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Study of some autophagy markers in type 2 diabetic patients with and without diabetic nephropathy

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Abstract
Background: One of the most prevalent and serious side effects of diabetes mellitus (DM) is diabetic nephropathy (DN), which is linked to higher mortality and morbidity rates in diabetic individuals. Autophagy is essential for the survival of podocytes. This work was designed to assess autophagy in individuals with type 2 diabetes mellitus (T2DM) with and without diabetic nephropathy (DN).
Methods: This case-control work was performed on 50 patients with T2DM. They were divided according to urinary albumin creatinine ratio (UACR) into the following subgroups; Group IA: 25 individuals without DN and Group IB: 25 individuals with DN. Every individual who participated had a medical taking of history and laboratory tests including routine investigations of Fasting blood sugar (FBS), HbA1c, Urinary albumin creatinine ratio (UACR), Estimated Glomerular Filtration Rate (eGFR) and C-reactive protein (CRP) and specific Detection of LAMP2 relative gene expressions in peripheral leucocytes by quantitative reverse transcription-real time PCR (qRT-qPCR).
Results: LAMP2 mRNA expression levels were substantially decreased in diabetic individuals with nephropathy than in those without.
Conclusions: These data suggest that there was inhibition of the autophagy pathway in diabetic individuals with DN than in those without and that impaired autophagy may be involved in the progression to DN.

Keywords: Autophagy, type 2 diabetes, diabetic nephropathy, LAMP2

Introduction
One of the greatest epidemics to ever affect the world is type 2 diabetes mellitus (T2DM). According to the International Diabetes Federation's (IDF) most current estimates, 449 million individuals worldwide have type 2 diabetes in 2017 by 2045, this number is predicted to increase to 702 million [1].
One of the most common and severe consequences of diabetes mellitus (DM) is diabetic nephropathy (DN), DN is linked to greater mortality and morbidity in those with diabetes. Developing innovative approaches for treating DN requires a better knowledge of its pathogenic processes [2].
In order to support homeostasis, autophagy is a crucial cellular process that removes degraded or old molecules and cellular components, such proteins, nucleic acids, lipids, and organelles [3] Autophagosome development involves five main stages: initiation, elongation, closure, fusion, and breakdown. Over 30 autophagy-related genes are expressed during these processes [4].
The majority of cellular stress response pathways interact with the autophagy mechanism, which may have an impact on disease etiology. The etiology of kidney damage caused by diabetes has been linked to an autophagy deficiency in tissues of the kidney [5].
Diabetes may severely reduce autophagy's ability to protect kidney cells, especially the proximal tubular cells and podocytes [6]. In the late stage of the autophagic process, the lysosomal membrane protein LAMP2 is necessary for the correct fusing of lysosomes with autophagosome [7]. Current therapies for DN are not satisfactory. Thus, the identification of new therapeutic targets is a top priority for controlling and preventing of DN. Recent research has focused on autophagy as it has an impact on the stress-responsive machinery which is involved in the pathophysiology of diabetes and diabetes-related kidney damage [8].

Subjects and Methods

Study population

The present work was performed in the Tanta University Hospital's clinical pathology and internal medicine departments on 50 subjects. They were classified as follows: Group IA: 25 individuals without DN (UACR < 30 mg albumin/ gram creatinine) and Group IB: 25 individuals who have DN (UACR ≥ 30 mg albumin/ gram creatinine). subjects with coronary heart diseases, thyroid disorders, and malignant diseases were excluded from this study. After a thorough explanation of the research's advantages and hazards, the Ethical Committee of Tanta University's Faculty of Medicine authorized the trial, and each participant provided their informed permission.

Biochemical measurements

A morning urine sample was collected from each subject for estimating UACR. The urine samples were centrifuged at 3000 rpm for 15 minutes before analysis. Six milliliters of peripheral Blood was drawn from each patient in a fully sterile precaution; 4 mL were delivered into two EDTA vacutainer tubes (2 mL in each tube) for haemoglobin A1C (HbA1c) estimation and molecular investigations. The remaining was delivered into sterile vacutainers with a clot activator, centrifuged at 1000 x g for a fifteen-minute period after being allowed to clot for thirty minutes. The serum was then separated and used immediately for estimating fasting blood sugar (FBS), serum creatinine and C- reactive protein (CRP) levels.

The FBS level, The CRP level, and the serum creatinine level was estimated by automated chemistry analyzer Konelab60 (Thermo Scientific, Finland). The urine microalbumin was measured by immunoturbidimetri method on semi-automated chemistry analyzer BTS 350 (BioSystems, Spain). High-performance liquid chromatography (HPLC) was used to test the HbA1c on an HPLC G8 auto analyzer (TOSOH, Japan). UACR was calculated according to the following formula: microalbumin level in urine (mg/dl) / creatinine level in urine (g/dl). eGFR was calculated as follows: eGFR (mL/min/1.73 m2) = 175 x Serum Cr-1.154 x age-0.203 x 1.212 (if patient is black) x 0.742 (if female).

Total RNA extraction, reverse transcription, and real-time PCR

Using TriRNA Pure Kits, total RNA has been extracted from peripheral leucocytes, Catalog No. TRP100, (Geneaid, Taiwan). The extracted RNA was subsequently converted into complementary DNA (cDNA) utilizing TOP script ™ cDNA Synthesis kit, catalog No. EZ005M, (Enzynomics, Korea) on thermo-mixer T-Shaker (EuroClone, Italy). The cDNA was used for quantitative estimation of LAMP2 relative gene expression using TOPreal™ qPCR 2X PreMIX (SYBR Green) PCR Panels, catalogue No: P750, (Enzynomics, Korea) on Quanti Studio 5 Real-Time PCR System (Thermo fisher Scientific, China).

The following primers were used in the reaction: LAMP2 forward 5′-CGTTCTGGTCTGCCTAGTCC-3′ and reverse 5′-CAGTGCCATGGTCTGAAATG-3′, and β-actin forward 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′.

The cycle threshold for target and reference genes was determined using the software supplied with QuantiStudio 5. Utilizing the formula 2-ΔΔCT, the target gene expression was comparatively measured and normalized to the reference gene.

Statistical analysis

SPSS software (Statistical Package for the Social Sciences, version 19, SPSS Inc. Chicago, IL, USA) was used to conduct the statistical evaluation. The range, mean, and standard deviation were computed for quantitative parameters. Analysis of the ROC curve was also carried out. Statistics were judged significant at $p<0.05$.

Results

Laboratory findings

As shown in Table 1, Significantly increased UACR concentrations were discovered in diabetic individuals with nephropathy (Group IB) than in diabetic patients without nephropathy (Group IA) ($p<0.001$), while eGFR was substantially decreased ($p<0.001$), with no substantial variation among the two groups in FBS and CRP levels as well as HbA1c (Table 1).

Table 1: Comparison of the laboratory findings between the studied groups (Group I vs. Group II).

		Group I	Group IB	P
FBS (mg/dL)	Median (IQR)	169.0 (32.0)	175.0 (81.0)	0.684
CRP (mg/L)	Median (IQR)	3.98±0.99	3.98±0.89	0.988
HbA1c (%)	Median (IQR)	8.38±1.39	8.51±1.33	0.741
UACR (mg albumin/g creatinine)	Median (IQR)	16.80 (4.30)	472.0 (638.5)	< 0.001*
eGFR (mL/min/1.73m^2)	Median (IQR)	88.60 (11.50)	33.40 (24.40)	< 0.001*

CRP, C-reactive protein; FBS, fasting blood sugar; UACR, urinary albumin creatinine ratio; HbA1c, hemoglobin A1c; eGFR, estimated glomerular filtration rate; IQR, interquartile range

LAMP2 expression in T2DM patients with and without DN: As shown in Figure 1, the relative LAMP2 expression was substantially decreased in diabetic individuals with nephropathy (Group IB) than in those without nephropathy (Group IA) ($p<0.001$).

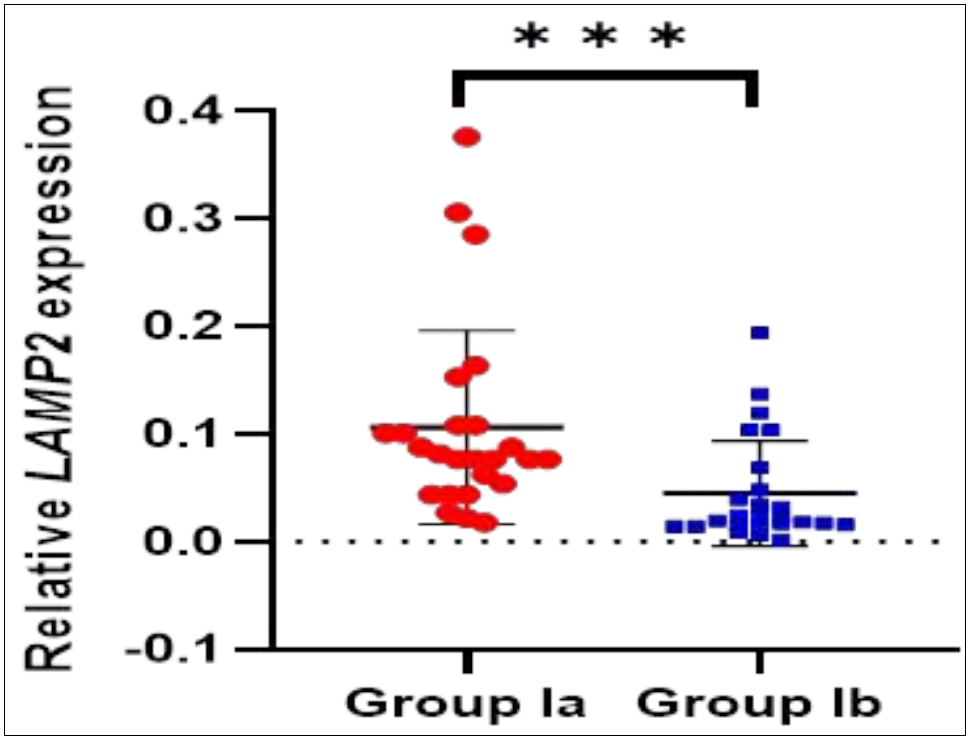

Fig 1: Comparison of relative *LAMP2* expression among the studied groups. ***, $p<0.05$; LAMP2, lysosomal associated protein 2

LAMP2 and prediction of diabetic nephropathy

As demonstrated in Table 2, the Univariate logistic regression analysis showed that relative *LAMP2* expression levels were significantly associated with DN progression in patients with DM ($p=0.015$) and they remained significantly related in the multivariate analysis ($p=0.003$).

Table 2: Univariate and multivariate logistic regression analysis of LAMP2 for DN prediction in patients with DM

	Univariate		Multivariate	
	OR (95% CI)	**P**	**OR (95% CI)**	**P**
LAMP2	0.001 (0.001 – 0.032)	0.015*	0.001 (0.001 – 0.002)	0.003*

Using the ROC curve analysis, the AUC of relative *LAMP2* expression for differentiating patients with DN from those without was 0.790, indicating that relative *LAMP2* expression had a diagnostic efficacy for DN in patients with DM (Table 3 and Fig. 2).

Table 3: Performance characteristics of LAMP2 in differentiating patients with DN (Group IB) from those without (Group IA).

	Cut off	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy
LAMP2	< 0.04	0.790	72	88	70	74	72

AUC, area under curve; PPV, positive predictive value; NPV, negative predictive value

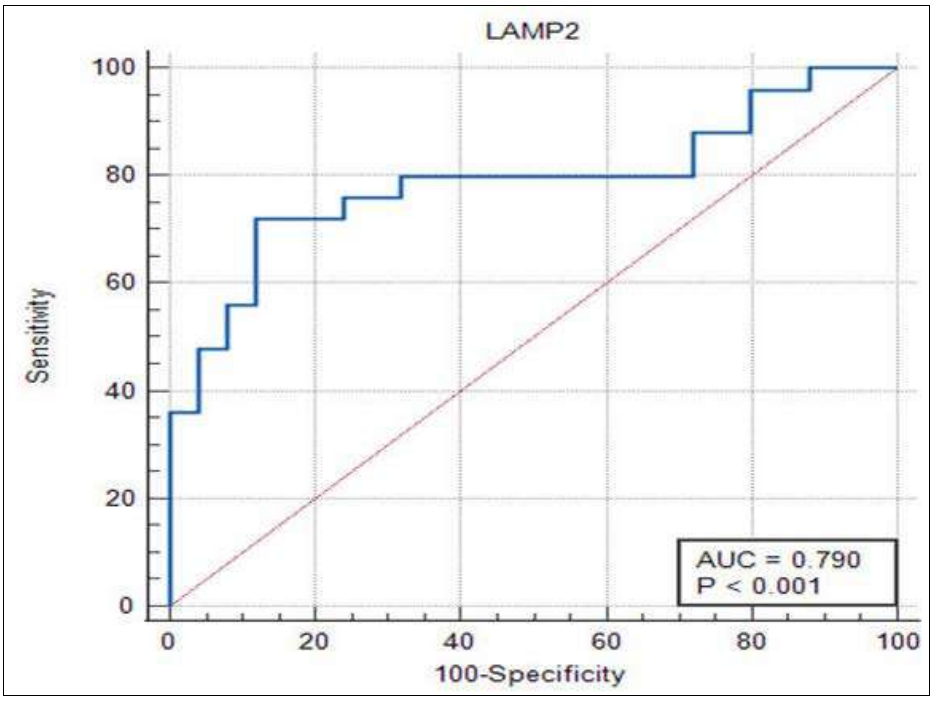

Fig 2: ROC curve analysis of LAMP2 in the patients with DN (Group Ib) and those without (Group Ia).

Discussion

A metabolic condition caused by abnormalities in insulin secretion is diabetes mellitus (DM), action, or both [9]. T2D and its associated complications contribute to 11.3% of deaths worldwide, placing a significant burden on healthcare facilities [10]. DN is a significant DM consequence that leads to chronic kidney damage [11]. According to IDF, 40% of diabetics may get end-stage renal failure [12].

Autophagy is the process by which cells break down their own long-lived proteins and damaged organelles, such as mitochondria [12]. Autophagy has been linked to the evolution of T2D via compromised pancreatic beta-cell activity and the emergence of insulin resistance. Numerous studies indicate that elevated autophagy functions as a defense mechanism in pancreatic beta cells against oxidative stress [13].

Podocytes, the cells form the kidney filtration barrier, have a relatively limited capacity for cell proliferation and replacement, making the maintenance of homeostasis dependent on systems of self-repair like autophagy. Evidence indicated that basal autophagy is very active in podocytes, promoting cell survival and its impairment is implicated in the pathogenesis of DN [13].

In this case control work, we aimed to assess LAMP2 as autophagy marker in T2DM individuals with and without DN in order to study the function of autophagy in the the progression of DM to DN. This research included 50 individuals with T2DM who were classified into two categories based on the UACR: diabetics without nephropathy (Group IA) and diabetics with nephropathy (Group IB).

In the present study, as expected, UACR levels were discovered to be substantially greater in diabetic individuals with DN (Group IB) than in diabetic patients without DN (Group IA). eGFR was substantially decreased in diabetic individuals with DN (Group IB) than in diabetic patients without DN (Group IA).

In this study, the autophagy pathway was investigated by measuring the expression levels of *LAMP2* in diabetic cases with and without DN. The study's findings showed that mRNA relative expression of *LAMP2*, which is involved in later phases of autophagy, was substantially lower in the individuals with DN (Group IB) than in those without (Group IA).

Our findings were in line with Kaburagi *et al.* [13], who noticed that individuals with DN had lower urine LAMP2 levels compared to those with T2DM implying that further functional investigations should be conducted to determine if the reduced urine LAMP2 level in DN individuals reflects the disease's altered autophagic activity. In addition, Guo *et al.* [13] revealed that urinary *LAMP2* levels significantly decreased in individuals with DN.

LAMP2 is part of the lysosomal membrane and has been demonstrated to be crucial in the activity of chaperone-mediated autophagy (CMA) and autophagosome maturation [13].

Given the essential role of *LAMP2* in autophagy and the function that autophagy plays in the development of DN, it is unsurprising that reduced expression of *LAMP2* mRNA was observed in patients with DN, which suggests changes in late steps of the autophagic machinery [13]. In this study, the ROC curve analysis for the prediction of DN showed that the AUC of relative *LAMP2* gene expression was significant. Which suggest that LAMP2 has a high diagnostic accuracy for DN.

It is evident that DN pathogenesis involves defective autophagy, which implies that autophagy activation could be a potential therapy for prevention and treatment of DN [13].

Conclusions

The present study revealed decreased levels of LAMP2, which is involved in later phases of autophagy suggesting that there was inhibition of the autophagy pathway in diabetic individuals with DN contrasted with individuals without and that the pathophysiology of DN may be influenced by defective autophagy. In addition, *LAMP2* mRNA expression could predict the development of DN in patients with DM and may be used as early markers for the diagnosis of DN.

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Conflict of Interest: Nil

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