Diabetic tubulopathy: effect of toll-like receptor 2, toll-like receptor 4, and nuclear factor κb in elderly type 2 diabetes mellitus patients

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Objective
The aims of the current study were to investigate the role of toll-like receptor (TLR)2, TLR4, and nuclear factor κB (NF-κB) expression in the pathogenesis of diabetic nephropathy (DN) in elderly type 2 diabetics, and also to determine the functional role of TLR2, TLR4, and NF-κB in tubular inflammation.

Background
Chronic kidney disease is one of the major complications of type 2 diabetes mellitus (T2DM) and is the leading cause of end-stage renal disease. There is growing evidence indicating that chronic low-grade inflammatory response is a recognized factor in the pathogenesis and progression of diabetic renal injury.

Patients and methods
Our study included a human part and an animal part, in the human part, participants were divided into four groups; old patients: 30 T2DM patients aged 65 years and above with documented DN in stage 2 incipient nephropathy and stage 3 overt nephropathy, old control: 20 age- matched and sex-matched healthy persons aged 65 years and above serving as a control group, young patients: 30 T2DM patients aged less than 65 years with documented DN in stage 2 incipient nephropathy and stage 3 overt nephropathy and young control: 20 age-matched and sex-matched healthy persons aged less than 65 years serving as a control group. We took a full history of all patients and concluded complete physical examination. Mean arterial pressure, BMI and fundus examination were done. We measured fasting plasma glucose, 2 h postprandial plasma glucose, glycated haemoglobin, complete urine analysis, serum creatinine, blood urea nitrogen, C-reactive protein, urinary albumin/creatinine ratio, estimated glomerular filtration rate using Modification of Diet in Renal Disease Abbreviated Equation, level of TLR2, TLR4, and nuclear NF-κB. In the animal part, the study was conducted on 20 aged (1.5-year old) male wistar rats, the rats were divided into two main groups; group I (control): 10 normal healthy male rats, group II (diabetic): 10 rats with high-fat diet)/streptozotocin-induced diabetes. Blood samples were collected for the determination of fasting plasma glucose, fasting serum insulin, insulin resistance was assessed by calculating the homeostatic model assessment of insulin resistance, blood urea nitrogen, serum creatinine and C-reactive protein and analysis of TLR2, TLR4, and NF-κB in renal tissue was done.

Results
In the human part of our study: the level of TLRs2, TLR4, and NF-κB was significantly higher in old diabetic patients group than young diabetic patients group and control group. In the animal part of our study: the level of TLRs2 and 4 and NF-κB was significantly higher in diabetic rat group than healthy rat control group.

Conclusion
TLRs2, TLR4, and NF-κB were higher in old diabetic patients compared with young diabetic patients and normal individuals. These observations significantly added to the emerging role of TLRs in T2DM development and its possible role in the pathogenesis and progression of DN. Also, the present study showed that the TLR system was closely related to the ageing of the kidneys.

Keywords:
diabetes mellitus, diabetic nephropathy, nuclear factor kappa beta, toll-like receptor 2, toll-like receptor 4

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Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD) worldwide, which is mainly due to the increasing prevalence of type 2 diabetes [1]. Although DN is traditionally viewed as a nonimmune disease, emerging evidence suggests that inflammatory mechanisms play an important role in disease pathogenesis and progression [2]. Indeed, the decline of renal function in diabetic patients with proteinuria is positively associated with tubulointerstitial inflammation [3]. From a therapeutic perspective, blockade of the renin–angiotensin system coupled with strict glycaemic and blood pressure control is the best documented treatment strategy for DN. Despite the initial promises shown in early clinical trials [4,5], many patients still progress to ESRD. Therefore, investigations into the mechanisms underlying intrarenal inflammation may provide new therapeutic targets for anti-inflammatory strategies against DN.

Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors that play a fundamental role in the innate immune system by triggering proinflammatory signalling pathways in response to microbial pathogens and also activated by endogenous agonists of non-microbial origin and are involved in noninfectious inflammatory conditions [6]. Recently, TLRs have been implicated in the pathogenesis of acute and chronic renal disorders [7]. Among the 11 human TLRs, TLR2 and TLR4 might be a molecular link between inflammation and diabetes [8]. Therefore, we studied the relation and the levels of TLR2 and TLR4 in end-stage DN.

Patients and methods

The study was conducted in two parts:

Human part

Participants were divided into four groups:

(1) Old patients: included 30 patients with type 2 diabetes mellitus (T2DM) patients aged 65 years and above with documented DN in stage 2 incipient nephropathy and stage 3 overt nephropathy.
(2) Old control: included 20 healthy persons of matched sex and aged 65 years and above serving as a control group.
(3) Young patients: included 30 patients with type 2 DM patients aged less than 65 years with documented DN in stage 2 incipient nephropathy and stage 3 overt nephropathy.
(4) Young control: included 20 healthy persons of matched sex and aged less than 65 years serving as a control group.

All patients were enrolled from the Geriatric and Nephrology Units of the Department of Internal Medicine Main Alexandria University Hospital. Diagnosis of DN depended on thorough history taking, clinical examination including elevated arterial blood pressure and laboratory investigations including persistent albuminuria measured by urinary albumin creatinine ratio (>300 mg/g) and decline in glomerular filtration rate (GFR) measured by eGFR by Modification of Diet in Renal Disease Abbreviated Equation (MDRD) equation. An informed consent was taken from all participants.

Exclusion criteria

Patients having cancer, major organ disease and autoimmune and/or inflammatory disease or recently hospitalized for infectious diseases or taking corticosteroids or immunosuppressant drugs were excluded.

All patients were subjected to the following:

(1) Full medical history.
(2) Complete physical examination.
(3) Measuring blood pressure, weight and height. Mean arterial pressure will be calculated as [2X diastolic blood pressure (mmHg)+systolic blood pressure (mmHg)/3] and waist circumference.
(4) BMI was calculated as weight (kg)/height (m^2).
(5) Fundus examination.
(6) Fasting plasma glucose, 2 h postprandial plasma glucose and glycated haemoglobin.
(7) Urine analysis.
(8) Serum creatinine, blood urea nitrogen and C-reactive protein (CRP) by enzyme-linked immunosorbent assay.
(9) Urinary albumin/creatinine ratio which was measured by early morning spot urine sample.
(10) GFR was estimated using MDRD – abbreviated equation (MDRD): [GFR=175× (serum Cr)—1.154× (age)—203× (0.742 if female)].
(11) The surface expression of TLR-2 from monocytes of younger and older individuals was quantified by flow cytometry.
(12) Principle of the test.

Flow cytometry measures the optical and fluorescence characteristics of a single cell (or of any other particle, including nuclei, microorganisms chromosome preparations and latex beads). Physical properties

such as size (represented by forward-angle light scatter) and internal complexity (represented by right-angle scatter) can identify certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA [9].

(1) Procedures:
   (a) Isolation of peripheral blood mononuclear cells: blood was collected in sterile collection tubes containing EDTA. Two millilitres of Ficoll were placed in a centrifuge tube and a layer of 1 ml of blood was placed on top very carefully, ensuring that the blood and Ficoll did not mix; white blood cells were isolated by Ficoll (Sigma, Saint Louis, Missouri, USA) gradient and centrifugation was performed at 1800 rpm for 20 min. The cell suspension was washed three times in PBS and centrifuged for 5 min at 3200 rpm.
   (b) Staining: a volume of 100 µl of cell suspension in PBS was incubated with 10 µl of phycoerythrin-conjugated antibodies for labelling of monocytes; peripheral blood mononuclear cells were costained with fluorescein isothiocyanate–conjugated anti-TLR2.
   (c) Flow cytometric analysis: data were acquired on a FACS caliber flow cytometer (Becton Dickinson Immune Cytometry Systems; Becton Dickinson, San Jose, California, USA). The results were expressed as percentages of monocytes expressing the TLR2 marker [10,11].
(2) Peripheral mononuclear cells expression of nuclear factor-κB (NF-κB) p65 and TLR-4 was detected by using reverse transcriptase–polymerase chain reaction (RT-PCR).

Gene expression analysis of nuclear factor-κB using RT-PCR

Quantitative analysis of NF-κB and TLR4 genes expression in Polymorph Nuclear Cell (PMNC) using quantitative RT-PCR (qRT-PCR). The isolated RNA firstly reverse transcribed using reverse transcriptase enzyme into complementary DNA (cDNA) and then amplified using specific primers by PCR.

Animal part

The rats were divided into two main groups:

(1) Group I (control): 10 normal healthy male rats.
(2) Group II (diabetic): 10 rats with high-fat diet/streptozotocin-induced diabetes.

After the last day of treatment period, rats were fasted overnight for 8 h, weighed and anaesthetized. Blood samples were collected for the determination of fasting blood glucose, fasting serum insulin, insulin resistance was assessed by calculating the homeostatic model assessment of insulin resistance using equation homeostatic model assessment of insulin resistance=fasting insulin (microU/l) × fasting glucose (nmol/l)/22.5, blood urea nitrogen, serum creatinine and CRP by enzyme-linked immunosorbent assay. Then, animals were sacrificed by deep anaesthesia and the two kidneys were dissected out to be used for total RNA isolation and analysis of TLR2, TLR4, and NF-κB gene expression using RT-PCR.

Statistical analysis

All data are presented as mean±SD. All statistical analyses were performed using SPSS statistical software version 18 (SPSS; SPSS Inc., Chicago, Illinois, USA). For human data, a one-way analysis of variance was performed on each variable followed by Tukey post-hoc test for multiple comparisons between groups and for experimental data, *t*-test was used to compare control rats and diabetic rats. The Kolmogorov–Smirnov test was used to study the normal distribution of the studied parameters. Differences were considered significant at P less than 0.05.

Results

Human part

TLR2 in peripheral blood

In the patients young group, theTLR2 mean is 91.6 ±2.3, in the control young group, the TLR2 mean is 21.0±6.3, in the patient old group, the TLR2 mean is 96.1±3.6 and in the control old group, the TLR2 mean is 29.1±12.6.

The patients young group showed significant higher TLR2 levels in comparison to the control old group.

The patients old group showed significant higher TLR2 levels in comparison to the control young and control old groups (Table 1 and Fig. 1).

Gene expression of NF-κB in the PMNC

In the patients young group, the fold-change of NF-κB mean is 1.33±0.54, in the control young group, the fold-change of NF-κB mean is 1±0.12, in the patients old group, the fold-change of NF-κB mean is1.54 ±0.42 and in the control old group, the fold-change of NF-κB mean is 1.00±0.30.

The patients young group showed significant higher fold-change of NF-κB levels in comparison to the control young group.

The patients old group showed significant higher fold-change of NF-κB levels in comparison to the control young and control old groups.

Gene expression of TLR4 in the PMNC

In the patients young group, the fold-change of TLR4 mean is 1.56±0.6, in the control young group, the fold-change of TLR4 mean is 1±0.38, in the patients old group, the fold-change of TLR4 mean is 1.74±0.46 and in the control old group, the fold-change of TLR4 mean is 1.00±0.18.

The patients young group showed significant higher fold-change of TLR4 levels in comparison to the control young group.

The patients old group showed significant higher fold-change of TLR4 levels in comparison to the control young and control old groups (Table 2 and Figs 2, 3).

Animal part

Renal expression of nuclear factor-κB

In the diabetic rats group, the Fold-change of NF-κB mean is 8.29±0.97, in the control group, the Fold-change of NF-κB mean is 1.0±0.14.

The diabetic rats group showed significant higher Fold-change of NF-κB levels in comparison to the control group (Table 3 and Fig. 4).

Table 1 Toll-like receptor 2 in peripheral blood of studied groups

	Control young (*n*=20)	Patients young (*n*=30)	Control old (*n*=20)	Patients old (*n*=30)
TLR2	21.0±6.3	91.6±2.3[a]	29.1±12.6	96.1±3.6[a,b]

Data presented as mean±SD. ANOVA, analysis of variance; TLR2, toll-like receptor 2. [a]Significantly different from control younger than 65 years by ANOVA. [b]Significantly different from control older than 65 years by ANOVA. [c]Significantly different from diabetic patients younger than 65 years by ANOVA.

Renal expression of toll-like receptor 2

In the diabetic rats group, the fold-change of TLR2 mean is 6.71±1.16, in the control group, the fold-change of TLR2 mean is 1.0±0.16.

The diabetic rats group showed significant higher fold-change of TLR2 levels in comparison to the control group (Table 4 and Fig. 5).

Renal expression of toll-like receptor 4

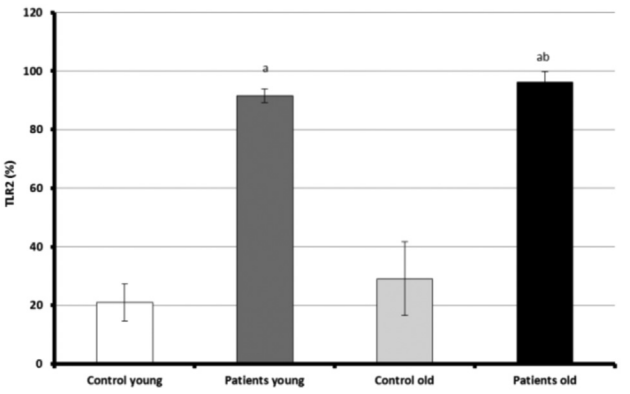
In the diabetic rats group, the fold-change of TLR4 mean is 3.22±0.58, in the control group, the fold-change of TLR4 mean is 1.0±0.21.

The diabetic rats group showed significant higher fold-change of TLR4 levels in comparison to the control group (Table 5 and Fig. 6).

Discussion

DN is traditionally viewed as nonimmune disease; emerging evidence suggests that inflammatory mechanisms play an important role in disease pathogenesis and progression. Indeed, the

Figure 1



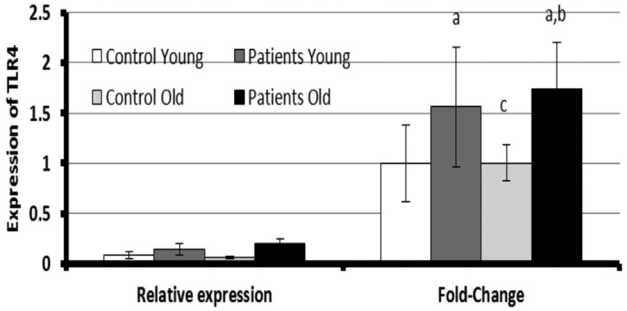
Monocytes level of Toll-like receptor 2 by flow cytometry of the studied groups. Data presented as mean±SD. (a) Significantly different from control younger than 65 years by analysis of variance (ANOVA). (b) Significantly different from control older than 65 years by ANOVA. (c) Significantly different from diabetic patients younger than 65 years by ANOVA.

Table 2 Gene expression of nuclear factor-κB and toll-like receptor 4 in the PMNC of the studied groups

Parameters	Control young (*n*=20)	Patients young (*n*=30)	Control old (*n*=20)	Patients old (*n*=30)
NF-κB				
Relative expression	0.279±0.036	0.374±0.151[a]	0.306±0.085	0.465±0.125[a,b,c]
Fold-change	1.00±0.12	1.33±0.54[a]	1.00±0.30[c]	1.54±0.42[a,b]
TLR4				
Relative expression	0.082±0.036	0.142±0.055[a]	0.061±0.012[c]	0.196±0.053[a,b,c]
Fold-change	1.00±0.38	1.56±0.60[a]	1.00±0.18[c]	1.74±0.46[a,b]

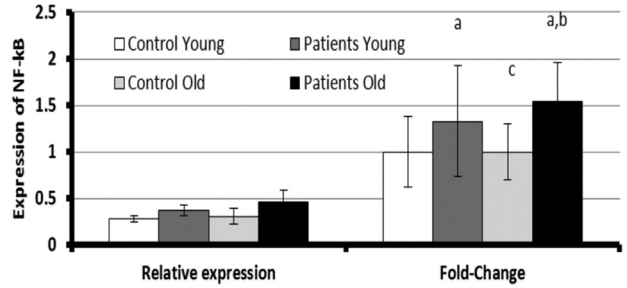
Data presented as mean±SD. ANOVA, analysis of variance; NF-κB, nuclear factor-κB; TLR4, toll-like receptor 4. [a]Significantly different from control younger than 65 years by ANOVA. [b]Significantly different from control older than 65 years by ANOVA. [c]Significantly different from diabetic patients younger than 65 years by ANOVA.

Figure 2



Gene expression of toll-like receptor-4 (TLR4) in the PMNC of the studied groups. Data presented as mean±SD. (a) Significantly different from control younger than 65 years by analysis of variance (ANOVA). (b) Significantly different from control older than 65 years by ANOVA. (c) Significantly different from diabetic patients younger than 65 years by ANOVA.

Figure 3



Gene expression of nuclear factor–κB in the PMNC of the studied groups. Data presented as mean±SD. (a) Significantly different from control younger than 65 years by analysis of variance (ANOVA). (b) Significantly different from control older than 65 years by ANOVA. (c) Significantly different from diabetic patients younger than 65 years by ANOVA.

deterioration of renal function in diabetic patients with proteinuria is positively associated with tubulointerstitial inflammation [8]. Inflammatory response is a recognized factor in the pathogenesis of DN. Numerous experimental and clinical studies have shown the participation of different inflammatory molecules and pathways in the pathophysiology of this complication [8].

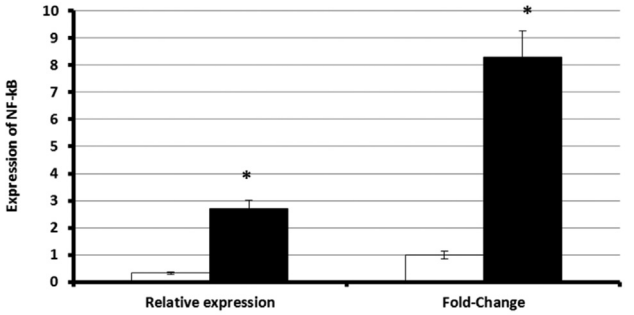
Toll-like receptors (TLRs) recognize molecular patterns relating to a variety of microbial infections. Stimulation through TLRs leads to activation of antigen-presenting cells, production of inflammatory cytokines creating inflammation and production of type 1 interferons (IFNs) that include IFN-α and IFN-β, and exerts direct effects on regulatory cells. These effects can direct the immune response, dealing with the immediate problems of infection and activating more specific responses of the adaptive immune system [12]. The recognition of microbial

Table 3 Renal expression of nuclear factor-κB of control and diabetic rats

	Control rats (n=10)	Diabetic rats (n=10)
Relative expression	0.32±0.05	2.69±0.32*
Fold change	1.0±0.14	8.29±0.97*

Data presented as mean±SD. *$P<0.05$, significantly different from control by t-test.

Figure 4



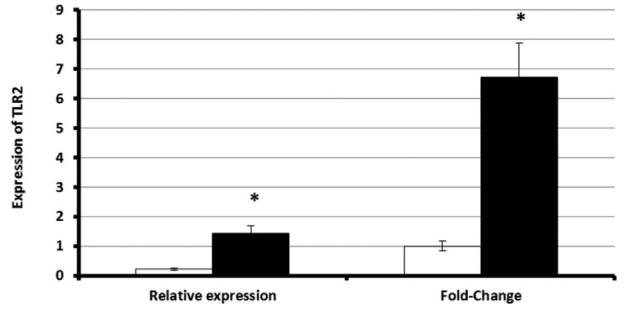
Change in renal nuclear factor-κB gene expression of control and diabetic rats. Data presented as mean±SD. *Significantly different from control by t-test ($P<0.05$).

Table 4 Renal expression of toll-like receptor 2 of control and diabetic rats

	Control rats (n=10)	Diabetic rats (n=10)
Relative expression	0.21±0.03	1.43±0.25*
Fold change	1.0±0.16	6.71±1.16*

Data presented as mean±SD. *$P<0.05$, significantly different from control by t-test.

Figure 5



Change in renal TLR2 gene expression of control and diabetic rats. Data presented as mean±SD. *Significantly different from control by t-test ($P<0.05$).

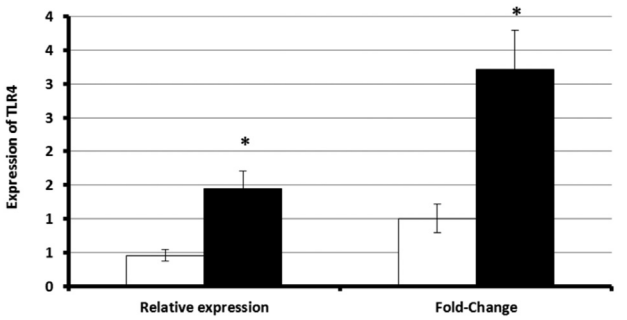
components by mammalian TLRs plays an important role in the activation of the innate immune response and subsequent proinflammatory reactions. TLRs also interact with ligands generated at sites of injury [13]. TLRs present on the cell surface recognize bacterial and fungal components, whereas intracellular TLRs recognize viral or microbial nucleic acids. In addition, TLRs also interact with endogenous

Table 5 Renal expression of toll-like receptor 4 of control and diabetic rats

	Control rats (*n*=10)	Diabetic rats (*n*=10)
Relative expression	0.46±0.09	1.45±0.26*
Fold change	1.0±0.21	3.22±0.58*

Data presented as mean±SD. *$P<0.05$, significantly different from control by *t*-test.

Figure 6



Change in renal TLR4 gene expression of control and diabetic rats. Data presented as mean±SD. *Significantly different from control by *t*-test ($P<0.05$).

ligands, such as oxLDL (oxidized LDL), HSPs (heat-shock proteins) 60 and 70 and fibrinogen and fibronectin, which are also elevated in diabetes mellitus [14]. Thus, different TLRs are amenable to targeting by different types of agents [14]. Among the TLRs, TLR2 and TLR4 might be a molecular link between inflammation and diabetes, in both experimental and clinical conditions [15]. The interactions among increased glucose levels, elevated nonesterified ('free') fatty acids and the resultant proinflammatory cytokines in diabetes have clear implications for the immune system [16].

In the human part of our study: the level of TLRs 2 and 4 and NF-κB was significantly higher in old diabetic patients group than young diabetic patients group and control group.

In the animal part of our study: the level of TLRs 2 and 4 and NF-κB was significantly higher in diabetic rat group than healthy rat control group.

These results agree with Dounousi *et al.* [17], their study concluded that chronic kidney disease (CKD) patients and patients with DN are characterized by increased expression of TLRs 2 and 4, on monocytes, that may contribute to their increased inflammatory state. He divided the participants into: Group 1 included 37 CKD patients, not having diabetes mellitus. Group 2 included 19 CKD patients with DN. Both groups were compared with age-matched controls, (control group). His results showed that patients of group 1 exhibited increased membrane expression only of TLR2 in monocytes compared with the control group ($P<0.02$). Patients of group 2 presented increased membrane expression of both TLR2 and TLR4 compared with the control group ($P<0.003$ and <0.001, respectively) and increased expression of TLR4 compared with group 1 ($P<0.02$).

Our results also agree with Lin *et al.* [18], who showed that the renal cortical TLR4 but not TLR2 was elevated and correlated with infiltrating CD68+ monocytes/macrophages in diabetic nephropathy biopsies. Their study was on kidney biopsy tissues obtained from nine participants with type 2 diabetes and biopsy-proven DN, nine participants with type 2 diabetes lacking nephropathy, nine normal controls and nine participants with no diabetic nephrotic syndrome. Also, our results partially agree with Dasu *et al.* [19], who concluded that TLR2 and TLR4 expression, their ligands, signalling, and functional activation are increased in recently diagnosed T2DM and contribute to the pro-inflammatory state. In their study, they measured the freshly isolated monocytes from healthy human controls (*n*=23) and T2DM patients (*n*=23) using real-time RT PCR, Western blot and flow cytometric assays. Their results showed that T2DM patients had significantly increased TLR2, TLR4 mRNA and protein in monocytes compared to controls ($P<0.05$). Their results agree with our result.

In a study held by Xi *et al.* [20], they investigated the expression levels of the endogenous ligands of TLRs and TLR signalling pathway molecules in the process of rat kidney ageing. The results they documented, the expression levels of the endogenous TLR ligands heat shock protein 70 (HSP70) and high-mobility group box-1 (HMGB1) were significantly increased, as were those of TLR1, 2, 3, 4, 5 and 11, they examined the expression and activation of the TLR signalling pathway downstream molecules myeloid differentiation factor 88(MyD88) and interferon regulatory protein 3 and found that MyD88 and interferon regulatory protein 3 levels were significantly elevated in the ageing kidneys. Recent studies have shown that immunological inflammation may be associated with ageing and ageing-related diseases. Serum levels of inflammatory cytokines, such as TNF-α, IL-6 and CRP, are significantly increased in elderly populations. However, the mechanisms by which inflammation is involved in tissue and organ ageing are not very clear. There two theories have been proposed, that is, oxidative stress theory and cytokine theory. Accumulated data suggest that during the ageing process of an organism,

excessive oxidative stress may activate many pro-inflammatory signalling pathways, including the NF-κB signalling pathway, and induce continuous (chronic) upregulation of pro-inflammatory mediators, leading to 'inflamm-aging' of tissues and organs [21–23].

The present study showed that the TLR system was closely related to the ageing of the kidneys, which provides a new direction for the further investigation of the renal ageing mechanism.

Conclusion

TLRs 2 and 4 and NF-κB were higher in old diabetic patients compared with young diabetic patients and normal individuals. These observations significantly add to the emerging role of TLRs in T2DM development and its possible role in the pathogenesis and progression of DN.

Also, the present study showed that the TLR system was closely related to the ageing of the kidneys.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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