Journal of Diabetes Research

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Contents

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Review Article

Recent Progress in Genetics and Epigenetics Research on Diabetic Nephropathy in Malaysia

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Diabetic nephropathy is a multifactorial disease. Gene susceptibility, as well as environmental exposure, plays an important role in disease progression. Malaysia is reported to be among the world's second-fastest-growing rates of kidney failure. Diabetic nephropathy has become the main cause of end-stage renal disease in Malaysia. This article is aimed at reviewing genetic studies conducted among diabetic nephropathy patients in the Malaysian population. This review was conducted by searching PubMed, MEDLINE, and Google Scholar databases to identify all relevant papers published in English from March 2022 to April 2022, using the following keywords: diabetes, type 2 diabetes, diabetic nephropathy, diabetic kidney disease, and Malaysia. The case-control study among diabetic patients with and without diabetic nephropathy showed a significant association with diabetic nephropathy in CNDP1, NOS3, and MnSOD genes. In the ethnic subgroup analysis, significant differences for diabetic nephropathy in terms of diabetes duration (≥10 years) were observed for CCL2 rs3917887, CCR5 rs1799987, ELMO1 rs74130, and IL8 rs4073. The IL8 rs4073 was associated only with the Indians, while the CCR5 rs1799987 was associated with the Chinese. In Malays, SLC12A3 Arg913Gln polymorphism and ICAM1 K469E (A/G) polymorphism were found to be associated with diabetic nephropathy. Studies on gene-environment interactions have suggested significant genetic and environmental factors such as smoking, waist circumference, and sex for eNOS rs2070744, PPARGC1A rs8192678, KCNQ1 rs2237895, and KCNQ1 rs2283228 with kidney disease. The genetic variants' contributions differed across ethnic groups. Therefore, a study to validate the genetic variants that are found to be associated with different ethnicities in Malaysia may be important in future studies.

1. Introduction

Malaysia is a country in Southeast Asia with a total population of 32.4 million, as reported by the Department of Statistics Malaysia, 2018. As a multiethnic country, the Malaysian population consists of Malays (69.1%), Chinese (23.0%), Indians (6.9%), and others (1.0%) [1]. The majority of the population in West (Peninsular) Malaysia is Malays, Chinese, and Indians.

The rising of dialysis patients due to diabetes complications is a primary concern for developing countries like Malaysia. According to the United States Renal Data Systems (USRDS) report 2021 (Figure 1), Malaysia has become the third highest country of incidents of treated end-stage renal disease (ESRD) attributed to type 2 diabetes (T2D) after Singapore and the Republic of Korea [2]. Diabetic nephropathy (DN) has become a significant public health problem in Malaysia due to the associated high morbidity and mortality, which parallels the increased prevalence of diabetes and hypertension among Malaysians.

In Malaysia, about 58% of new ESRD patients were diabetic. Findings from a nationwide population-based cross-sectional study in 2020 reported the prevalence of chronic kidney disease (CKD) in Malaysia has increased

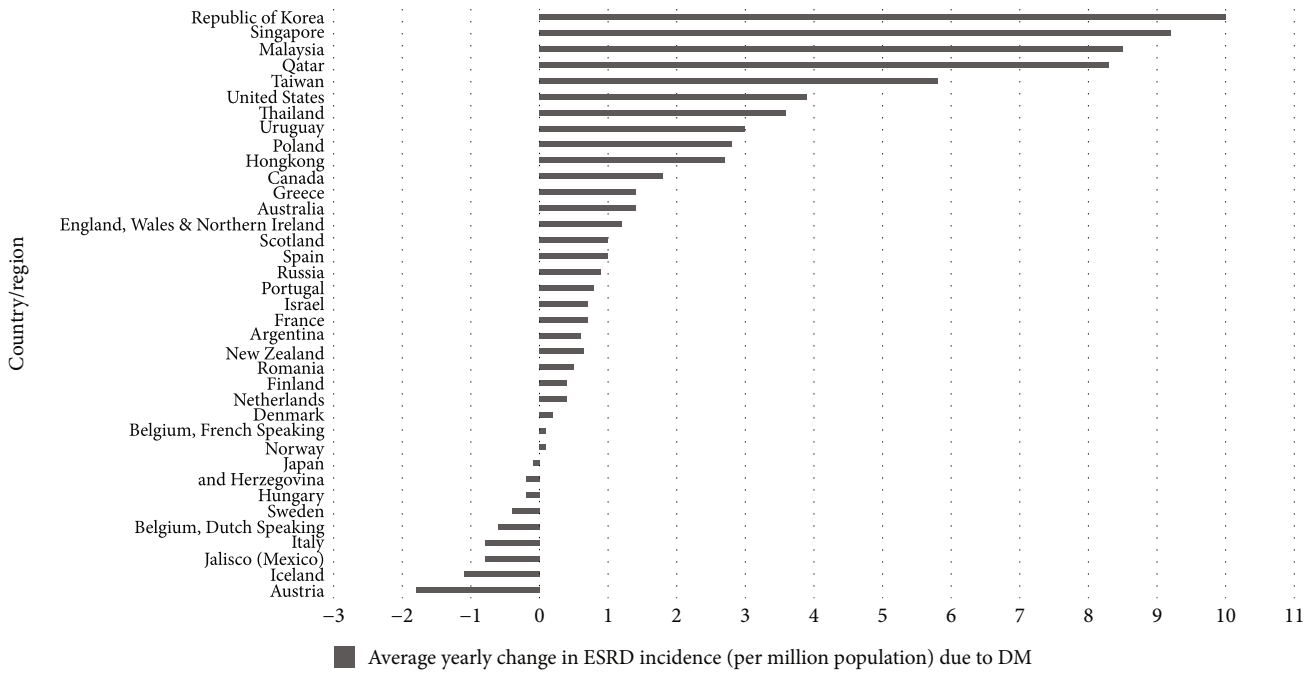

Figure 1: Displays the average yearly change in the incidence of treated ESRD attributed to diabetes, by country or region. Malaysia became the 3rd highest after Singapore and the Republic of Korea.

by 6.41%, from 9.07% in 2011 to 15.48% (95% CI: 12.30, 19.31) in 2018 [3]. The number of dialysis patients in Malaysia is reported to be greater in men compared to women. Most of the dialysis patients were in the age group of 45 years or older. The highest percentage of dialysis patients was in 55-64 years of age [4]. These can be explained as most diabetic patients develop DN after 10-15 years of having T2D.

The DN has not only become a burden to Malaysia's public and healthcare systems but it also affected our economy. In Malaysia, dialysis centre is managed either by the government or the private sector. For over 10 years, the number of private haemodialysis centres has been increasing rapidly. Although private haemodialysis centre accounts for 49% of the total number of a dialysis centre in Malaysia, the main source of funding continues to come from the government. According to the MDTR report in 2018 [5], the Malaysian government continued to provide more than 50% of dialysis funding therapy for new and existing patients. This includes subsidies to NGO centres, as well as government dialysis centres.

The DN is a microvascular complication due to diabetes. Albuminuria has been used widely in the clinic to indicate kidney disease. However, the prediction of DN using albuminuria is poor as it is not a specific biomarker. The extensive research to find a new biomarker for the early prediction of DN has become many scientists' interest worldwide. A genetic-altered factor has been suggested to influence an individual to develop DN in the future. Evidence is accumulating to support those genetic mechanisms that may also contribute to the progression of kidney disease among diabetic patients. With the advancing technology in biomedical research, many genetic variants have been identified and reported in different populations.

Modifiable risk factors such as dyslipidemia, hypertension, and glycaemic control and unmodifiable risk factors are age, race, and genetics are suggested to contribute to the pathogenesis of DN. The severity of kidney disease among diabetic patients differs from one to another. About 30-50% of diabetic patients will progress to kidney disease with some patients experiencing a relatively rapid decline in renal function despite good glycaemic control. This proposes the fact that genetics are among the main contributors to DN besides environmental factors.

This article is aimed at carrying out a literature review on genetic and epigenetic studies that have been conducted among DN patients in Malaysia. This update may be important for a better understanding of the genetics of diabetic kidney disease in our population.

2. Materials and Methods

Previously published articles were searched using PubMed, MEDLINE, and Google Scholar. The keyword terms used are diabetes, type 2 diabetes, diabetic nephropathy, and diabetic kidney disease with limited studies conducted among the Malaysian population. The search was carried out from May 2022 until June 2022. In total, 13 articles were included after reviewing the titles and abstract. The full text of all articles was further reviewed, and 5 of 13 research articles were excluded because they were review articles or did not report genetic findings. Only 6 research articles were finally included in this review (Figure 2).

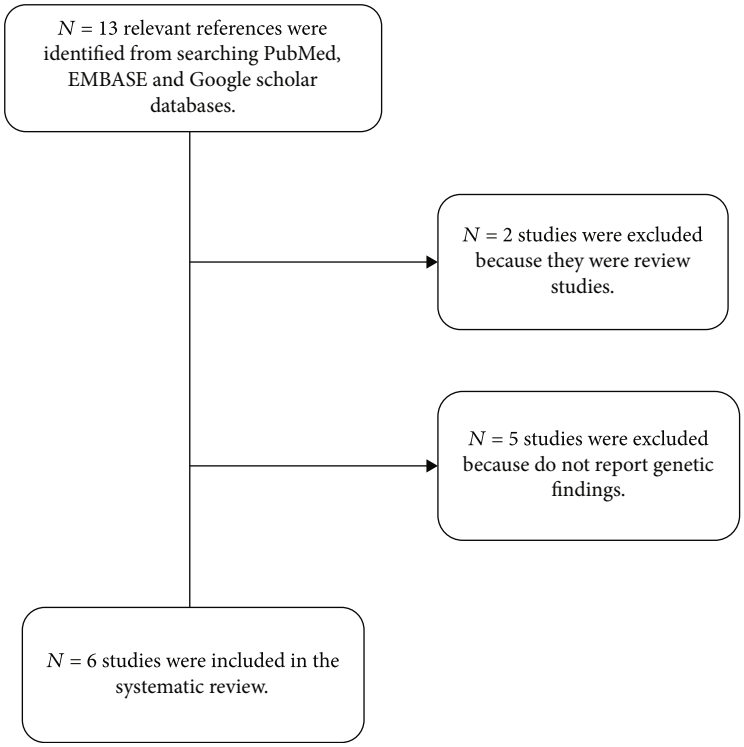

Figure 2: The flow chart summarized the outcomes of the search strategy. The keyword terms used are diabetes, type 2 diabetes, diabetic nephropathy, and diabetic kidney disease with limited studies conducted among the Malaysian population.

Table 1: Shows a summary of the included articles in the review.

Study design	Case definition	Method	Author, year
Case-control study	DN ($n = 171$) vs. ESRD (25)	Affymetrix GeneChip 1.0	Lokman et al., 2011 [12]
Case-control study	NDC ($n = 784$) vs. T2D with/without DN (633)	TaqMan genotyping	Abu Seman et al., 2014 [6]
Case-control study	NDC ($n = 90$) vs. T2D (n =52) vs DN (38)	TaqMan genotyping	Abu Seman et al., 2015 [14]
Case-control study	T2D ($n = 300$) vs. DN (325)	Sequenom MassARRAY iPLEX	Yahya et al., 2019 [10]
Case-control study	T2D ($n = 327$) vs. DN (325)	Sequenom MassARRAY iPLEX	Yahya et al., 2019 [11]
Case-control study	Healthy ($n = 300$) vs. DN (300)	Mass spectrometry genotyping	Ahmad et al., 2020 [13]

3. Results and Discussion

3.1. Genetics Studies of DN. In total, 7 research articles were included in this review, as presented in Table 1. All research articles included were using case-control study designs.

Abu Seman et al. [6] conducted a case-control study among Malays with T2D and DN. Patients were collected from various hospitals in Peninsular Malaysia. They divided T2D patients into 2 groups: T2D patients without DN and those with DN. Subjects in the normal glucose tolerance (NGT) group are defined as controls. They found that (solute carrier family 12 members 3) SLC 12A3 Arg913Gln polymorphism was associated with T2D ($p = 0.028$, OR = 0.772 (0.612-0.973), 95% CI) and DN ($p = 0.038$, OR = 0.547 (0.308-0.973), 95% CI) [6]. Meta-analysis using data from Malaysians together with Japanese [7, 8] and Caucasians [9] shows that the SLC12A3 Arg913Gln polymorphism has a protective effect in DN among T2D patients (Z value $= -1.992$, $p = 0.046$, OR = 0.792 (0.629-0.996) 95% CI).

When comparing T2D without and with DN, [10] found that the chemokine receptor 5 (CCR5) rs1799987 A allele has a significant and strong association with DN in Chinese with OR = 6.71 (2.55-17.68) 95% CI, while interleukin-8 (IL8) rs4073 showed association only in Indians with OR = 1.57 (0.66-3.71) 95% CI. They also studied the relationship between the oxidative stress-related polymorphism effects on the development of DN [11]. They found that manganese superoxide dismutase (MnSOD) rs4880 has an association with DN in 3 major ethnicities: the Chinese had an OR = 2.8 (0.53–14.94) 95% CI, the Indians had an OR = 2.4 (0.69–2.84) % CI, and the Malays had an OR = 2.16 (0.54–8.65) 95% CI; while for NOS3 rs1799983, the Indians had the highest risk with OR = 3.16 (0.52–17.56) 95% CI, followed by the Chinese with OR = 3.55 (0.36–35.03) 95% and the Malays with OR = 2.8 (0.29–28.32) 95% CI. Besides that, (carnosinase gene) CNDP1 D18S880 was also found to be associated among all the three major races with the Malays having the strongest association with

OR = 2.46 (1.48–4.10) 95% CI, the Chinese with OR = 2.26 (1.34–3.83) 95% CI, and the Indians with OR = 1.77 (1.18–2.65) 95% CI. Meanwhile, CNDP1 rs2346061 was found significantly associated with DN only among Indians with OR = 1.94 (1.76–3.20) 95% CI.

One study profiled the gene expression in the Malays' ethnic with T2D, with or without DN using the Affymetrix GeneChip 1.0 ST array. The study has identified several genes that showed upregulation in the DN group including (major histocompatibility complex) HLA-C, (component 3a receptor 1) C3AR1, (solute carrier family 16) SLC16A3, and (solute carrier family 9) SLC9A8 [12]. Consistently downregulated genes included (bone morphogenetic phosphatase kinase) BMP2K, (Solute carrier family 12) SLC12A1, (solute carrier family 7) SLC7A2, and (protein phosphatase 1 regulatory (inhibitor unit)) PPP1R1C.

Interactions between genetic variants and environmental factors with DN have been studied by Ahmad et al. [13]. They used Agena mass spectrometry to genotype 32 single nucleotide polymorphisms in T2D without and with DN. Their data showed that gene-environment interaction analyses have a significant on changeable risk factors such as smoking ((endothelial nitric oxide synthase) eNOS rs2070744, (PPARG coactivator 1 alpha) PPARGC1A rs8192678 and (potassium voltage-gated channel subfamily Q member 1) KCNQ1 rs2237895)), waist circumference (eNOS rs2070744, PPARGC1A rs8192678, KCNQ1 rs2237895, and KCNQ1 rs2283228), and HDL (eNOS rs2070744 and PPARGC1A rs8192678) [13]. When comparing the high-risk group on DN with the reference group male vs. female, males have higher probabilities of chronic kidney disease (PPARGC1A rs8192678). The predicted rate of newly detected DN progression in gene-environment interactions was significantly observed between KCNQ1 rs2283228 and two environmental factors (sex and BMI). The genetic association of the SNPs is summarized in Table 2.

3.2. Epigenetics Study of DN. Not much research has been done on the Malaysian population's DNA methylation in diabetic nephropathy. Abu Seman [14] conducted the DNA polymorphism and methylation in the intercellular adhesion molecule 1 (ICAM1) gene using TaqMan allelic discrimination and pyrosequencing. They reported that the ICAM1 K469E (A/G) rs5498 ($p = 1.7 \times 10^{-6}$, OR = 2.909 (1.857–4.556) 95% CI was significantly associated with DN. However, no association of ICAM1 DNA methylation with DN was detected.

The same authors measured the DNA methylation levels of SLC30A8 in T2D patients with DN. They analyzed 6 CpG sites in the solute carrier family 30 member 8 (SLC30A8) gene among Malays of ethnic Malaysia. Results showed that the DNA methylation levels of SLC308 were higher in T2D (82.9%) but not those in T2D patients with DN when compared to NGT controls (80.1%) ($p = 0.0014$). The study provides the first evidence that increased DNA methylation of SLC30A8 is associated with T2D but DN in a Malay ethnic [15].

From our literature search, there are still limited studies conducted on the genetics of DN among the Malaysian population. Out of 13 research articles found, only 7 of them are related and have published results on the genetics of DN.

In this review, we can see that the genetic findings presented are not congruent. This may be due to different definitions of cases and controls. 4 of them choose healthy populations as controls, and 2 articles used T2D without DN as controls. The case definition also varies between articles such as T2D with DN or ESRD patients.

To advance reproducibility in different populations, longitudinal studies are compulsory. This involves the prospective recruitment of a large cohort of healthy individuals at baseline and the follow-up for over several decades to track DN incidence among T2D patients. However, due to higher costs, and the fact that the patients usually develop DN after 10-15 years of having T2D, longitudinal studies for complex diseases such as T2D and DN remain uncommon to do. Nevertheless, replication using other populations may be useful to validate the previous finding, either using larger samples or other cohorts.

Most of the research conducted on epigenetics used DNA extracted from peripheral blood to study changes in DNA methylation in DN. This is most likely because kidney tissues are hard to get and the biopsy procedure is invasive. Despite having a few limitations such as mixed cell DNA methylation data from human kidney tissue would give a shred of strong evidence to support the role of epigenetics in the pathogenesis of DN. Compared to tissue biopsy, whole blood is much easier to get and gives similar data. These have been shown in the study published by Dayeh TA [16]. Therefore, a combination of studies using DNA extracted from blood and tissue samples would support and better understand the association of genetic variation and environmental factors in DN.

3.3. SLC12A3 Arg913Gln Polymorphism. The SLC12A3 gene is located at chromosome 16 at position exon 23 [17]. The systematic review comprising 2106 individuals with DN have summarized research articles that study the SLC12A3 rs11643718 polymorphism. This review showed a significant genetic association in the Arg913Gln variation of the SLC12A3 gene with the DN, suggesting that the mutations of the SLC12A3 rs11643718 polymorphism could be a significant predictor of ESRD [18]. This polymorphism has also been reported to be significantly associated with T2D patients with DN in the Chinese [19] and South Indian populations [20].

3.4. CCR5 rs1799987 an Allele. The CCR5 is located at chromosome 3 in the chemokine receptor gene cluster region [21]. This protein is expressed by T cells and macrophages and is known to be an important coreceptor for the macrophage-tropic virus entering the host cells. Pokrzywnicka et al. recently evaluated the CCR5 rs1799987 polymorphism association with DN among T2D [22]. They reported there was no association of CCR5 rs1799987 polymorphism in Polish populations. Further replication in other populations with larger sample sizes may be needed to confirm the association of this polymorphism.

Table 2: Shows differences in the odd ratio of the allele distribution among genetic variants.

SNP	Chromosome	Ensembl	Genes	Risk allele	Odd ratio (OD) 95% CI	Author, year
rs11643718	16q13	ENSG00000070915	SLC12A3	G/A	0.547 (0.308-0.973)	Abu Seman et al. 2014 [6]
rs1799987	3p21	ENSG00000160791	CCR5	G/A	6.71 (2.55-17.68) (Chinese)	Yahya et al., 2019 [10]
rs4073	4q13.3	ENSG00000169429	IL-8	T/A	1.57 (0.66-3.71) (Indians)	Yahya et al., 2019 [10]
rs4880	6q25.3	ENSG00000112096	MnSOD	C/T	2.16 (0.54–8.65) Malays, 2.8 (0.53–14.94) Chinese, 2.4 (0.69–2.84) Indians	Yahya et al., 2019 [11]
rs1799983	7q36.1	ENSG00000164867	NOS3	T/C	2.8 (0.29–28.32) Malays, 3.55 (0.36–35.03) Chinese, 3.16 (0.52–17.56) Indians	Yahya et al., 2019 [11]
rs2070744	7q36.1	ENSG00000164867	eNOS	T/C	0.64 (0.11-1.18) smoking, 0.57 (0.26-0.88) waist circumference	Yahya et al., 2019 [10]
D18S880 microsatelite	18q22.3	ENSG00000150656	CNDP1		2.46 (1.48–4.10) Malays, 2.26 (1.34–3.83) Chinese, 1.77 (1.18–2.65) Indians	Yahya et al., 2019 [11]
rs2346061	18q22.3	ENSG00000150656	CNDP1	A/C	1.94 (1.76–3.20) Indians	Yahya et al., 2019 [11]
rs8192678	4p15.2	ENSG00000109819	PPARGC1A	C/T	0.85 (0.64-1.05) sex, 0.68 (0.36-1.00) smoking, 1.90 (1.11-2.68) waist circumference	Ahmad et al., 2020 [13]
rs2237895	11p15.5	ENSG00000053918	KCNQ1	A/C	0.42 (0.01-0.84) smoking, 1.21 (0.66-1.78) waist circumference	Ahmad et al., 2020 [13]
rs2283228	11p15.5	ENSG00000053918	KCNQ1	A/C	1.21 (0.55-1.87) waist circumference	Ahmad et al., 2020 [13]
rs5498	19p13.2	ENSG00000090339	ICAM1	A/G	1.27 (1.01-1.61)	Abu Seman et al., 2015 [14]

3.5. Interleukin-8 (IL8) rs4073. Interleukin-8 (IL-8) is a chemoattractant cytokine produced by a diversity of tissue and blood cells. The protein encoded by this gene is a member of the CXC chemokine family and is a major mediator of the inflammatory response [23]. The development and progression of DN involve immune and inflammatory mechanisms. The IL-8 is known as relevant for the development of DN, as they are potentially involved in the onset of disease complications [24]. A systematic review showed that IL-8 rs4073 was associated with increased susceptibility to DN [25].

3.6. Manganese Superoxide Dismutase (MnSOD) rs4880. The oxidative stress-related polymorphism has been suggested to affect the development of DN. Manganese superoxide dismutase (MnSOD) protects the cells from oxidative damage by scavenging free radicals. Research in 1,510 Finnish and Swedish patients with type 1 diabetes studied the effects of MnSOD rs4880 alone and in combination with smoking on DN development [26]. Their results indicated that smoking together with MnSOD rs4880 homozygous had 2.52 times increased risk of DN (95% CI: 1.73-3.69).

3.7. NOS3 rs1799983. Nitric oxide is a reactive free radical that acts as a biological mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities [27]. The gene encoding eNOS (NOS3) is located in chromosome 7q35-36 and consists of 26 exons with a total size of 21 kb [28]. The rs1799983 polymorphism results are resulting changes in the eNOS protein sequence, which leads to degradation and malfunction of enzyme activity [29, 30]. The rs1799983 T allele was shown to be significantly associated with DN in both T2D and DN [31]. Similar results were observed in Egyptian [32, 33] and Indian populations [34]. However, the rs1799983 variant of NOS3 was found to not be associated with CKD in Canarian subjects [35]. The same results were found in the Brazilian [36], Iranian [37], Saudi Arabian [38], and Egyptian populations [39].

3.8. Carnosinase Gene (CNDP1) D18S880 Microsatellite and rs2346061 Polymorphism. CNDP1 is located on chromosome 18q. The CNDP1 encodes a dipeptidase that hydrolyses the substrate L-carnosine (β-alanyl-L-histidine) [40]. Carnosine serves as a scavenger of oxygen radicals and thus can inhibit the formation of AGEs [41, 42], It is therefore believed to play a protective role in DN. Meta-analysis of the carnosinase D18S880 microsatellite polymorphism confirmed the association with DN susceptibility [43]. The CNDP1 rs2346061 has been shown to play a role in susceptibility to kidney disease in patients with T2D in the Swedish population [44].

There are a few limitations to the research conducted to study the genetics of DN among the Malaysian population as included in this review. First, most of the research articles mentioned that the number of samples included in their study is small. This is due to the limited resources for DN cases, although it was larger compared to previous local studies, as discussed by Ahmad [13]. Larger sample sizes are needed to achieve sufficient power [45]. Although the calculated sample size is statistically enough for the study, there is still a possibility to lose the low-frequency variants that need a larger sample size to be detected [46].

Moreover, not many of the research articles discuss or did biological studies to support the results. Biological processes and molecular mechanisms underlying the effect of the genetic variants are worth to be explored. Studies in vivo and in vitro can be done using commercialized cells or suitable animal models that are available on the market. Effects of the nonsynonymous SNPs that involved changes in the amino acid could be studied using genetic engineering. The nonspecific insertions, deletions, or other mutations (indels) could be reversed using the CRISP technique [47], and the function of the genetic variants in the disease could be further evaluated.

All the published genetics data on DN in Malaysia were using the case-control approach. This study design is used when researchers want to compare those with the disease or condition under study (cases), and a similar group of people who do not have the disease or condition (controls). The case-control study design has a few advantages and disadvantages or limitations. The case-control approach allows us to study a rare disease that requires less sample size [48]. This approach requires less time and is less expensive than the prospective study approach [49]. However, this study design is not able to determine the incidence and prevalence of the disease [50]. It also cannot be used to evaluate the causality. Yet, the study design is practically chosen based on the study objective.

4. Conclusions

This paper reviews the update of genetic and epigenetic studies of DN conducted in Malaysia. Although it became the third-highest country for incidents of ESRD due to diabetes, research conducted on this disease is still limited. It is a heterogeneous and polygenic disease with several genes, proteins, and environmental factors that may contribute to the disease. Multiple genetic variants have been suggested for the progression of DN. The case-control and candidate gene-based association studies are the main approaches that have been explored to identify the susceptibility of the genetic variants. Therefore, further research on confirming the potential biomarkers of DN among Malaysian populations is required.

Data Availability

No underlying data was collected or produced in this study.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Renal Histologic Findings in Necropsies of Type 2 Diabetes Mellitus Patients

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Background. Very few studies have analyzed early histologic lesions of diabetic nephropathy (DN) in patients without signs of clinical involvement (microalbuminuria). In this study, we analyzed renal histologic lesions in necropsies of diabetic patients with or without previous signs of DN. *Methods*. Histological material was analyzed from 21 autopsies of type 2 diabetes mellitus (T2DM) patients (9 with albuminuria and 12 without albuminuria) and 4 controls. Histologic lesions were evaluated according to the Tervaert classification. *Results*. Kidneys of diabetic patients presented significantly higher scores in most histologic indices analyzed (glomerular basal membrane thickening, mild and severe mesangial expansion, nodular sclerosis, interstitial fibrosis, and tubular atrophy) than in nondiabetic controls ($p < 0.01$ in all cases). In contrast, no significant differences were detected between histologic scores when comparing the 21 diabetic patients with and without albuminuria. A significant percentage of cases without albuminuria showed moderate to severe histologic lesions, particularly severe mesangial expansion and severe glomerular vascular lesions. No significant differences were found in age, blood pressure, diabetes vintage, BMI, HbA1c, cholesterol, triglycerides, or treatments between the two (albuminuric vs. nonalbuminuric) T2DM patient groups. *Conclusions*. Our data suggest that histologic lesions of DN are present in the early stages of the disease, even without albuminuria presence. More precise and earlier metabolic control is recommended in T2DM, and monitoring of risk factors can play a role in DN development.

1. Introduction

The prevalence of diabetes mellitus (DM) is around 425 million people worldwide, and this figure is predicted to increase to over 600 million by 2045 [1]. Diabetic nephropathy (DN) is the leading cause of morbidity and mortality in patients with DM, and estimations indicate that 30–40% of DM patients will develop DN [2]. In this context, excess all-cause mortality in DM patients is associated with chronic kidney disease (CKD). Beyond its diagnostic and prognostic value, microalbuminuria is used as a marker of endothelial dysfunction, indicating the existence of glomerular compromise. Patients who express microalbuminuria also have a higher risk of progression to proteinuria and end-stage kidney disease (ESKD). This urinary biomarker represents an important variable for assessing DN progression [3].

Table 1: General patient characteristics: most recent clinical and analytical data (median and interquartile range, percentage).

Variable	Type 2 diabetic patients		Control ($n = 4$)	p value alb vs no alb	p value control vs T2DM
	Albuminuric ($n = 9$)	Nonalbuminuric ($n = 12$)	Control ($n = 4$)		
Age (years)	75 [64–90.5]	77.5 [68.25–83.75]	81 [58.5–82.5]	0.890	0.803
Male sex (%)	77.8	75.0	75.0	1	1
Diabetes mellitus vintage (mo)	108 [56–124.5]	90.5 [63–109.5]	NA	0.422	NA
Weight (kg)	68.5 [63–NA]	78.5 [69.5–102.25]	75 [47–NA]	0.198	0.885
Height (cm)	161 [156–168.5]	165 [159.75–171.25]	160 [159–NA]	0.500	0.875
BMI (kg/m^2)	31.8 [27.45–36.4]	30.35 [26.7–34.6]	29.7 [18.7–NA]	0.559	0.559
Systolic BP (mmHg)	130 [87–148.5]	110 [100–138]	124.5 [117–140.25]	0.519	0.477
Diastolic BP (mmHg)	65 [55–72]	61 [54–72]	63.5 [51–75.25]	0.815	0.852
Mean blood pressure (mmHg)	90.33 [65.67–95.5]	72.33 [69.25–90.75]	NA	0.754	NA
Last albuminuria (mg/g Cr)	223.33 [153–393.7]	10.53 [3.08–17.69]	2.6 [2.6–2.6]	<0.001	0.200
Albuminuria 1 year before (mg/g Cr)	220.22 [82–294–36]	7.33 [3.34–14.22]	NA	0.001	NA
Albuminuria 2 years before (mg/g Cr)	217.02 [86.40 332.64]	6.72 [3.64–19.45]	NA	<0.001	NA
Last serum creatinine (mg/dL)	1.89 [1.03–4]	1.02 [0.72–2.01]	0.68 [0.41–1.73]	0.032	0.068
Serum creatinine 1 year before (mg/dL)	1.49 [0.96–2.03]	1 [0.82–1.49]	NA	0.111	NA
Serum creatinine 2 years before (mg/dL)	1.16 [0.96–1.81]	0.91 [0.73–1.14]	NA	0.058	NA
eGFR <60 mL/min/1.73 m^2 (%)	83.3	31.3	25.0	0.056	0.594
Last eGFR (mL/min/1.73 m^2)	22.96 [15.87–65.08]	70.84 [45.01–85.65]	85.55 [43.13–96.4]	0.037	0.231
eGFR 1 year before (mL/min/1.73 m^2)	44.26 [31.95–68.05]	74.99 [51.13–85.87]	NA	0.095	NA
eGFR 2 years before (mL/min/1.73 m^2)	50.07 [35–77.96]	82.47 [59.12–88.78]	NA	0.095	NA
Serum albumin (day/dL)	3.3 [2.75–4.15]	3.4 [3.2–3.85]	3.2 [3–NA]	0.250	0.569
Cholesterol (mg/dL)	142 [122–173]	169.5 [132.5–176.75]	151 [122.5–173.5]	0.276	0.695
Triglycerides (mg/dL)	100 [72–147.5]	138 [70–156]	70 [48–NA]	0.599	0.172
Glycemia (mg/dL)	143 [96–182.5]	119.5 [107.5–161]	102 [89–111.25]	0.760	0.031
Last HbA1c (%)	7.56 [6.53–9.43]	6.75 [6.13–7.55]	NA	0.238	NA
HbA1c 1 year before (%)	7 [6.33–8.9]	6.9 [6.4–7.35]	NA	0.734	NA
HbA1c 2 years before (%)	6.6 [6.1–8.4]	6.65 [6.4–7.35]	NA	0.917	NA
HbA1c <7.5% (%)	33.3	33.3	NA	1.000	1.000
Diabetic retinopathy	11.1	0	NA	0.429	1.000

Variables are expressed as median and interquartile ranges. eGFR <60 mL/min/1.73 m^2, male sex, retinopathy, and HbA1c <7.5% in %. NA: not available (not calculable).

However, the currently accepted paradigm is that degree of albuminuria/proteinuria alone is not sufficient for prognostic evaluation, since not all patients with type 2 (T2) DM who develop renal failure have albuminuria/proteinuria [4].

Following the Tervaert pathological classification for DN, studies have shown that both glomerular and interstitial involvements are independently associated with CKD [5]. Tubulointerstitial damage is related to glucotoxicity, which accelerates CKD progression and is closely related to renal function decline; indeed, studies point to tubular damage as a better predictor of CKD progression than classic markers such as albuminuria [6]. Of interest, in earlier stages of CKD, the presence or absence of certain pathological findings such as nodular and exudative lesions and/or mesangiolysis is predictive of adverse renal events (dialysis, doubling of serum creatinine, or sustained decrease in estimated glomerular filtration rate [eGFR]). These pathological lesions are relevant in T2DM patients with normal and abnormal

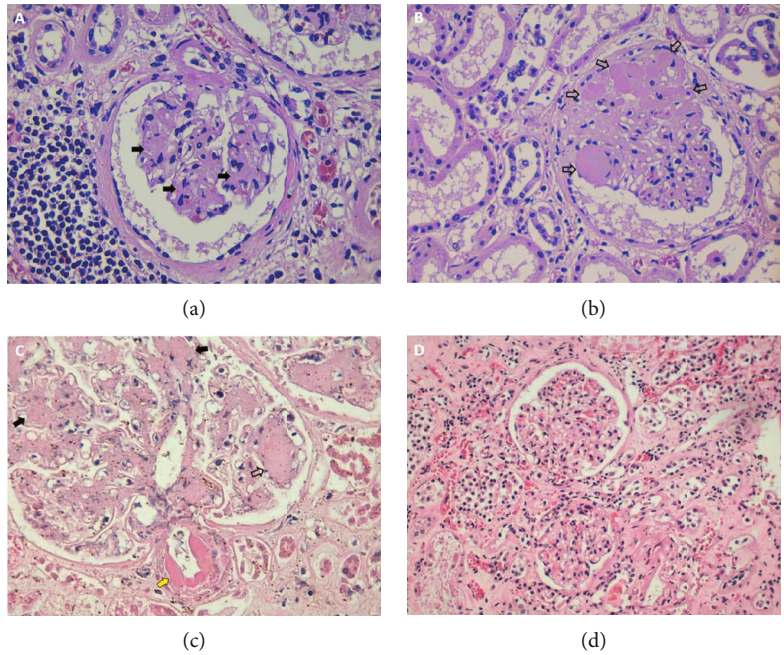

Figure 1: Kidney histological findings. (a) HE 400x, glomeruli with mild mesangial expansion (black arrows) and Bowman's capsule fibrosis. Findings of chronic interstitial fibrosis and tubular atrophy (IFTA) on the left side of the figure, in a nonalbuminuric diabetic subject. (b) HE 400x, nodular lesions (Kimmelstiel-Wilson) (empty arrows) that correspond to a class III: nodular sclerosis in the histologic classification system, in a diabetic albuminuric subject. (c) HE 400x, severe mesangial expansion, Kimmelstiel-Wilson nodules, and hyalinosis of efferent arteriole (yellow arrow) in a diabetic albuminuric subject. (d) HE 200x, glomeruli with nonspecific changes from a sample of the control group.

eGFR or microalbuminuria, becoming predictors of cardiovascular and adverse renal events [7].

Prevention of DN is the key to avoiding disease progression, and most therapeutic measures have focused particularly on patients with incipient nephropathy (microalbuminuria) or eGFR decrease. Recent KDIGO guideline recommendations on diabetes management are centered on T2DM patients with CKD (urine albumin-to-creatinine ratio >30 mg/g or eGFR <60 mL/min/73 m^2) [3], yet DN prevention measures should be established in earlier stages before microalbuminuria develops.

Renal biopsy in T2DM patients is usually indicated in those with significant renal manifestations such as severe proteinuria, microscopic hematuria, rapid unexplained worsening of kidney function, or over 30% decline in eGFR after initiating RAAS inhibition [8]. In this regard, some studies have analyzed early DN lesions in patients without clinical signs of this involvement (microalbuminuria) [9]. Nonetheless, although not a routine technique, renal biopsy remains the gold standard of DN diagnosis and recent evidence supports determining renal histologic involvement in this disease. For this reason, very few studies to date have analyzed renal histologic alterations in the early stages of T2DM.

The main aim of the study was to analyze whether T2DM patients without microalbuminuria present histologic lesions of DN. For this purpose, histologic samples of renal tissues were analyzed in necropsies from diabetic patients with or without microalbuminuria, and these findings were compared with renal samples from autopsies of nondiabetic patients as a control group.

2. Methods and Material

Samples were selected from autopsies performed at the Hospital Clínico Universitario of Valencia between 2015 and 2017. A total of 25 autopsies were analyzed: 21 in T2DM patients (9 in albuminuric and 12 in nonalbuminuric patients) and 4 nondiabetic matched controls. Histological data were therefore included for all cases according to the work protocol and classification of histologic lesions. Once selected, the data of all these clinical and pathological variables were retrieved from hospital electronic medical records.

2.1. Processing of Histologic Material. Histological data were reviewed by one expert pathologist in renal histology who was blinded to the study data. Given that most autopsies show a greater or lesser degree of acute tubular necrosis (ATN), we decided to evaluate only vascular lesions, although analysis of interstitial lesions was also attempted since most autopsies could establish scores for fibrosis with associated tubular atrophy or inflammatory findings.

Renal autopsy histology material was fixed in 10% formalin and embedded in paraffinic tissue. All samples were stained by Dako Cover Stainer (Dako) Autostainer for hematoxylin-eosin (H-E) staining and Artisan Link Pro

Table 2: Renal histologic findings in necropsies of T2DM (albuminuric and nonalbuminuric) patients and nondiabetic controls.

	Albuminuric ($n = 9$)	Nonalbuminuric ($n = 12$)	Controls [4]	p value alb vs no alb	p value control vs T2DM
Total number of glomeruli in the sample	113 [94–228.5]	159.5 [120.75–213.5]	129.5 [82.25–186.5]	0.207	0.452
GBM thickening, n	100 [59–130]	111 [30.25–171.25]	0 [0–12.75]	0.846	<0.001
GBM thickening (%)	84 [43.5–92.5]	86.5 [21–93.75]	0 [0–12.75]	0.890	<0.001
Mild mesangial expansion, n	100 [59–130]	111 [30.25–171.25]	0 [0–12.75]	0.846	<0.001
Mild mesangial expansion (%)	41.2 [26–107.5]	47.1 [11.25–134.25]	10 [0–23.75]	0.890	0.015
Severe mesangial expansion (%)	11 [0.5–42.5]	3 [0–67.25]	0 [0–0]	0.637	0.015
Advanced glomerular sclerosis (>50%) (n)	8 [5.5–21.5]	8 [4.25–15.75]	1 [0–30.5]	0.677	0.113
Nodular sclerosis (KW) (yes/no) (%)	66.7	18.8	0	0.031	0.260
Arteriolar hyalinosis (yes/no) (%)	66.7	56.3	0	0.691	0.017
Presence of arteriosclerosis (%)	88.9	91.7	75.0	0.841	0.408
Presence of interstitial lesions (%)	28.6	18.8	0	0.621	0.539
IFTA (%)	10 [5–27.5]	5 [1–22.5]	1 [0–3.75]	0.456	0.006
IFTA					
(i) No IFTA	12.5	23.1	72.0		
(ii) <25%	62.5	53.8	25.0		
(iii) 25–50%	12.5	23.1	0		
(iv) >50%	12.5	0	0	0.653	0.037
Inflammation					
(i) Absent	11.1	22.2	75.0		
(ii) Infiltration only related to IFTA	55.6	77.8	0		
(iii) Infiltration in areas without IFTA	22.2	11.1	25.0	1.000	0.023

GBM, glomerular basement membrane; KW, Kimmelstiel-Wilson; IFTA, interstitial fibrosis and tubular atrophy.

(Dako) for special stains such as PAS, Masson's trichrome, and Jones stain (silver).

The histologic score was based on the Tervaert classification of the American Society of Pathological Anatomy [5]:

(i) Glomerular lesions. Total number of glomeruli, the number of glomeruli with thickening of the glomerular basement membrane, the number of glomeruli with the mild expansion of the mesangium, the number of glomeruli with severe expansion, the presence of nodules, the number of glomeruli with sclerosis, and glomeruli with global sclerosis (>50%)

(ii) Tubulointerstitial lesions. Interstitial fibrosis and tubular atrophy (IFTA), subdivided into four points according to the affected percentage: 0 (no fibrosis), 1 (<25%), 2 (25–50%), and 3 (>50%), and the presence of interstitial inflammation and whether or not it is associated with areas of IFTA: 0 (absence of interstitial inflammation), 1 (presence, also associated with areas of IFTA), and 2 (presence, not associated with areas of IFTA)

(iii) Vascular lesions. Arteriolar hyalinosis, arteriosclerosis (establishing two degrees based on the predominant thickness of the intima and media layers between), and arteriolar drops

(iv) Others. Capsular drops, glomerular and tubular adhesions, tubular glomeruli (atrophy of the proximal convoluted tubule (TCP) in the urinary pole), and the presence of urinary cysts and fibrinolysis

2.2. Clinical Data Collection. Clinical data were retrieved from hospital electronic records, including the following clinical characteristics: age (years), duration of diabetes until death (months), sex, height (cm), weight (kg), body mass index (BMI) (kg/m^2), blood pressure (mmHg), analytical data, eGFR (mL/min/1.73 m^2), previous diagnosis of retinopathy, history of cardiovascular disease (ischemic heart disease, transient ischemic attack (TIA)/stroke, peripheral vascular disease, and heart disease), arterial hypertension (HTN), and drug treatments: antiaggregate, oral anticoagulants, RAAS blockers, and hypoglycemic agents.

2.3. Statistical Analysis. The Shapiro-Wilk test was used to determine sample distribution. Given the sample size and distribution, quantitative variables are expressed as a median and interquartile range and qualitative variables as percentages. The Mann-Whitney U test was used to compare differences between continuous variables in the two independent

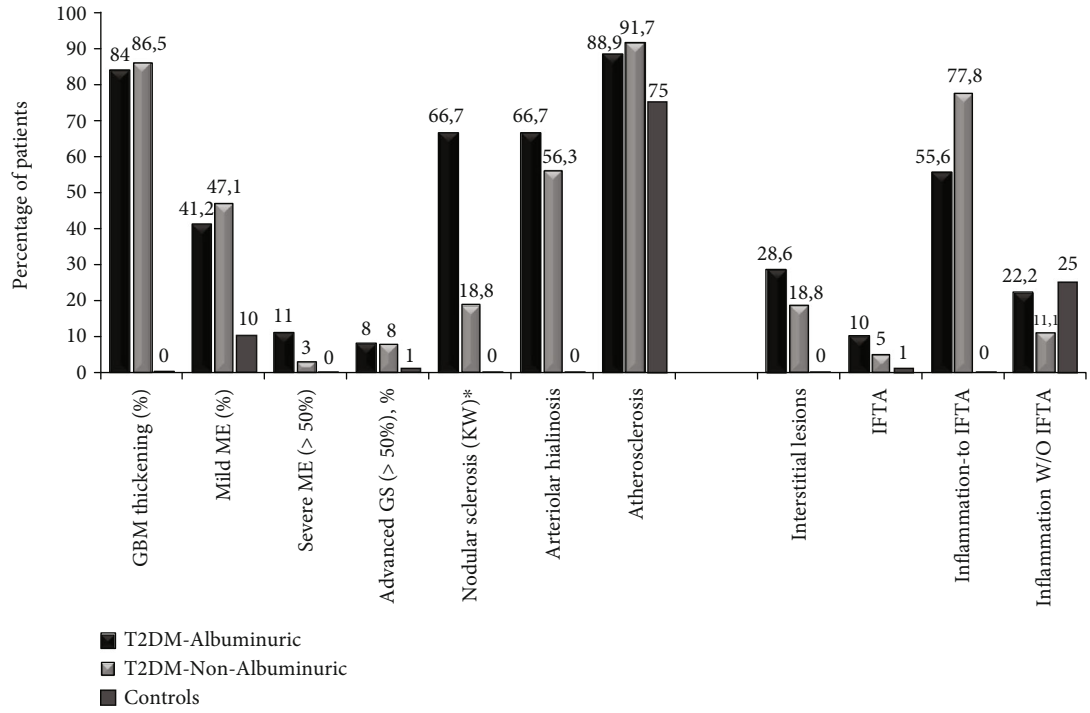

Figure 2: Percentage of patients with different histologic lesions. (a) Glomerular lesions. (b) Interstitial lesions. The number of glomeruli evaluated in each group was albuminuric patients 113 [94–228.5], nonalbuminuric patients 159.5 [120.75–213.5], and controls 129.5 [82.25–186.5]. There were significant differences in all lesions when comparing diabetic patients and nondiabetic controls. No significant differences were found when comparing albuminuric and nonalbuminuric T2DM patients except for the presence of nodular sclerosis∗ ($p = 0.031$).

groups. Statistical significance was set at $p < 0.05$. For categorical variables, the comparison was made using Fisher's test as counts were too low to use the chi-squared test. Descriptive analysis and the Mann-Whitney U test were performed using the IBM SPSS Statistics version 19.0 software, while the R software was used to apply Fisher's exact test. All statistical tests with p values below 0.05 were considered significant.

3. Results

Clinical and analytical data from autopsies of diabetic patients with and without albuminuria and controls are summarized in Table 1 and Supplemental Table 1 (S1). Albuminuria values in patients with and without this condition differentiated the characteristics of the two groups since those patients without albuminuria showed a very low range (mean 10.53 mg/g with an interquartile range of 3.08–17.69), which contrasted with the much higher ranges observed in the albuminuria patient group (median 223 mg/g with an interquartile range of 153–393). Some histological samples of the groups are shown in Figure 1.

Patients with albuminuria showed higher serum creatinine ($p = 0.032$) levels and lower eGFR ($p = 0.037$) than nonalbuminuric ones. Moreover, the latter group received more insulin and fewer metformin doses ($p = 0.031$ in both cases). No significant differences between groups were found in clinical or analytical data or treatments received (Tables 1 and S1). These data show that the three patient groups (nonalbuminuric, albuminuric, and controls) had similar and therefore comparable clinical characteristics, despite the limited number of patients.

3.1. Comparison of Diabetics and Nondiabetic Controls. The patients' clinical and histologic characteristics are shown in Table 2. For the histologic study, we analyzed a large number of glomeruli (median, 146) in each sample analyzed (interquartile range: 112–209).

Comparing findings of diabetic patients with nondiabetic controls, as expected, we observed that patients with diabetes presented significantly higher scores in glomerular basement membrane thickening, mesangial expansion, and arterial hyalinosis ($p < 0.015$ in all cases), all with signs related to glomerular lesions of T2DM. Likewise, diabetic patients had higher scores in interstitial lesions, inflammation, and tubular atrophy than nondiabetic controls ($p < 0.05$). There were no significant between-group differences related to arteriosclerosis lesions in the scores (Figure 2).

3.2. Comparison of Diabetic Patients with and without Albuminuria. When histologic lesions were compared between albuminuric and nonalbuminuric patients, we found significant differences regarding the presence of nodular sclerosis (presence of Kimmelstiel-Wilson nodules): 66.7% in albuminuric vs. 18.8% in nonalbuminuric ($p = 0.031$), but none were found for the remaining glomerular lesions. No significant differences were detected in

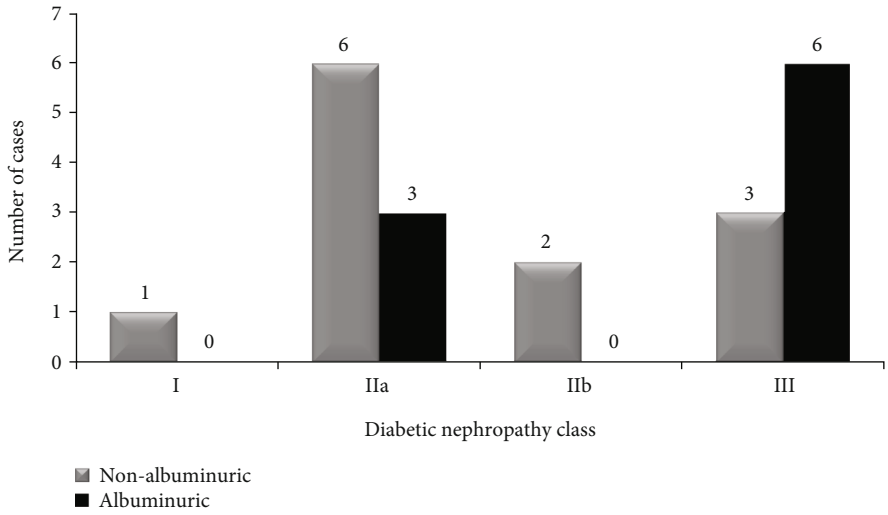

Figure 3: The number of cases in the different classes of glomerular diabetic nephropathy lesions. Glomerular lesions in all diabetic patients. We show albuminuric (black) against nonalbuminuric (gray) ($n = 21$) patients.

interstitial lesions, although albuminuric patients showed greater severity of IFTA lesions and higher infiltration in areas without IFTA, both of which are assigned a higher score in the Tervaert score classification [5] (Table 2 and Figures 2–4).

Note that no significant differences were found between albuminuric and nonalbuminuric subjects in variables such as BMI, degree of blood pressure control, or lipid parameters. Although albuminuric patients had higher HbA1c over the previous year, this difference did not reach statistical significance as HbA1c levels were practically identical across the two groups in the two preceding years. Neither were any significant differences found in associated comorbidities or treatment received, with the exception that albuminuric patients received a higher percentage of insulin and metformin ($p = 0.031$ in both cases). It is striking that no patient in the albuminuric group had diabetic retinopathy (Table 1).

These data suggest that patients without albuminuria already have DN-associated histologic lesions despite the absence of clinical manifestations of the disease (microalbuminuria). Particularly noteworthy is that normoalbuminuric patients presented a similar degree of mild mesangial expansion (41.2% vs. 41.7%) and thickening of GBM to those with diabetes (84% vs. 86.5%); moreover, in some glomeruli, they showed severe mesangial expansion, although to a lesser degree than in albuminuric subjects, even though the median course of diabetes in nonalbuminuric patients was 90.5 months (interquartile range: 63–109.5).

4. Discussion

The first finding of our study is that a significant percentage of patients not displaying early clinical signs of DN such as microalbuminuria already show moderate to severe DN-related histologic involvement, although there were no significant differences in comorbidities and renal progression factors compared with the albuminuria patient group. These findings are suggestive of early renal involvement in nonalbuminuric T2DM patients, also supported by the fact that in our sample, no patient had diabetic retinopathy (nor in cases with albuminuria). These findings gain further importance considering that histologic studies cannot be performed in patients with early DN expression.

The prognostic implication of Tervaert classification scores [5] has been well established since An et al.'s work [10], supporting the predictive value of the previously described pathological classification of DN, especially for interstitial lesions. In most histologic studies performed in DN patients, the samples included predominantly grades IIb and IV and high proteinuria levels [10, 11] and thus had greater renal compromise than those analyzed in our study. Similarly, other studies analyzing kidney biopsies from DN patients also found considerable changes in arteriosclerosis [12].

In one of the few published studies including a large sample of patients in initial DN stages, Quinn et al. analyzed the histologic material of nephrectomy [13], evaluating 859 samples from partial or total nephrectomies from kidney neoplasia (304 (35.4%) patients with T2DM). Their findings demonstrate that including renal histologic variables with baseline eGFR contributes to a more precise estimation of kidney function decline, especially in the early stages of DN. The degree of histologic damage was highly variable in patients with CKD stages 1–2, and some individuals displayed relatively severe structural damage despite preserved eGFR. These findings were a high degree of fibrosis, tubular atrophy, and glomerulosclerosis.

On the other hand, our findings show clinical and pathological dissociation, further supporting recent controversy over the emerging concept of "DN without proteinuria" [14], which complicates the interpretation of histologic data in patients with different degrees of DN. Findings point toward the existence of two phenotypes of renal involvement in T2DM (with or without proteinuria), of which the latter can progress to ESKD without a significant degree of proteinuria [15].

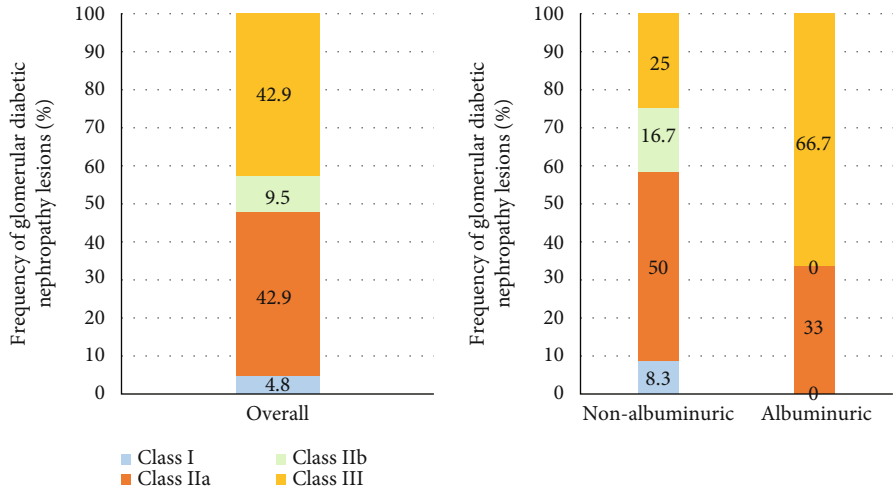

Figure 4: Frequency of different classes of glomerular diabetic nephropathy lesions. Frequency of different classes of glomerular diabetic nephropathy lesions in all diabetic patients (left panel). The right panel shows the different classes according to albuminuric status ($n = 21$).

The second interesting finding of our study was the high prevalence of arteriolar hyalinosis (66.7% in albuminuric and 56.3% in nonalbuminuric patients). Quinn et al. also reported a high degree of arterial hyalinosis, at 32.7%, although without distinguishing between diabetic and nondiabetic patients [13]. This has also been demonstrated in kidney biopsies from DN-affected patients, where 85% displayed arteriolar hyalinosis and 60% arteriolosclerosis [16]; this condition can also affect the arteries, with a preponderant role in glomerular hemodynamic changes. The high prevalence of these histologic alterations may have considerable implications for the renal adaptive mechanisms involved in glomerular hemodynamics, since arterioles with hyalinosis may have hampered mechanisms of vasoconstriction and vasodilation through which they exert their role in intraglomerular HTN, which could affect current nephroprotective treatments in T2DM such as renin-angiotensin-aldosterone system (RAAS) blockers and sodium/glucose cotransporter 2 inhibitors (SGLT2i) [17, 18].

This may partially account for the variability of response to nephroprotective treatments in T2DM. In the RENAAL (Reduction in End Points in Noninsulin-Dependent Diabetes Mellitus with the Angiotensin II Antagonist Losartan) study, the albuminuria reduction rate at 6 months showed that 41.6% of patients experienced no reduction in albuminuria; in 25.9%, albuminuria was lowered by 0–30%, and 32.3% saw an albuminuria decrease of over 30% [19]. Kraus et al. analyzed the initial eGFR dip after SGLT2i treatment in the EMPA-REG OUTCOME study [20]. They showed that in patients treated with empagliflozin, 31% showed no eGFR decline; in 41%, it dropped between 0 and 10%, and in 28%, the dip was between 10 and 30%. Only 1.4% showed an eGFR decline greater than 30%. These different responses to nephroprotective treatments may be due to multiple factors, including the extent of arterial hyalinosis in afferent and efferent arterioles. The high prevalence of these histologic alterations may have important implications for the renal adaptive mechanisms involved in glomerular hemodynamics.

Thirdly, our findings add weight to the importance of early and strict metabolic control in T2DM. Two post hoc analyses of the UKPDS study have shown that the glycemic legacy effects seen in T2DM are largely explained by historical HbA1c values, which have a greater impact on renal and cardiovascular outcomes than recent values; microvascular complications and mortality also increased in those patients who presented HbA1c >6.5% in the first year after diagnosis [21, 22]. Based on our findings and supported by these post hoc studies, we could suggest that early detection of diabetes and intensive glucose control from the time of diagnosis are essential for maximum reduction of long-term risk of glycemic complications.

The VERIFY study recently demonstrated that early intervention with combination therapy of vildagliptin plus metformin provides greater and more durable long-term benefits in newly diagnosed T2DM patients compared with metformin as monotherapy [23]. This result also supports that early and intense intervention can improve metabolic control in these patients. In this regard, the DECLARE study in T2DM patients with or at risk of atherosclerotic cardiovascular disease found that treatment with dapagliflozin was associated with a 24% reduction in the renal endpoint (hazard ratio, 0.76; 95% CI, 0.67–0.87). These findings demonstrate the effect of an SGLT2i in earlier stages of renal disease since in this study, 65% of patients showed no expression of any renal markers (eGFR < 60 mL/min/1.73 m^2, microalbuminuria or macroalbuminuria: 93% showed eGFR > 60 mL/min/1.73 m^2 and 69.1% showed no albuminuria) and 59.4% of patients had no previous cardiovascular disease [24, 25]. Interestingly, the use of SGLT2i seems to prevent and reduce diabetic kidney disease progression compared to placebo in this large and diverse population study in T2DM patients with or without established atherosclerotic cardiovascular disease, most of whom had preserved renal function. Although the possible

mechanisms of benefit were multiple and incompletely understood, in most cases, this benefit has been independently associated with glycemic control. Based on current evidence, KDIGO guidelines recommend that glycemic management for T2DM and CKD patients should include lifestyle therapy and first-line treatment with metformin and an SGLT2i [3].

Our study has several limitations, such as the low number of samples used for analysis, the presence of tubulointerstitial lesions inherent to acute tubular necrosis or nephrotoxic agent history in some histologic samples, and the lack of additional pathologists to avoid single observer bias. Furthermore, there were just nine autopsies in patients with albuminuria and a slight imbalance between age in the groups and other variables. That HbA1c analysis was carried out in the previous two years could be considered a limitation of the study, as could interpretation of these results without considering metabolic control history. Nonetheless, the exhaustive histologic analysis applying updated scores and the high number of glomeruli analyzed in each sample allowed us to obtain significant results.

5. Conclusion

Our data suggest that renal involvement in T2DM patients is present in earlier stages of the disease (patients without albuminuria), highlighting the importance of early metabolic control as well as control of other risk factors which may influence DN development. Large sample size studies are warranted to confirm these data and to further study new therapeutic options such as SGLT2i and/or glucagon-like peptide-1 receptor agonists which show great promise in DN prevention. These findings can serve as a basis to support early and strict metabolic control in patients with DM and the use of drugs with demonstrated renal and cardiovascular benefit in the treatment of this disease, all of which will undoubtedly improve prognosis in affected patients.

Data Availability

Data are available upon request.

Ethical Approval

Protocol for the research project has been approved.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Authors' Contributions

All authors contributed equally to this work.

Supplementary Materials

The supplementary table shows the general characteristics of the patients. Thus, the last clinical and analytical data known are presented in median values, interquartile range, and percentage. *(Supplementary Materials)*

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