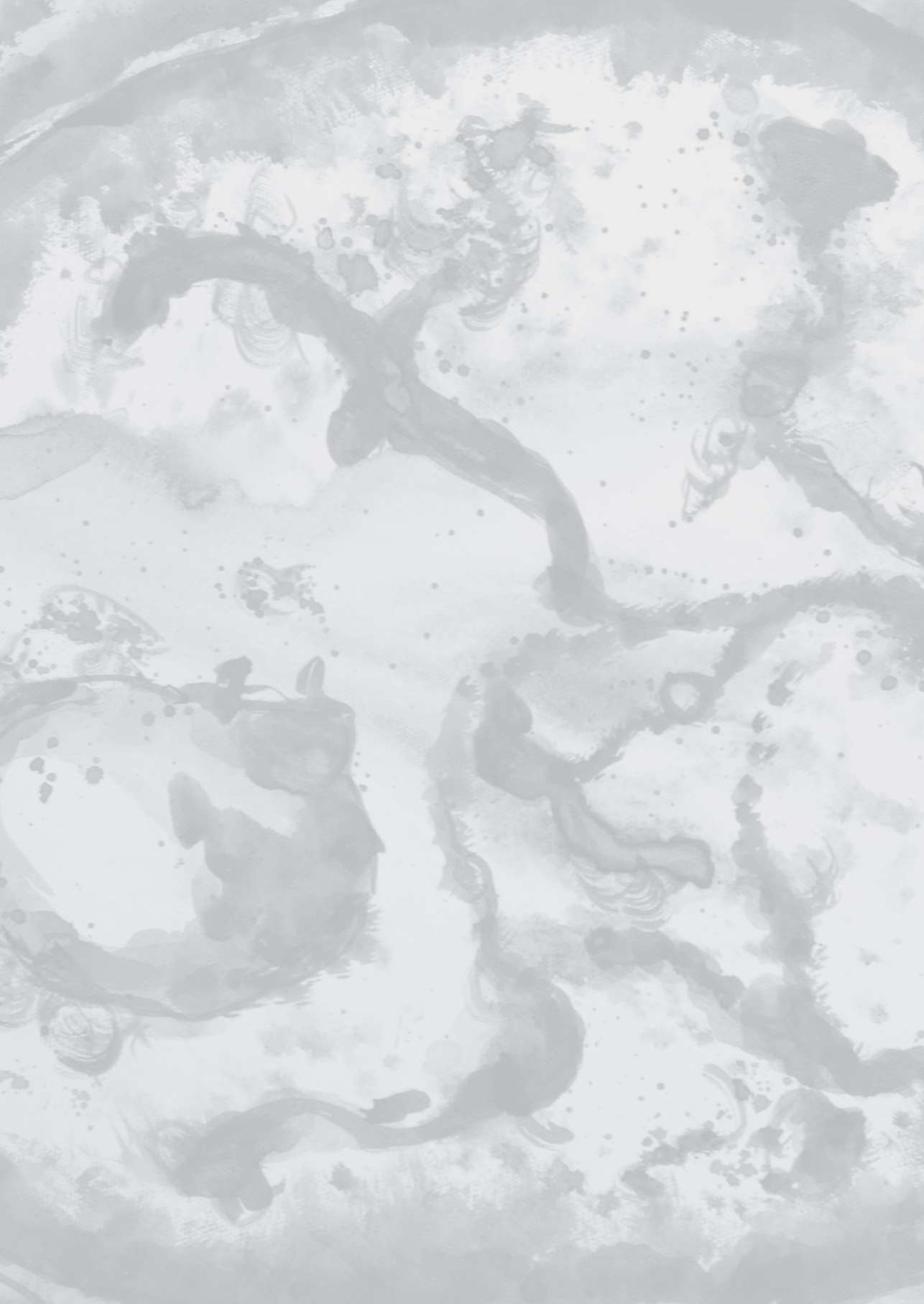
Author: Klessens, C.Q.F.
Title: Diabetic nephropathy : from histological findings to clinical features
Issue Date: 2017-11-22



Macrophages in diabetic nephropathy in patients with type 2 diabetes

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Nephrology Dialysis Transplantation, 2017; 32(8): 1322-1329

ABSTRACT

Inflammation plays a role in the development of diabetic nephropathy (DN) in type 2 diabetics. Although macrophages have been found in experimental models of DN, little is known regarding the presence of macrophages in patients with DN. Therefore, we investigated the presence and phenotype of glomerular and interstitial macrophages in relation to clinical and histopathological parameters in patients with DN.

Renal autopsy samples were obtained from eighty-eight type 2 diabetic patients with histologically proven DN and stained for CD68 and CD163 as general and M2/anti-inflammatory markers of macrophages. Renal damage was scored based on histopathological classification of DN. Control renal autopsy samples were obtained from patients without renal abnormalities and from diabetic patients without DN. Positive cells per glomerulus were counted. Interstitial macrophages were counted semi-quantitatively.

Macrophages were present in all groups. In the DN group, the mean number of CD68+ cells/glomerulus and CD163+ cells/glomerulus was 4.2 (range 0-19) and 2.1 (range 0-14.47), respectively. The distribution was similar between all histopathological classes. Glomerular CD163+ macrophages were positively associated with DN class, interstitial fibrosis and tubular atrophy, and glomerulosclerosis. Interstitial CD68+ macrophages were correlated with GFR stage and albuminuria.

Our results demonstrate that macrophages are present in the glomeruli and interstitium of type 2 diabetic patients with DN and of controls. Although patients and controls had similar numbers of glomerular macrophages, glomerular anti-inflammatory CD163+ macrophages were associated with pathological lesions in DN. Taken together with the correlation between interstitial macrophages and IFTA, DN class, and renal function, this finding suggests that macrophages may play a role in DN progression. Therefore, targeting macrophages may be a promising new therapy for inhibiting the progression of DN.

INTRODUCTION

The global prevalence of type 2 diabetes (T2D) is increasing rapidly [1]. A severe complication associated with T2D is the development of diabetic nephropathy (DN), a major cause of end-stage renal disease [2]. Despite current therapy, many patients with DN progress to renal failure [3]. Increasing our understanding of the pathophysiology that underlies DN can help to develop therapeutic regimens for inhibiting the development and/or progression of DN. Traditionally, DN was believed to result from interactions between hemodynamic factors and metabolic factors [4, 5]. Recent studies, however, found that inflammatory mediators play an important role in the early stages of the disease [6]. Thus, newly emerging treatment options focus on inhibiting this inflammatory pathway [7].

Experimental studies have shown that the inflammatory response that precedes and helps promote insulin resistance in diabetes depends largely upon the accumulation of macrophages in tissue [8]. Furthermore, experimental models of DN have shown that DN is accompanied by an influx of macrophages in response to an upregulation of monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) [8, 9]. In these models, inhibiting the influx of macrophages reduces albuminuria, reduces glomerular damage, and slows the progression of renal disease [10, 11]. The influx of macrophages has also been observed in other chronic kidney diseases, including anti-GBM nephritis and ANCA-associated pauci-immune crescentic glomerulonephritis [12, 13]. Animal models of these renal diseases revealed that renal injury can be mitigated by depleting macrophages and/or by disrupting macrophage recruitment [12]. Nevertheless, whether these macrophages affect the course of the disease or merely represent a response to injury remains unknown [10, 14]. In addition, infiltrating macrophages can differentiate into distinct phenotypes in response to the microenvironment, thereby expressing pro-inflammatory, anti-inflammatory, or profibrotic cytokines [8, 15, 16].

In patients with DN, MCP-1 is upregulated in renal tissue, and MCP-1 levels are increased in the urine, suggesting that an influx of macrophages plays a pathogenic role in the development of proteinuria, in glomerular damage, and in the progression of renal disease in humans [17-20]. However, relatively little is known about the presence or phenotype of macrophages in the kidneys of patients with diabetes. To date, only one study investigated the presence of macrophages in patients with DN; this relatively small study found macrophages in the glomeruli and interstitium of diabetic patients but did not investigate the phenotype of these macrophages [21].

The aim of this study was to investigate the presence and phenotype of macrophages in renal autopsy samples obtained from a cohort of patients with histologically proven DN. In addition, we examined the association between these macrophages and both histopathological and clinical parameters. Renal damage was scored in accordance with the histopathological classification of DN, which is linked to renal outcome [22-24].

METHODS

In this retrospective autopsy study, renal autopsy tissue specimens were obtained from patients with type 2 diabetes. The samples were retrieved from the pathology archives at Leiden University Medical Center. A total of 88 patients were included retrospectively from autopsies performed in 1984 through 2004 via the database of the Department of Pathology for autopsy material. The primary inclusion criterion was the presence of T2D in patients who were over the age of 18 years at the time of death. We also used two control groups. One control group consisted of renal autopsy material obtained from non-diabetic patients with no other renal abnormalities but with other comorbidities, including hypertension, heart failure, or atherosclerosis (N=5). The second control group consisted of diabetic patients with no histological evidence of DN (N=18).

We classified renal damage in the diabetic patients using the histopathological classification of DN, which is based on defined glomerular damage [25]. This classification system was used to investigate whether the presence of renal macrophages was associated with glomerular damage. The presence of CD68+ and CD163+ cells was used to indicate total macrophages and M2 (anti-inflammatory) macrophages, respectively [16].

Clinical data

Clinical data were obtained from the medical records available at Leiden University Medical Center and from the patients' general practitioners. Approval to obtain relevant clinical data from the patients' practitioners was obtained from the medical ethics committee of Leiden University Medical Center. The following laboratory results were obtained: serum creatinine; eGFR (calculated using the MDRD formula); microalbuminuria (i.e., 30-300 mg/L) or macroalbuminuria (i.e., >300 mg/L), measured via 24-hour urine or dipstick tests; systolic and diastolic blood pressure; serum hemoglobin, serum cholesterol; and serum HbA1c. GFR was staged in accordance with the stages of chronic kidney disease established by the KDIGO (Kidney Disease Improving Global Outcomes) guidelines. Dipstick tests were interpreted and staged as follows: absent when negative or trace; microalbuminuria when 1+ or 2+ (30-300 mg/L); or macroalbuminuria when 3+ or 4+ (>300 mg/L). These data were collected retrospectively from the period start-

ing one year before the patient's death. Data that reflected a stable representation of the patient's serum and/or urine levels were included. We also obtained data regarding comorbidity, duration of T2D, medication history, hypertension, smoking history, and diabetic complications such as retinopathy, cardiomyopathy, polyneuropathy, and diabetic foot ulcers. For each patient, the cause of death was obtained from the autopsy report.

Histopathology

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 1-µm and 3-µm thickness and stained with hematoxylin and eosin (HE), periodic-acid Schiff (PAS), and silver stain (for the 1-µm thick sections).

Renal tissue specimens containing ≥100 glomeruli were scored by two investigators who were blinded with respect to the patients' clinical data. Glomerular lesions, interstitial lesions, and vascular lesions were scored in accordance with the established histopathological classification for DN [25]. Specifically, a score of 0 was given if interstitial fibrosis and tubular atrophy (IFTA) was not present in the cortex. A score of 1 was given if less than 25% IFTA was present. A score of 2 was given when at least 25% but less than 50% IFTA was present. Finally, a score of 3 was assigned when at least 50% IFTA was present [25]. In addition, the following glomerular lesions were noted and scored as either present or absent: focal segmental glomerulosclerosis (FSGS), cholesterol emboli, any other glomerular lesions, capsular drops, and hyalinosis of the glomerular vascular pole.

Immunohistochemistry

To detect and characterize the macrophages presence in the kidney samples of DN patients and controls, immunohistochemical staining was performed on sequential slides using monoclonal mouse antibodies against human CD68 (1:2000, DakoCytomation, Glostrup, Denmark), a general macrophage marker, and monoclonal mouse antibodies against human CD163 (1:10, Abcam, Cambridge, UK), an anti-inflammatory macrophage marker (M2) [16]. As a control for CD68+ and CD163+ cells, we used a monoclonal antibody against CD45 (1:800, DakoCytomation, Glostrup, Denmark), a pan-leukocyte marker. Paraffin-embedded kidney samples were sectioned and then deparaffinized. Antigen retrieval was performed using Tris-EDTA buffer, pH 9.0 (for CD45 and CD68 immunostaining) or citrate buffer, pH 6.0 (for CD163 immunostaining). After blocking endogenous peroxidase, the sections were incubated in the relevant primary antibody for 1 hour. As a negative control, mouse IgG1 negative control fraction (DakoCytomation) was used at the same concentration as the respective primary antibody. Followed by antibody detections using the DAKO Envision+ System and was visualized

using diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin and coverslipped.

Immunohistochemistry analysis

The immunostained sections were scanned in an IntelliSite Pathology Ultra-Fast Scanner (Philips Healthcare, Eindhoven, the Netherlands). Using a Philips Image Viewer, the number of macrophages was counted in 50 glomeruli per section. Only CD68+ and CD163+ cells with a visible nucleus and well-defined cytoplasm were counted as positive in the glomeruli, similar to previous studies [26, 27]. The mean number of macrophages in the glomeruli was determined by dividing the total number of macrophages by 50. The investigators were blinded with respect to DN class and clinical parameters and scored the number of CD68+ and CD163+ cells per glomerulus. Interstitial macrophages were quantified in a separate session.

Interstitial CD68+ macrophages in the cortex were counted in a total of ten high-power fields (20x) and graded semi-quantitatively on a 0-3 scale. Grade 0 corresponded to macrophages in <10% of the interstitium; grade 1 corresponded to macrophages in 10-30% of the interstitium; grade 2 corresponded to macrophages in 30-50% of the interstitium; and grade 3 corresponded to macrophages in >50% of the interstitium [28].

Statistical analysis

The SPSS statistical software package, version 20.0 (IBM, Armonk, NY) was used for all statistical analyses. Statistical differences between groups were analyzed using the Jonckheere-Terpstra test for trends, the Kruskal-Wallis test, or the Mann-Whitney *U* test. Correlations for non-normally distributed data were evaluated by calculating Spearman's rank correlation (rho). Regression models (linear and ordinal) were used to investigate the influences of independent variables. Kappa score (k) was determined using the method of Landis and Koch and was used to quantify intraobserver and interobserver agreement of interstitial macrophage scoring [28]. Differences with a p-value ≤ 0.05 were considered statistically significant. The data in the tables are presented as either a percentage or the mean value (± SEM).

Ethics

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Declaration of Helsinki.

RESULTS

The clinical characteristics of the 88 patients with type 2 diabetes (T2D) and histologically proven DN are summarized in Table 1. Mean age was 71 years, and 53.4% were male. The mean duration of T2D was 12.7 years. Kidney samples obtained from these patients were classified according to the histopathological classification of DN [25]. Nineteen patients had class I DN, which is characterized by a thickened glomerular basement membrane. Twenty-nine patients had class II DN, which is characterized by mesangial expansion; 18 of these patients had class IIa DN, and 11 patients had class IIb DN. Thirty-four patients had class III DN, which is characterized by the presence of nodular sclerosis. Finally, the remaining six patients had class IV DN, which is characterized by global sclerosis in more than 50% of glomeruli. The five control samples obtained from non-diabetic subjects had no other renal abnormalities, and the 18 control samples obtained from diabetic patients without DN had no histological lesions characteristic of DN based on light microscopy and electron microscopy. In the control group (N=23; 57% male), mean age was 69 years, and mean blood pressure was 131/76 mmHg. The eighteen diabetic controls had a mean duration of diabetes of 8 years. With respect to renal damage, no glomerular damage was observed in the renal tissue specimens of the control group. However, some damage was observed in the interstitium and vessels, and this damage was probably age-related (e.g., atherosclerosis, ischemia, etc.). Neither IFTA nor the number of globally sclerotic glomeruli differed significantly between the diabetic control group and the DN cases (p=0.441 and 0.474, respectively).

Table 1. Clinical characteristics of the study group with histologically proven DN

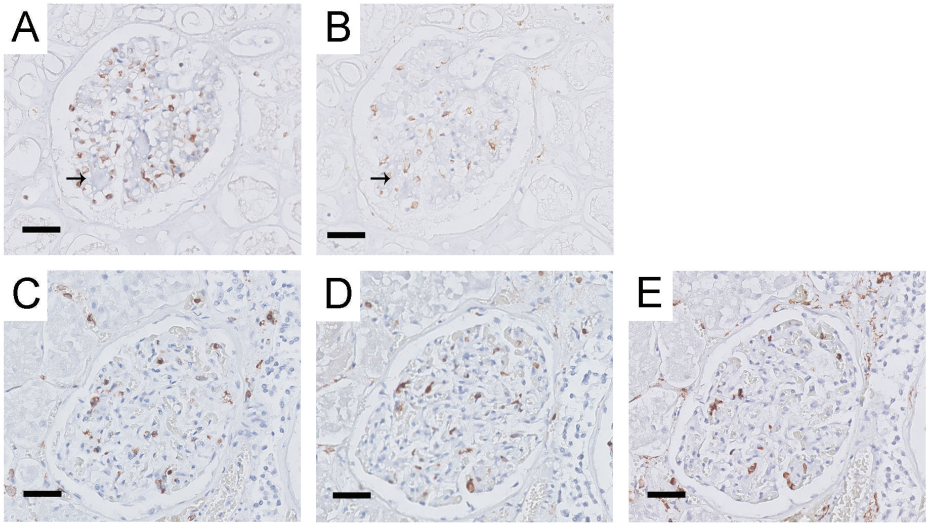
Clinical characteristics (N=88)	
Gender (% male)	47/88 (53.4)
Age (years)	70.6 ± 10.8
Duration of T2D (years)	12.7 ± 1.286
eGFR (ml/min/1.73m^2)	48.98 ± 3.696
Serum creatinine (µmol/L)	163 ± 11.826
HbA1c (% units)	8.56 ± 0.45
Proteinuria present[a]	36/55 (40.9%)
Systolic blood pressure, mmHg	136 ± 3.54
Diastolic blood pressure, mmHg	76.7 ± 1.55
Hb, (mmol/L)	7.0 ± 0.178
Death by CV event (%)	53.4
Ante mortem sepsis and/or renal insufficiency, N (%)	26 (29.5)

[a] Data regarding proteinuria were available for 55 patients only. Except where indicated otherwise, data are expressed as mean ± SEM. T2D, type 2 diabetes; Hb, hemoglobin; CV event, cardiovascular event

Glomerular macrophages and histological and clinical findings

In the group of 88 patients with T2D-associated DN, we counted the number of cells that stained positive for CD68 (a general marker of macrophages and monocytes) and CD163 (a scavenger receptor that is used as a marker of M2 or anti-inflammatory macrophages) in not globally sclerotic glomeruli. In all four histopathological classes of DN, both CD68+ and CD163+ macrophages were present in the glomeruli (Figure 1). When all four classes of DN were pooled, the mean number of CD68+ cells was 4.2 cells per glomerulus (median: 2.9; range: 0-19), and the mean number of CD163+ cells was 2.1 cells per glomerulus (median: 1.63; range: 0-14.74); the distribution of the mean number of cells/glomerulus in each sample is shown in Figure 2A. Thus, in each sample, the ratio of CD163+/CD68+ cells was approximately 1:2, indicating that 50% of the macrophages had a M2 phenotype. CD68+ macrophages were also present in the glomeruli of the samples obtained from the non-diabetic and diabetic control groups, with 5.5 cells per glomerulus (range: 0.9-15) and 4.5 cells per glomerulus (range: 2.3-6.8), respectively (Figure 2 B). In a subgroup of nine patients with DN, we stained for CD45 in sequential sections in order to confirm that the CD68+ cells were infiltrating leukocytes; CD68+ cells co-localized with CD45+ cells, confirming that the CD68+ cells were indeed inflammatory cells (Figure 1 C-E).

Figure 1. Accumulation of macrophages in the glomeruli of patients with type 2 diabetes and histologically proven diabetic nephropathy



A and **B:** Example immunostained renal sections obtained from a patient with class III DN. Sequential sections for CD68+ cells (**A**) and CD163+ cells (**B**) of a class III DN case with nodular sclerosis (arrow). **C-E:** Example immunostained renal sections obtained from a patient with class I DN. Sequential sections for CD45+ cells (as a control marker for infiltrating leucocytes) (**C**), CD68+ cells (**D**) and CD163+ cells (**E**)

No associations were found between the number of glomerular CD68+ cells and DN class (p=0.63), interstitial fibrosis and tubular atrophy (IFTA) (p=0.09), or global glomerulosclerosis (p=0.16) in the cohort of diabetics with DN, and similarly no association was found between the number of CD68+ cells and IFTA (p=0.68) or global glomerulosclerosis (p=0.46) in the diabetic without DN control group. In contrast, glomerular CD163+ cells were positively correlated with DN class (p=0.03), interstitial fibrosis and tubular atrophy (p <0.001), and global glomerulosclerosis (p=0.05). Renal function was inversely associated with glomerular CD68+ cells (p=0.017), but not with CD163+ cells (p=0.399). Finally, neither glomerular CD68+ cells nor glomerular CD163+ cells were associated with the presence of albuminuria (p=0.23, p=0.49, respectively) or with any other clinical parameters, including T2D duration (p=0.59, p=0.29, respectively), HbA1c (p=0.53, p=0.60, respectively), and cholesterol (p=0.60, p=0.45, respectively). Therapeutic regimens, including the use of RAAS blockers or oral diabetic medication, had no significant effect on the type or number of glomerular macrophages in our cohort. However, the use of RAAS blockers was correlated with interstitial macrophages (p=0.046) and

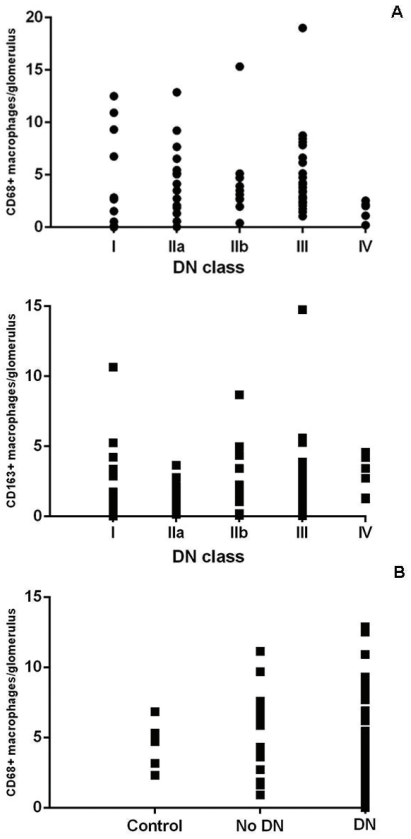


Figure 2. Glomerular macrophages in all four DN classes and control groups

Autopsy renal samples were sectioned and immunostained for CD68 or CD163, after which CD68+ and CD163+ cells were counted in 50 not globally sclerotic glomeruli per section. **A**) Distribution of the average number of CD68+ cells (upper plot) and CD163+ cells (lower plot) per glomerulus (N=88 patients). **B**) Average number of CD68+ cells per glomerulus in control non-diabetic subjects with no other renal abnormalities (N=5), diabetic patients without histologically proven DN ("No DN"; N=18), and type 2 diabetic patients with histologically proven DN (N=88). In each plot, each symbol represents an individual patient.

GFR (p=0.028). To correlate pathological lesions (e.g., IFTA, FSGS, cholesterol embolus, hyalinosis, and/or global glomerular sclerosis) with hypertension, we divided the cases into two groups containing hypertensive cases and non-hypertensive cases. Only the number of globally sclerotic glomeruli differed significantly between these two groups (p=0.004); however, these glomeruli were already excluded from further analysis.

Interstitial macrophages and histological and clinical findings

Next, we counted interstitial CD68+ macrophages using a semi-quantitative method. The semi-quantitative analysis of interstitial macrophages had substantial interobserver and intraobserver agreement (k=0.66 and 0.74, respectively). Our analysis revealed a significant positive correlation between the presence of these cells and the histopathological class of DN (p=0.026). The histological parameters (i.e., DN class, IFTA, and global glomerulosclerosis) correlated positively with interstitial CD68+ macrophages in the DN patient cohort (Table 2). With respect to clinical parameters, interstitial macrophages were significantly correlated with GFR stage, serum creatinine, and albuminuria (Table 3). In the diabetic control group, interstitial CD68+ macrophages were correlated with IFTA, but were not correlated with global glomerulosclerosis (Table 2). The multiple regression model showed that sepsis did not predict type or the number of glomerular CD68+ and CD163+ cells in patients with similar class of DN (p=0.995 and p=0.304, respectively). The multiple regression model showed that interstitial macrophages were associated with sepsis in patients with similar amount of IFTA (p=0.018). However, the correlations between interstitial macrophages and clinical parameters remained significant when cases with sepsis were excluded (Supplementary Table 1). Next, we divided the cause of death into cardiovascular (53.4% of cases), inflammatory (33%), and other (13.6%). No significant difference was found between the causes of death with respect to the number of macrophages in the glomeruli or interstitium (mean glomerular CD68+ cells, p=0.650; mean glomerular CD163+ cells, p=0.115; interstitial macrophages p=0.580).

Table 2. Correlation between histology and interstitial CD68+ macrophages

	T2D patients with DN (N=88)		Diabetic controls[a] (N=18)	
	rho[b]	*p*-value	rho[b]	*p*-value
DN class	0.236	0.03*	NA	NA
IFTA	0.494	<0.0001*	0.650	0.01*
Global sclerosis	0.444	<0.0001*	0.423	0.13

[a] Patients with diabetes but without DN (based on light and electron microscopy). [b] Spearman correlation coefficient. * Statistically significant ($p \leq 0.05$). DN class, histopathological DN classification; IFTA, interstitial fibrosis and tubular atrophy; NA, not applicable

Table 3. Correlation between clinical parameters and interstitial CD68+ macrophages

	rho[a]	*p*-value
GFR stage	0.302	0.009*
Serum creatinine, µmol/L	0.317	0.006*
Presence of albuminuria	0.292	0.03*
Microalbuminuria and Macroalbuminuria	0.293	0.03*
HbA1c (% units)	-0.266	0.14
Diabetes duration	-0.051	0.72
RAAS blockers	0.255	0.046*
Oral diabetic medication	-0.129	0.32

[a] Spearman correlation coefficient. * Statistically significant ($p \leq 0.05$). GFR stage, estimated glomerular filtration rate based on the KDIGO CKD guidelines; HbA1c, glycated hemoglobin; RAAS blockers, renin-angiotensin-aldosterone system blockers; NS, not significant, might be due to insufficient data

DISCUSSION

This study demonstrates the presence of both glomerular and interstitial macrophages in all four histopathological classes of DN in a relatively large cohort of T2D patients with histologically proven DN. Similarly, macrophages were also observed in control subjects. Interestingly, a subset of macrophages (specifically, anti-inflammatory CD163+ cells) was also seen in patients from all four histopathological classes of DN. The presence of glomerular CD163+ cells was positively associated with DN class, IFTA, and global sclerosis. Therefore, we speculate that the function of infiltrating macrophages becomes increasingly anti-inflammatory when the histopathological parameters become more severe. Based on our results, we cannot conclude whether the presence of macrophages is a reaction to or a mediator of renal damage. A higher number of interstitial CD68+ macrophages was correlated with higher histopathological class, increased IFTA, increased global glomerulosclerosis, decreased eGFR, and the presence of albuminuria.

The functional role of macrophages in the development of DN is not completely understood. Nevertheless, our results show the presence of macrophages with different phenotypes in the glomeruli of patients with DN. Interestingly, macrophages were present in the glomeruli of all three groups, including diabetic patients with DN, diabetic patients without histologically proven DN, and control subjects with no histological renal abnormalities. However, the patients in the two control groups had comorbid conditions, including hypertension, heart failure, and/or atherosclerosis, and these conditions could have played a role in the presence of macrophages in their renal samples; nevertheless, these groups were suitable controls, as these comorbid

conditions were also present among our cohort of patients with DN. These findings are consistent with the findings of Nguyen *et al.* [21], who reported the presence of macrophages in a small cohort of patients with diabetes as well as in non-diabetic controls. Moreover, Nguyen *et al.* found no difference between diabetic patients and controls with respect to glomerular CD68+ macrophages. Nguyen *et al.* also found a correlation between both glomerular and interstitial macrophages and progression to renal failure. However, no additional analysis was performed between subsets of macrophages. In contrast, our study, which was based on a large autopsy cohort containing more than 50 glomeruli per case, revealed that anti-inflammatory CD163+ macrophages were present in patients from all DN classes, including class I and class IIa.

The histopathological classification of DN has not been proposed as a model for progressive diabetic damage; rather, it has been offered as a description of the various histological lesions that can present in patients with DN. Several studies reported a robust association between the DN classification and renal outcome [22-24]. Moreover, Mauer *et al.* studied patients with type 1 DN and found a correlation between thickening of the glomerular basement membrane, the index of mesangial expansion, and albuminuria [29, 30]. In contrast, in our study of patients with type 2 diabetes and histologically proven DN, no correlation between thickening of the glomerular basement membrane (i.e., class 1 DN) and albuminuria was found. Moreover, we found no correlation between albuminuria and mesangial expansion (i.e., class II DN). This discrepancy may be explained by an increased heterogeneity of lesions among patients with T2D [31] due to the increased prevalence of additional damaging factors in T2D, including hypertension, aging, atherosclerosis, and dyslipidemia [32]. Because the influx of macrophages was similar in all four DN classes, inflammation may play a role in all diabetes-related lesions in T2D.

Despite the relatively low number of human studies performed to date [21, 33], compelling evidence obtained using experimental models of DN supports the accumulation of macrophages. For example, in both streptozotocin-induced and db/db mice, the accumulation of renal macrophages has been associated with the progression of glomerular and tubular damage [9, 14]. In the early stages of streptozotocin-induced DN, depleting macrophages with irradiation inhibits both the development of glomerular hypertrophy and the production of collagen IV [34]. In addition, a recent study found that M2-like (i.e., anti-inflammatory) macrophages can facilitate renal repair by increasing the expression of enzymes involved in matrix degradation [35].

The interstitial accumulation of macrophages is believed to positively or negatively affect the progression of renal disease [16, 35]. In several renal diseases, the accumulation

of these cells is closely correlated with progressive renal failure [36-38]. In addition, interstitial macrophage accumulation is an adverse prognostic finding in DN and correlates closely with the progression of renal insufficiency [21]. Interestingly, reducing the number of interstitial macrophages in experimental models decreases both tubulointerstitial injury and interstitial fibrosis [39]. In our patient cohort, the interstitial macrophages correlated significantly with decrease in renal function, suggesting that the influx of interstitial macrophages is associated with the progression of DN.

Given the consistent finding of macrophage involvement in both experimental models and human studies, we hypothesize that the influx of macrophages plays a role in the inflammatory pathway underlying DN. However, because no correlation was found between the number of glomerular macrophages and clinical findings or histological damage, their numbers cannot fully account for the difference in damage. Thus, glomerular macrophages may simply be innocent bystanders in the kidney. Alternatively, the expression profile of the macrophages—rather than their absolute numbers—may mediate glomerular damage. For example, in several renal diseases the differentiation of macrophages into pro-inflammatory, anti-inflammatory, or profibrotic cells can influence inflammation [40].

Our study has a few limitations that warrant discussion. First, because we used autopsy material, the presence of interstitial macrophages could have been influenced by ante mortem comorbidity. However, we believe this is unlikely, as interstitial macrophages were significantly correlated with clinical parameters measured long before death. In addition, no significant differences were observed between the cause of death and either glomerular or interstitial macrophages. Second, some clinical data were not available for some patients, thereby limiting our power with respect to measuring correlations between groups for some clinical parameters (e.g., albuminuria).

Despite the abovementioned limitations, we believe that this study has high clinical relevance, as emerging therapeutic approaches focus on inhibiting the inflammatory pathway as a mean to prevent renal failure in DN. Although, currently available treatments can slow the progression of diabetic complications, still many patients develop end-stage renal disease. Recently, two clinical trials revealed that treating T2D patients with an MCP-1 inhibitor or an inhibitor of its receptor (C-C chemokine receptor type 2, CCR2) attenuated proteinuria [41, 42]. This finding underscores the important role that inflammation plays in DN, and it highlights the potential of anti-inflammatory therapy. Therefore, obtaining insight into the presence and the various phenotypes of macrophages in the kidneys of T2D patients is essential for optimizing this therapeutic approach.

In conclusion, this study showed that macrophages were present in both the glomeruli and interstitium of a clinically heterogeneous cohort of patients with type 2 diabetes, regardless of the stage of DN. Despite the lack of difference in the number of glomerular macrophages between patients and controls, the number of glomerular CD163+ macrophages (M2-like, i.e., anti-inflammatory) is associated with nodular sclerosis, global glomerulosclerosis, and interstitial fibrosis. Together with the correlation between the number of interstitial macrophages and IFTA, DN class, and renal function, this finding suggests that the number of macrophages may play a role in the progression of DN. Although future studies are needed to determine the precise role that macrophages play in DN, macrophages may serve as an effective therapeutic target for inhibiting the progression of renal damage in patients with DN.

DISCLOSURES

None.

ACKNOWLEDGEMENTS

This study was supported by a Junior Postdoc (Project code: 14OKG06) and Kolff Student Research Grant (Project code: 14OKK12) from the Dutch Kidney Foundation. CQFK and DHTIJ collected and analyzed data. MZ and CQFK carried out experiments. DHTIJ, CQFK and IMB analyzed histological data. RW helped designing and performed statistical analyses together with CQFK. RW, JAB, TJR, IMB, and DHTIJ were involved in the study design. All authors were involved in writing the paper and had final approval of the submitted and published versions. The authors have no conflict of interest.

REFERENCES

1. International Diabetes Federation. IDF Diabetes Atlas, Sixth Edition. 2013
2. Wild S, Roglic G, Green A, et al. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, 2004; 27: 1047-53
3. Svensson M, Sundkvist G, Arnqvist HJ, et al. Signs of nephropathy may occur early in young adults with diabetes despite modern diabetes management: results from the nationwide population-based Diabetes Incidence Study in Sweden (DISS). *Diabetes Care*, 2003; 26: 2903-9
4. Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet*, 1998; 352: 213-9
5. Raptis AE and Viberti G. Pathogenesis of diabetic nephropathy. *Exp Clin Endocrinol Diabetes*, 2001; 109 Suppl 2: S424-37
6. Navarro-Gonzalez JF, Mora-Fernandez C, Muros de Fuentes M, et al. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nat Rev Nephrol*, 2011; 7: 327-40
7. Donath MY. Targeting inflammation in the treatment of type 2 diabetes: time to start. *Nature Reviews Drug Discovery*, 2014; 13: 465-476
8. Tesch GH. Role of macrophages in complications of type 2 diabetes. *Clin Exp Pharmacol Physiol*, 2007; 34: 1016-9
9. Chow F, Ozols E, Nikolic-Paterson DJ, et al. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. *Kidney Int*, 2004; 65: 116-28
10. You H, Gao T, Cooper TK, et al. Macrophages directly mediate diabetic renal injury. *Am J Physiol Renal Physiol*, 2013; 305: F1719-27
11. Ninichuk V, Clauss S, Kulkarni O, et al. Late onset of Ccl2 blockade with the Spiegelmer mNOX-E36-3'PEG prevents glomerulosclerosis and improves glomerular filtration rate in db/db mice. *Am J Pathol*, 2008; 172: 628-37
12. Wang Y and Harris DC. Macrophages in renal disease. *J Am Soc Nephrol*, 2011; 22: 21-7
13. Zhao L, David MZ, Hyjek E, et al. M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol*, 2015; 10: 54-62
14. Chow FY, Nikolic-Paterson DJ, Atkins RC, et al. Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis. *Nephrol Dial Transplant*, 2004; 19: 2987-96
15. Ricardo SD, van Goor H, and Eddy AA. Macrophage diversity in renal injury and repair. *J Clin Invest*, 2008; 118: 3522-30
16. Anders HJ and Ryu M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int*, 2011; 80: 915-25
17. Tarabra E, Giunti S, Barutta F, et al. Effect of the monocyte chemoattractant protein-1/CC chemokine receptor 2 system on nephrin expression in streptozotocin-treated mice and human cultured podocytes. *Diabetes*, 2009; 58: 2109-18
18. Camilla R, Brachemi S, Pichette V, et al. Urinary monocyte chemotactic protein 1: marker of renal function decline in diabetic and nondiabetic proteinuric renal disease. *J Nephrol*, 2011; 24: 60-7
19. Wada T, Furuichi K, Sakai N, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int*, 2000; 58: 1492-9

20. Verhave JC, Bouchard J, Goupil R, et al. Clinical value of inflammatory urinary biomarkers in overt diabetic nephropathy: a prospective study. *Diabetes Res Clin Pract*, 2013; 101: 333-40
21. Nguyen D, Ping F, Mu W, et al. Macrophage accumulation in human progressive diabetic nephropathy. *Nephrology (Carlton)*, 2006; 11: 226-31
22. Oh SW, Kim S, Na KY, et al. Clinical implications of pathologic diagnosis and classification for diabetic nephropathy. *Diabetes Res Clin Pract*, 2012; 97: 418-24
23. An Y, Xu F, Le W, et al. Renal histologic changes and the outcome in patients with diabetic nephropathy. *Nephrol Dial Transplant*, 2015; 30: 257-66
24. Mise K, Hoshino J, Ubara Y, et al. Renal prognosis a long time after renal biopsy on patients with diabetic nephropathy. *Nephrol Dial Transplant*, 2014; 29: 109-18
25. Tervaert TW, Mooyaart AL, Amann K, et al. Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol*, 2010; 21: 556-63
26. Amirbekian V, Lipinski MJ, Briley-Saebo KC, et al. Detecting and assessing macrophages in vivo to evaluate atherosclerosis noninvasively using molecular MRI. *Proc Natl Acad Sci U S A*, 2007; 104: 961-6
27. Shaikh S, Brittenden J, Lahiri R, et al. Macrophage subtypes in symptomatic carotid artery and femoral artery plaques. *Eur J Vasc Endovasc Surg*, 2012; 44: 491-7
28. Landis JR and Koch GG. The measurement of observer agreement for categorical data. *Biometrics*, 1977; 33: 159-74
29. Mauer SM, Steffes MW, Ellis EN, et al. Structural-functional relationships in diabetic nephropathy. *J Clin Invest*, 1984; 74: 1143-55
30. Huang C, Kim Y, Caramori ML, et al. Cellular basis of diabetic nephropathy: II. The transforming growth factor-beta system and diabetic nephropathy lesions in type 1 diabetes. *Diabetes*, 2002; 51: 3577-81
31. Gambara V, Mecca G, Remuzzi G, et al. Heterogeneous nature of renal lesions in type II diabetes. *J Am Soc Nephrol*, 1993; 3: 1458-66
32. Fioretto P and Mauer M. Histopathology of diabetic nephropathy. *Seminars in Nephrology*, 2007; 27: 195-207
33. Furuta T, Saito T, Ootaka T, et al. The role of macrophages in diabetic glomerulosclerosis. *Am J Kidney Dis*, 1993; 21: 480-5
34. Sassy-Prigent C, Heudes D, Mandet C, et al. Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. *Diabetes*, 2000; 49: 466-75
35. Belliere J, Casemayou A, Ducasse L, et al. Specific macrophage subtypes influence the progression of rhabdomyolysis-induced kidney injury. *J Am Soc Nephrol*, 2015; 26: 1363-77
36. Hooke DH, Gee DC, and Atkins RC. Leukocyte analysis using monoclonal antibodies in human glomerulonephritis. *Kidney Int*, 1987; 31: 964-72
37. Ootaka T ST, Yusa A, Munakata T, Soma J, Abe K. Contribution of cellular infiltration to the progression of IgA nephropathy: A longitudinal, immunohistochemical study on repeated biopsy specimens. *Nephrology* 1995: 135-142
38. Yang N, Isbel NM, Nikolic-Paterson DJ, et al. Local macrophage proliferation in human glomerulonephritis. *Kidney Int*, 1998; 54: 143-51
39. Morii T, Fujita H, Narita T, et al. Association of monocyte chemoattractant protein-1 with renal tubular damage in diabetic nephropathy. *J Diabetes Complications*, 2003; 17: 11-5

40. Tian S, Zhang L, Tang J, et al. HMGB1 exacerbates renal tubulointerstitial fibrosis through facilitating M1 macrophage phenotype at the early stage of obstructive injury. *Am J Physiol Renal Physiol*, 2015; 308: F69-75

41. Haller HG, Menne J, Eulberg D, et al. Anti-inflammatory and Renoprotective Effects of CCL2 Inhibition with Emapticap Pegol (NOX-E36) in Type 2 Diabetic Patients with Albuminuria in Renal Week 2014. 2014: 74A

42. de Zeeuw D, Bekker P, Henkel E, et al. The effect of CCR2 inhibitor CCX140-B on residual albuminuria in patients with type 2 diabetes and nephropathy: a randomised trial. *Lancet Diabetes Endocrinol*, 2015; 3: 687-96

SUPPLEMENTARY TABLES

Supplementary Table 1. Correlation between clinical parameters and interstitial CD68+ macrophages in patients without sepsis

	rho[a]	*p*-value
GFR stage	0.303	0.035*
Serum creatinine	0.265	0.068
Presence of albuminuria	0.487	0.003*
Microalbuminuria and Macroalbuminuria	0.497	0.002*
HbA1c (% units)	-0.147	0.524
Diabetes duration	-0.034	0.848
RAAS blockers	0.358	0.023*
Oral diabetic medication	0.047	0.775

[a] Spearman correlation coefficient. Statistically significant ($p \leq 0.05$)

GFR stage, estimated glomerular filtration rate based on the KDIGO CKD guidelines; HbA1c, glycated hemoglobin; RAAS blockers, renin-angiotensin-aldosterone system blockers