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Histological evidence of diabetic kidney disease precede clinical diagnosis

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Abstract

BACKGROUND: In the absence of a histological diagnosis, persistent albuminuria is globally accepted as the main diagnostic criteria for diabetic kidney disease.

METHODS: In the present retrospective study, we evaluated data from an Italian cohort of 42 deceased diabetic donors (mainly with type 2 diabetes). Using the kidney biopsies obtained at the time of donation to evaluate single or double allocation based on Karpinski score, we determined the prevalence of histological lesions attributable to diabetes.

RESULTS: All 42 donors presented with proteinuria in the normal range and an estimated Glomerular Filtration Rate (CKD-EPI) >60 ml/min/1.73m². A kidney biopsy was available for 36 patients; of these, one was not interpretable and 32 showed histopathological lesions consistent with diabetic kidney disease and encompassing all histological classes. Thus, we found a relatively high proportion of histologically proven diabetic kidney disease that had been clinically undiagnosed, as none of the patient had significant proteinuria and eGFR <60 cc/min/1.73 m².

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Author Contributions

GC, DM, AA, and FV did the data analyses and wrote the manuscript. ADE helped in data interpretation and critically revised the manuscript. EF critically helped interpreting the results and reviewed/edited the manuscript. SV and GP helped in performing and analyzing TEM. MR did pre transplant nephrectomy and performed procurement biopsies. IC and FA helped in collecting data. GLM conceived and oversaw the project and interpreted the results.

Disclosure

All the authors declared they have no competing interests.

CONCLUSIONS: The data we present here support the need to implement routine kidney biopsies in normoalbuminuric diabetic subjects in the early stages of Chronic Kidney Disease. Such strategy may help to improve risk stratification in diabetic patients and guide therapeutic decisions during the early stages of the disease.

Keywords

Albuminuria diabetes mellitus; diabetic nephropathy; diabetic kidney disease; kidney biopsy

Introduction

Chronic kidney disease (CKD) is a public health problem worldwide and represents one of the main causes of cardiovascular mortality [1]. Diabetic kidney disease (DKD), which develops in approximately 10% to 30% of patients with diabetes mellitus [2], is one of the most important long-term complications of diabetes and among the leading causes of end stage kidney disease (ESRD) in developed countries; the overlap of diabetes mellitus and renal injury exponentially amplifies the risk of cardiovascular events and death [3]. DKD is characterized by the development of albuminuria, often followed by a decline in glomerular filtration rate (GFR) over a long period of time (10–20 years) and evolution towards ESRD [4–6]. Peculiar histological features that typically characterize DKD are the thickening of the glomerular basement membrane, nodular or diffuse mesangial sclerosis, arteriolar hyaline sclerosis, micro aneurysms and exudative lesions [7]. However, increasing evidence indicates that many patients with diabetes, erroneously diagnosed as having progressive forms of DKD, are in fact developing non-diabetic renal diseases (NDRD) or ‘mixed’ conditions, where typical features of DKD overlap with other pathological diagnoses [7–11]. Therefore, while clinically indicated kidney biopsies in DKD are likely to reveal NDRD, routine kidney biopsies for diabetic patients provide a better understanding of the natural history of DKD, as clearly demonstrated by studies that implemented research kidney biopsies during the early stages of kidney disease. The thickening of the glomerular basement membrane, a unique feature of DKD, predicts DKD progression in a normoalbuminuric cohort of patients with type 1 diabetes (T1D) [12], while reduction in podocytes number is observed at later stages [13]. In type 2 diabetes (T2D), several histological lesions including reduction in podocytes number precede albuminuria [14–16]. Furthermore, lesions in T2D are rather heterogeneous, and only typical diabetic nephropathy features were described to contribute to loss of GFR [17]. The correct classification of such patients would be crucial to predict the natural course of the disease and to allow its early treatment. According to the classical paradigm, the natural history of DKD consists in an early phase of microalbuminuria (albumin excretion of more than 30 mg/g of creatinine in 2 out of 3 random urine samples collected in within a 6 months period), which precedes overt nephropathy characterized by macroalbuminuria and a progressive decline of GFR [18]. Despite such a large body of evidence on how histology may predict DKD progression, current guidelines for DKD screening are solely based on the detection of persistent albuminuria in two out of three morning urine collections over a six months period of time [19]. This is a major limitation, as many diabetic patients who reach a persistent GFR <60cc/min remain normoalbuminuric [20–24]. Therefore, the definition of DKD that

recognizes the clinical appearance of albuminuria as specific of microangiopathic damage may not be exhaustive [8, 25].

In this retrospective study we analyzed data from a cohort of patients with positive history of diabetes, enrolled as candidate cadaveric donors for kidney transplant. The aim was to investigate the prevalence and classification of histopathological lesions of DKD in diabetic patients with clinically silent nephropathy.

Methods

We performed a retrospective analysis on deceased kidney donors, who therefore showed normal renal biochemical parameters, enrolled from 2004 to 2015 at S. Orsola Hospital-University of Bologna, Italy. Among them, we selected deceased donors with a history of diabetes.

At the time of donation, kidneys from diabetic patients were biopsied to evaluate single or double allocation [26] based on Karpinski score [27], in accordance to the Italian Clinical Guidelines for Organ Transplantation from Deceased Donors published by the Italian National Transplant Centre (CNT) of the National Institute of Health (ISS) (<http://www.trapianti.salute.gov.it>). We considered both T1D and T2D candidate donors who were older than 18; we selected those cases where data from the kidney biopsy performed at time of death was available.

Clinical data

Clinical information was obtained through the medical records made available by the Intensive Care Units where the patients had been hospitalized and subsequently provided to the Regional Transplant Center of Emilia Romagna in Bologna once they became eligible as donors. Due to the retrospective nature of this study, registration or approval by the ethics committee was waived. The study was carried out in conformity with the Declaration of Helsinki. The following laboratory and clinical measurements were included: serum creatinine at time of hospitalization, eGFR (calculated using the CKD- EPI formula), proteinuria (>30 mg/dl, determined through spot urine collections), serum hemoglobin and serum cholesterol. Data indicating a stable representation of the serum and urine levels during terminal hospitalization were included. We also obtained data regarding comorbidities, medication history, hypertension, smoking, and complications secondary to diabetes. The cause of death was also obtained from the hospitalization records.

Histopathological analysis

Renal tissue was fixed in Serra solution and embedded in paraffin. Slices were cut at 3- μ m thickness and stained with hematoxylin and eosin, periodic acid-Schiff (PAS) and Masson's trichrome stain. Renal tissue specimens were scored by 2 pathologists not in contact with each other and unaware of the patients' clinical data. The rare discordant cases were discussed collegially. Glomerular lesions, interstitial lesions, and vascular lesions were scored in accordance to the established histopathologic classification for DKD. Diabetes score were assigned according to Tervaert *et al.* [10, 28]:

- patients without histologic lesions, and with no thickening of the glomerular basal membrane at Transmission Electron Microscopy (TEM), were designated as class 0 DKD;
- patients without histologic lesions, but with thickening of the glomerular basal membrane (>430 nm in males and >395 nm in females) at TEM, were designated as class I DKD;
- patients showing mild mesangial expansion were designated as class IIa DKD;
- patients showing moderate/severe mesangial expansion were designated as class IIb DKD;
- patients with patent diabetic glomerular nodules and $<50\%$ globally sclerotic glomeruli were designated as class III DKD;
- patients with $\geq 50\%$ globally sclerotic glomeruli were designated as class IV DKD.

The four histological parameters of the Karpinski score [27] were separately assessed as well: glomerulosclerosis score, tubular atrophy score, interstitial fibrosis and vascular damage (Supplementary Material). Finally, further histopathological variables were evaluated for each kidney as reported in detailed in the Supplementary Material section.

Transmission Electron Microscopy

In the present study, TEM was used in order to measure the thickness of the glomerular basal membrane and distinguish class 0 from class I DKD. Small specimens of renal tissue were retrieved from paraffin blocks, de-paraffined in xylene, rehydrated in ethanol (100%, 95% and 70%) and washed in 0.15 M sodium cacodylate buffer. After post-fixation in 1% osmium tetroxide, the samples were washed with increasing concentration of ethanol (from 70 % to 100%), embedded in Araldite resin and cut with the ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate before the examination with Philips CM10 (FEI Company, Milan, Italy) Transmission Electron Microscope equipped with a Gatan camera. For each sample, five digital images were randomly acquired using the FEI proprietary software Olympus SIS Megaview SSD digital camera. The thickness of the Glomerular Basement Membrane (GBM) was measured in twelve different positions at 13500 of magnification. Class I DKD was defined as the presence of basal membranes with average thickness >430 nm in male patients and >395 nm in female patients [29].

Statistical analysis

Differences with a P value less than 0.05 were considered statistically significant. The histopathological data were analyzed using the chi-square test.

Results

A total of 42 cadaveric kidney donors were selected based on established diagnosis of type 1 or type 2 diabetes prior to expiration. Of those, 7 were excluded because their kidney biopsy was not available or was of poor quality. Only 35 diabetic subjects were therefore included

in the analysis. The characteristics of these patients are summarized in Table 1. Individual data are also provided in the Supplementary Material section, Table S1. The cohort was composed by 1 patient with T1D and 34 patients with T2D. The mean age was 69.7 and 51.4% of the subjects were female.

Clinical data

4 out of 35 patients were treated with insulin, 14 received oral anti-diabetic agents, while 7 (20%) achieved glycemic control through lifestyle changes, mostly diet. Multiple dipstick testing for protein in urine available prior to expiration excluded the presence of proteinuria in all patients. Ultrasonography examination reported normal kidney size ($11.63 \text{ cm} \pm 0.15$). The medical records revealed that 24 patients (68.6%) had a diagnosis of primary hypertension, and that 10 of them had received therapy with an angiotensin-converting enzyme inhibitor or an angiotensin-receptor blocker. None of the patients had diabetic retinopathy (Table 1). Hemorrhagic stroke was the most prevalent cause of death, followed by ischemic stroke and cranial trauma (Table S1).

Histological lesions

Histopathological characteristics are summarized in Table 2. Histopathological classification revealed that 6 patients had light microscopic changes: in these 6 cases, TEM was applied in order to distinguish class 0 from class I diabetic nephropathy [10]. The mean glomerular basal membrane (GBM) thickness obtained was $260.566 \pm 122.276 \text{ nm}$. In accordance with Tervaert *et al.* [28], 3 cases were assigned class 0 DKD due to an average GBM thickness under the cut-off (see the Methods section); 3 cases were categorized as class I DKD, 22 patients class IIa DKD, 3 patients class IIb DKD and 4 patients class III DKD (Figure 1). No class IV were observed in our series. Myointimal hyperplasia was observed in 45.7% of patients, multifocal arteriolar hyalinosis in 60%, Interstitial fibrosis and tubular atrophy (IFTA) in 62.8% (Table S1). NDRD lesions compatible with primary glomerulonephropathies were not reported. Noticeably, no electron dense deposits or deposition of immunocomplexes were observed under TEM (Figure 1).

Discussion

We retrospectively described a cohort of 35 subjects, with history of diabetes, declared eligible as deceased donors for kidney transplant for absence of pathological findings in serum and urine test. However, 32 subjects presented renal histopathological changes compatible with DKD. The absence of clinical signs and laboratory findings for DKD of course represents one of the primary steps to obtain eligibility as donors for kidney transplant.

As mentioned in the introduction, the classical paradigm of DKD is based on a progression from glomerular hyperfiltration, to microalbuminuria, to proteinuria and to lower GFR [18, 30]. In last decade this concept has been partially revised and several studies described more heterogeneous presentations of DKD [9, 11], not fully consistent with the expectations of this classical paradigm [22, 31–33]. In the DEMAND (Developing Education on Microalbuminuria for Awareness of Renal and Cardiovascular Risk in Diabetes) study, a

cross-sectional study involving 11,573 patients with type 2 diabetes, 6,072 individuals presented with normoalbuminuria and 17% of them (1,044 patients) also had eGFR <60 ml/min per 1.73 m² [33]. Previously, Kramer *et al.* [31], in a cross-sectional study, reported this number being as high as 33%. These data are consistent with a recent report [34] describing how the prevalence of CKD (eGFR <60 ml/min per 1.73 m²) in patients with T2D has increased from 9.2% to 14.1% in the last 20 years, despite albuminuria having decreased from 21% to 16%. Only a limited number of studies have previously described the presence of histologically proven DKD in absence of albuminuria and CKD. Reports by Fioretto *et al.* [35] and Caramori *et al.* [12] elegantly evaluated the histological characteristics of DKD in patients with T1D, describing how the width of glomerular basement membrane predicts the development of proteinuria and/or ESRD earlier than microalbuminuria.

Most of these histological studies were conducted on diabetic patients receiving a kidney biopsy for research purposes and only limited studies have validated these findings on organs from cadaveric donors. Among them, in an autopsy study on 168 subjects with diabetes, Klessens *et al.* [36] have recently described 106 subjects with histopathologic changes compatible with DKD, 20 of which did not present DKD-associated proteinuria within their lifetime. In contrast with our findings, many patients considered by Klessens and colleagues were in CKD stage 3 (eGFR <60 ml/min per 1.73 m²) [36]. Similarly to what reported by Klessens *et al.* [36], in our population the percentage of glomeruli with nodular mesangial sclerosis was minimal (average value of 11% in kidneys of patients in DKD class III) (Supplemental Table).

We determined histologically that the most represented DKD lesion was the class IIa, that means mild mesangial expansion. It's worth noting that our population is composed of marginal donors [37], with a mean age of 70 years. This may justify the non-diabetic alterations found, such as the mean total Karpinski score (3.9±1.9) and the presence of IFTA in nearly 90% of the sample. As far as vascular changes are concerned, a severe myointimal thickening was observed in 44.4% of the patients and a multifocal arteriolar hyalinosis in 58.3%. These findings are consistent with previous data estimating at around 80% the prevalence of not diabetic renal disease detected in renal biopsies of diabetic patients [38–40]. However, previous studies may suffer from a selection bias regarding the reasons for performing the renal biopsy [36]; in our study, due to the different technique used to perform the renal biopsy, we had the huge benefit of being able to study at least 30 glomeruli for patient, which are certainly many more than those usually available with needle biopsy. Considering the amount of renal tissue, we are certain about the presence or absence of DKD and, moreover, we are largely confident of the distribution across the DKD classes. Typical nodular lesions were focally concentrated in specific areas of tissue. Thus, it is plausible that their heterogeneous distribution may be partly responsible for the underrepresentation of DKD in previous renal biopsy studies.

A reasonable suggestion is that DKD lesions develop focally, in different steps, and that we observe the onset of proteinuria only when a given percentage of glomeruli are already damaged. Said and Nasr [41] suggested that in early DKD stages the tubules are not damaged and still able to reabsorb the leaked albumin from the injured glomeruli,

contributing to the absence of albuminuria [42, 43]. Only after interstitial damage occurs, the resulting loss of reabsorption capacity causes the albuminuria. This provides an explanation of why glomerular damage consistent with DKD can occur before the onset of albuminuria. Moreover, in some cases, renin angiotensin aldosterone inhibitors administration may have delayed the onset of proteinuria [44].

Currently, no standardized criteria for performing a kidney biopsy in diabetic patients are reported and the decision to perform it, or not, is responsibility of the physician alone. Until now, proteinuria with no proven diabetic retinopathy is the major recognized reason for performing kidney biopsies [7, 45]. In the present study we have shown that the histological classes of DKD are not directly associated with proteinuria, enforcing kidney biopsy as the gold standard for an early diagnosis of DKD. Kidney biopsies are not routinely performed in diabetic patients in absence of proteinuria and/or advanced renal failure. This happens not only for the possible risks related to the procedure, in particular bleeding, but also because the fact that clinical characteristics of DKD (diabetes duration, level of proteinuria and GFR <60 ml/min) do not accurately indicate the renal conditions is a recent finding, still to be completely clarified [46]. This concept has therefore not yet impacted the common clinical practice.

In recent years, thanks to technical advances, biopsies have evolved from indirect to real-time visualization through ultrasound guidance of the procedure [47, 48]. As a result this is now a really safe procedure, when provided in centers with relevant experience (>30 biopsies/year) [49, 50]. The data presented here support the need to detect early signs of DKD through kidney biopsy in patients with early stage renal failure (GFR 90–60 ml/min). This must be performed in centers with significative experience in dealing with kidney diseases.

As recently reported by several studies, there have been advances in the discovery and validation of non-invasive biomarkers for predicting disease progression and response to treatment in DKD [51]; unfortunately none of them have yet been validated for clinical practice. Therefore, as of now, kidney biopsy is fundamental to determine patterns for DKD in diabetic patients, also in the early stages of CKD.

To summarize, we presented a retrospective analysis of deceased kidney donors with a history of diabetes, thought to be in good clinical conditions and with no reported evidence of progressive and/or advanced stages of DKD before hospitalization. In contrast to the classical paradigm of DKD [18], our findings suggest that renal lesions consistent with DKD may develop before any clinical diagnosis is possible. Moreover, our data indicate that underdiagnosed DKD encompasses all histological classes including class III, which is typically characterized by the presence of Kimmelstiel-Wilson nodules. In two cases, low but significative levels of proteinuria (>30 mg/dl) were reported, which were not correlated with the severity of DKD histological lesions.

Despite the advantages previously discussed, some limitations require a separate discussion. Proteinuria was detected by dipstick, meaning that microalbuminuria may not have been detected. Moreover, the cohort may suffer from selection bias: the population was composed

in its entirety by white individuals, and extending these results to other populations is therefore not possible.

Nodular lesions, that characterize class III DKD, may also occur in non-diabetic patients with hypertension and a history of smoking (especially in elderly subjects) [52]. However it seems unlikely that our results were strongly influenced by this, as patients with nodules had a median age of 68 years and only 2 of 4 patients were in pharmacological treatment for hypertension.

As previously mentioned, clinical information was obtained through the medical records of the Intensive Care Units where the patients, mostly already unconscious, were hospitalized. Some personal information may therefore be lacking, as well as pathological signs impossible to evaluate, such as retinopathy.

A further minor limitation is connected to the fact that renal samples for TEM were retrieved from paraffin blocks, with possible shrinkage of GBM; as a consequence we might have underestimated the values of GBM thickness. However this would not have altered the class of DKD in our population, except for the 3 cases reported as class 0, which may be in the upper level.

Conclusions

Histological lesions proving DKD are clearly detected also in diabetic patients with no proteinuria and in the early stages of CKD, such as Class I and II. It is reasonable to think that, when proteinuria occurs, kidneys of diabetic patients may already manifest minor to severe glomerular injuries. The early identification of this underdiagnosed group of DKD is crucial to promptly provide a specific therapeutic regimen, such as inhibitors of the renin-angiotensin system or, more recently, agents preventing progression of kidney failure [3, 53–55]. We acknowledge that performing a kidney biopsy by default in all diabetic patients is logistically demanding; however, considering our findings, the aim may be performing a kidney biopsy at least in diabetic subject with CKD Class II. Further studies should be designed to better investigate the development of diagnostic algorithms to accurately detect currently underdiagnosed patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Examples of diabetic nephropathy classes at histology. Class 0 is characterized by normal-appearing glomeruli (a), without thickening of the glomerular basement membrane at transmission electron microscopy (b). Class I is characterized by a variable thickening of the glomerular basement membrane (c), requiring confirmation at transmission electron microscopy (d). Class II is characterized by mild (class IIa, square e) or moderate/severe (class IIb, square f) expansion of the glomerular mesangium. Class III is characterized by the presence of severe mesangial expansion, increased mesangial cellularity and Kimmelstiel-

Wilson nodules; note the arteriolar hyalinosis (arrows). PAS (a,c,e,f,g) and Trichrome (h) stains; magnification 40x. Magnification 13500x (b,d).

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Table1.

Baseline characteristics of the patients

Baseline characteristics	Mean $\pm$ SEM (%)
Sex (M/F)	17/18
Age (yrs)	69.7 $\pm$ 1.3
T1D/T2D	1/34
Proteinuria (mg/dl)	11.02 $\pm$ 3.2
Creatinine (mg/dl)	0.98 $\pm$ 0.07
CKD Class	Class I: 7 (20) Class II: 28 (80)
Retinopathy	0
Hypertension	24 (68.6)
Dislipidemia	21 (60)
Renal Longitudinal Size (cm)	11.63 $\pm$ 0.15
Diabetic Therapy	Insulin: 4 (11.4) OAD: 14 (40) LC: 6 (17.1) N/A: 11 (31.4)
Anti-Hypertensive Therapy	18 (51.4)
RAASi	8 (22.8)

eGFR, estimated glomerular filtration rate; LC, lifestyle changes; N/A, not available; OAD, oral anti-diabetic; RAASi, renin angiotensin aldosterone system inhibitor; SEM, standard error mean; T1D, type 1 diabetes; T2D, type 2 diabetes; yrs, years.

Table2.

Histological findings

	Number of patients (%)
DKD histological class	Class 0: 3 (8.6) Class I: 3 (8.6) Class IIa: 22 (62.8) Class IIb: 3 (8.6) Class III: 4 (11.4)
Total Karpinski score (mean±SD)	3.9±1.9
Percentage of global glomerulosclerosis (%mean ±SD)	11±8.3
Thickening TBM	7 (20)
Myointimal thickening	Absent: 6 (17.1) Mild: 10 (28.6) Severe: 16 (45.7) N/A: 3 (8.6)
Increase in MM	Absent: 5 (14.3) Mild: 20 (57.1) Moderate: 6 (17.1) Severe: 4 (11.4)
Nodules	4 (11.4)
Microaneurisms	1 (2.8)
Capsular Hyalins drops	2 (5.7)
Hyalinosis GBM	3 (8.6)
Vascular hyalinosis	Absent: 8 (22.9) Focal: 6 (17.1) Multifocal: 21 (60)
IFTA	Absent: 2 (5.7) Mild (<25%): 22 (62.9) Moderate (25–50 %): 10 (28.6) N/A: 1 (2.8)

DKD, diabetic kidney disease; GBM, glomerular basement membrane; IFTA, inflammatory fibrosis/tubular atrophy; MM, mesangial matrix; N/A, not available; SD, standard deviation; TBM: tubule basement