The relationship between serum apelin level and different grades of diabetic nephropathy in type 2 diabetic patients

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Background
Diabetic nephropathy (DN) is the leading cause of renal failure. Diabetic patients with microalbuminuria typically progress to proteinuria and overt DN. Similar to other microvascular complications of diabetes, there are strong associations between glucose control (as measured using HbA1c) and the risk of developing DN. Apelin (APLN), a peptide first isolated from bovine stomach tissue extracts, is the endogenous ligand for the G-protein-coupled APJ receptor that is expressed at the surface of some cell types. APLN and APJ are widely expressed in homogenates from animal organs in a pattern shared with angiotensinogen and the angiotensin receptor. APLN is widely distributed in the central nervous system and periphery, especially in the heart, kidney, lung, and mammary glands. The APLN–APJ system may be involved in the pathogenesis of DN, which may play a renoprotective role partially by antagonizing the angiotensin II–ATIR pathway.

Aim
The aim of this study was to investigate the relation between serum APLN and different grades of DN in type 2 diabetic patients.

Patients and methods
This study was conducted on 150 diabetic patients and 20 controls selected from the inpatient department and outpatient clinics of the Internal Medicine Department in Menoufia University Hospital. The selected participants were divided into four groups: group 1 included 20 healthy controls; group 2 included 50 type 2 diabetes mellitus (T2DM) patients with normoalbuminuria; group 3 included 50 T2DM patients with microalbuminuria; and group 4 included 50 T2DM patients with macroalbuminuria. Members of the study groups were subjected to thorough history taking with special emphasis on age, sex, and duration of diabetes mellitus. Investigations included liver profile, complete blood count, fasting and 2 h postprandial plasma glucose, glycosylated hemoglobin (HbA1c), complete urine analysis, kidney function tests (blood urea nitrogen and serum creatinine), urine albumin/creatinine ratio, estimated glomerular filtration rate, and serum APLN.

Results
Serum APLN was significantly higher in group 4 compared with the other groups, in group 3 compared with groups 1 and 2, and in group 2 compared with group 1. There was a significant positive correlation between serum APLN and serum creatinine, urine albumin/creatinine ratio, and HbA1c. Further, there was a significant negative correlation between serum APLN and estimated glomerular filtration rate in the studied diabetic patients. There was no correlation between serum APLN and BMI in diabetic patients.

Conclusion
From this study, we can conclude that serum APLN is significantly higher in patients with DN compared with diabetic patients without nephropathy, and there is a positive correlation between serum apelin and the degree of DN. Thus, APLN may play an important role in the development of DN.

Keywords:
apelin, diabetes mellitus, nephropathy

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Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease, which may require hemodialysis or even kidney transplantation. It is associated with an increased risk for death in general, particularly from cardiovascular disease. Albuminuria is a marker of greatly increased cardiovascular morbidity and mortality for patients with either type 1 or type 2 diabetes [1].

DN is the leading cause of renal failure in the USA. Without intervention, diabetic patients with microalbuminuria typically progress to proteinuria

and overt DN. This progression occurs in both type 1 and type 2 diabetes [2].

Pathological changes to the kidney include increased glomerular basement membrane thickness, microaneurysm formation, mesangial nodule formation (Kimmelstiel–Wilson bodies), and other changes [3].

Screening for DN or microalbuminuria may be accomplished with either a 24 h urine collection or a spot urine measurement of microalbumin. Measurement of the microalbumin-to-creatinine ratio may help account for concentration or dilution of urine, and spot measurements are more convenient for patients compared with 24 h urine collections [4].

Apelin (APLN) is a peptide, which in humans is encoded by the *APLN* gene [5]. APLN is the endogenous ligand for the G-protein-coupled APJ receptor that is expressed at the surface of some cell types. It is widely expressed in various organs such as the heart, kidney, liver, adipose tissue, gastrointestinal tract, brain, adrenal glands, endothelium, and human plasma [6,7].

The apelin receptor (APLNR) mRNA is highly expressed in the glomeruli, whereas its level of expression is lower in all nephron segments, including collecting ducts, that express vasopressin V2 receptors. APLNR mRNA was also found in endothelial and vascular smooth muscle cells of glomerular arterioles [8]. It appears that APLN had a direct receptor-mediated vasoconstrictive effect on vascular smooth muscle. These results show that APLN has complex data on the preglomerular and postglomerular microvasculatures regulating renal hemodynamics. Its role on tubular function (if any) remains to be determined [9].

Aim

The aim of this study was to investigate the relation between serum APLN and different grades of DN in type 2 diabetic patients.

Patients and methods

This study was conducted on 150 diabetic patients and 20 healthy controls selected from the inpatient department and outpatient clinics of the Internal Medicine Department in Menoufia University Hospital. The protocol of the study was approved by the ethical committee of Faculty of Medicine, Menoufia University. The selected participants gave consent for participation in the study before they were exposed to examination and investigations. The study was conducted from November 2014 to November 2015.

Diabetic patients were divided into normoalbuminuric patients, low-grade albuminuria (microalbuminuric) patients, and high-grade albuminuria (macroalbuminuric) patients.

The selected participants were divided into four groups:

(1) Group 1, which included 20 healthy controls.
(2) Group 2, which included 50 type 2 diabetes mellitus (T2DM) patients with normoalbuminuria.
(3) Group 3, which included 50 T2DM patients with microalbuminuria.
(4) Group 4, which included 50 T2DM patients with macroalbuminuria.

Exclusion criteria

Exclusion criteria included cerebrovascular diseases, metabolic diseases, inflammatory diseases, advanced liver disease, heart failure, pregnancy, and steroid usage.

Members of the study groups were subjected to thorough history with special emphasis on age, sex, duration of diabetes mellitus (DM), presence or absence of specific diabetic complications, and treatment modalities. Complete physical examination was carried out for all members, including weight, height, and signs of diabetic complications. Investigations included the following: liver profile, complete blood count, fasting and 2 h postprandial plasma glucose, HbA1c, complete urine analysis, kidney function tests (blood urea nitrogen and serum creatinine), urine albumin/creatinine ratio, and estimated glomerular filtration rate (eGFR).

GFR was estimated using Cockroft–Gault equation (ml/min):=[140−age (years)]×weight (kg)/[72×serum creatinine (mg/dl)]×0.85 (if a woman). The reference range of GFR values in young individuals is from 90 to 130 ml/min/1.73 m^2 [10].

Microalbuminuria (low-grade albuminuria) was considered if urinary albumin levels were between 30 and 300 μg per mg creatinine. Significant proteinuria (macroalbuminuria) was present if urinary albumin levels were greater than 300 μg per mg creatinine.

Serum APLN measure: The RayBio Apelin C-Terminus Enzyme Immunoassay Kit (RayBio Inc., Brea, California, USA) is designed to target the C-terminus of the 77-aa apelin peptide; thus, the active forms of APLN, including apelin-36 and apelin-13, can be detected. Blood samples obtained from the

patients after an overnight fasting were collected in plain test tubes without an anticoagulant, and these samples were immediately chilled in ice boxes. Thereafter, serum samples were separated by means of centrifugation and stored in a deep freeze at −20°C until they were analyzed collectively.

Statistical methodology

Data were analyzed using statistical package for the social science software computer program version 15 (SPSS; SPSS Inc., Chicago, Illinois, USA). Quantitative data were presented as mean and SD. Qualitative data were presented as frequency and percentage. To compare between groups we used the χ^2-test, analysis of variance test, and least significant difference. *P* value of 0.05 or less was considered significant.

Results

There was no significant difference between the studied four groups as regards age and sex. BMI was significantly higher in diabetic groups compared with the control group.

The duration of DM was significantly higher in group 4 compared with groups 2 and 3.

HbA1c was significantly higher in group 4 compared with the other diabetic groups.

Serum creatinine (mg/dl) was significantly higher in group 4 compared with the other groups and in group 3 compared with groups 1 and 2. Urine albumin/creatinine ratio was significantly higher in group 4 compared with the other groups and in group 3 compared with groups 1 and 2. GFR ml/min/1.73 m^2 was significantly lower in group 4 compared with the other groups and in group 3 compared with groups 1 and 2 (Table 1).

Serum APLN was significantly higher in group 4 compared with the other groups, in group 3 compared with groups 1 and 2, and in group 2 compared with group 1 (Table 2).

There was a significant positive correlation between serum APLN and serum creatinine, urine albumin/creatinine ratio, and HbA1c. Further, there was a significant negative correlation between serum APLN and eGFR in the studied diabetic patients. There was no correlation between serum APLN and BMI in diabetic patients (Table 3).

Discussion

DM is the leading cause of end-stage renal disease. Nephropathy is a major complication of type 2 diabetes [11]. In early DN, there are increases in the GFR and albuminuria. These pathological indices are, in part, the consequence of glomerular capillary damage [5]. Previous studies have demonstrated that increased glomerular filtration surface area in DN is associated with the formation of new glomerular capillaries and a slight elongation of pre-existing capillaries [12].

The glomerular filtration barrier includes endothelial cells, podocytes, and the basement membrane. The highly specialized glomerular endothelium contributes to the selective glomerular barrier [13].

Table 1 Comparison between the studied groups as regards serum creatinine, urinary albumin creatinine ratio, and glomerular filtration rate

	Group 1 (*n*=20) (mean±SD)	Group 2 (*n*=50) (mean±SD)	Group 3 (*n*=50) (mean±SD)	Group 4 (*n*=50) (mean±SD)	*F*-test	*P* value	LSD
Serum creatinine (mg/dl)	0.77±0.11	1.05±0.17	1.3±0.13	2.6±0.75	93.3	<0.001**	1 vs. 3, 42 vs. 3, 43 vs. 4
Urinary albumin/creatinine ratio (μg/mg creatinine)	–	19.1±3.7	210.8±36.06	1207.7±267.9	98.2	<0.001**	2 vs. 3, 43 vs. 4
Glomerular filtration rate (ml/min/1.73 m^2)	113.8±4.04	97.7±7.8	78.4±5.7	43.4±6.2	396.2	<0.001**	1 vs. 3, 42 vs. 3, 43 vs. 4

LSD, least significant difference. **Highly significant.

Table 2 Comparison between the studied groups as regards serum apelin

	Group 1 (*n*=20)	Group 2 (*n*=50)	Group 3 (*n*=50)	Group 4 (*n*=50)	*F*-test	*P* value	LSD
Serum apelin (mean±SD) (ng/ml)	0.61±0.13	3.7±0.9	4.8±1.9	5.8±2.3	28.3	<0.001**	1 vs. 2, 3, 42 vs. 3, 43 vs. 4

LSD, least significant difference. **Highly significant.

Table 3 Correlation between serum apelin and other parameters in the diabetic patients

	Serum apelin	
	R	*P* value
Serum creatinine	0.68	<0.05*
Urine albumin/creatinine ratio	0.81	<0.05*
eGFR	–0.71	<0.05*
HbA1c	0.77	<0.05*
BMI	0.15	>0.05

eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin. *Significant.

APLN is an adipokine secreted by the adipose tissue and by the endothelial cells in various parts of the body. APLN is expressed in two places in the kidney: the glomerular capillary cells and glomerular arteriolar rectus [14]. In-vitro studies have revealed that APLN and the APLNR can induce the sprouting of endothelial cells in an autocrine or paracrine manner, thus suggesting a role for APLN in angiogenesis [8,15–17]. Therefore, APLN may play an important role in DN.

The aim of the work was to study the relation between serum APLN and different grades of DN in type 2 diabetic patients.

The present study reported that the serum APLN was significantly higher in diabetic patients compared with nondiabetic individuals. In diabetic patients, serum APLN had a significant direct correlation with HbA1c.

This is similar to the findings of Billir *et al.* [18], who reported that APLN levels of DM patients with neuropathy and DM patients without neuropathy were found to be higher than that in healthy individuals. Similar results were obtained from other studies [19–22].

However, Yavuz *et al.* [14] reported that there were no differences between the diabetic patients and the nondiabetic ones in terms of their APLN levels.

APLN has been linked to states of insulin resistance (IR). In many clinical studies, compared with normal controls, plasma APLN concentration is increased in IR individuals [9], and in morbidly obese individuals with T2DM [23,24]. When considered as a continuous variable, APLN also has been found to correlate positively with HbA1c [25]. Notably, some have shown that plasma APLN levels are paradoxically decreased in newly diagnosed patients with T2DM [26,27]. Although it is difficult to reconcile these divergent findings, these data do suggest the possibility of alternative regulatory pathways for APLN production in the setting of IR.

At present, few genomic studies assessing for variants in the *APLN* and *AGTRL1* genes have been published. One study reported that, in male diabetic patients who carry the C allele of a SNP, rs2235306, in the *APLN* gene, there was a significantly higher fasting glucose in blood compared with those carrying the T allele. However, this finding was not found in female diabetic patients, and other measures of insulin sensitivity (e.g. 2 h oral glucose tolerance test, fasting insulin, homeostatic model assessment-IR) showed no correlation, raising the possibility of a spurious result [28].

In the last years, there has been a growing appreciation of APLN's involvement in the pathogenesis of IR. APLN secretion is regulated by insulin, and clinical studies have demonstrated elevated plasma APLN concentrations in individuals with IR. Moreover, direct administration of APLN has been shown to increase insulin sensitivity, peripheral glucose uptake, and adiponectin levels, as well as decrease adiposity, hyperinsulinemia, and free fatty acid levels. In a human study evaluating APLN's effects on insulin sensitivity, the available evidence nevertheless suggests that APLN ameliorates IR, positioning APLN/APJ signaling as a possible pharmaceutical target for the treatment of T2DM and the metabolic syndrome. Despite this early promise, however, unresolved issues remain as regards APLN and its association with insulin sensitivity. The intracellular mechanisms governing APLN-induced glucose uptake and its relationship with the classic insulin signaling cascade have yet to be fully characterized. Moreover, APLN's regulation of fatty acid homeostasis, as well as its significance relative to insulin sensitivity, needs to be further clarified. Nevertheless, targeting the APLN/APJ signaling may represent a potentially novel avenue in designing therapies for IR. These data indicate that APLN directly increases insulin sensitivity and suggest that the elevations in circulating APLN observed in states of IR are compensatory [29].

In the present study, there was no correlation between serum APLN and BMI. It has been clearly demonstrated by several studies that the increased APLN levels in type 2 diabetic patients seemed to be independent of obesity. Studies evaluating the link between APLN levels and obesity have reported conflicting results [23,24,29]. Soriguer *et al.* [23] showed that APLN levels in morbidly obese patients were significantly higher than that in controls only

when the obese patients were diabetic. In nondiabetic controls, they found a positive correlation between APLN levels and BMI. They showed that APLN concentrations were higher in diabetic patients independent of increased BMI. These data suggest that obesity is not the main determinant of plasma APLN levels in type 2 diabetic patients; this is in agreement with previous studies [23,30].

In the present study, serum APLN was significantly higher in diabetic patients with nephropathy (either those with low-grade albuminuria or high-grade albuminuria) compared with diabetic patients without nephropathy. Moreover, it was significantly higher in patients with high-grade albuminuria compared with those with low-grade albuminuria. Further, there was a significant direct correlation between serum APLN and albumin/creatinine ration in urine.

In a study comprising 60 patients with DN, the serum APLN 13 level was found to be higher in diabetic patients, and a positive correlation was documented between the APLN 13 level and albuminuria [19]. Similar results was obtained from the study by Billir *et al*. [18].

It is generally accepted that endothelial dysfunction is important for diabetic microvascular disease [31]. The growth of new blood vessels and increased permeability of microvessels in nephrons are believed to be the main pathogenesis of DN [32]. In the study by Zhang *et al*. [19], the observed increase in glomerular permeability with APLN may, therefore, be relevant during the early stages of DN, thereby supporting the concept that endothelial dysfunction is causally linked to DN.

Abnormal vessels are associated with increased glomerular hypertrophy and enhanced frequency of glomerular occlusion, fibrinoid lesions, tubulointerstitial injury, and urinary albumin excretion [33–35]. Blocking angiogenesis attenuates glomerular basement membrane thickening and mesangial expansion [36,37], thereby indicating that the increase in abnormal vessels contributes to the development of early features of DN.

If APLN induces glomerular capillary sprouting to form structurally immature vessels, the proliferation of glomerular endothelial cells will be the first step. Therefore, the proliferating effects of APLN on glomerular endothelial cells were measured in one study. This study revealed that APLN induced the proliferation of glomerular endothelial cells in a dose-dependent manner. These findings clearly suggested that APLN may be a crucial factor for pathological glomerular angiogenesis [19].

APLN performs angiogenic functions through endocrine or paracrine pathway. Glomerular endothelial cells have to migrate to the sites of APLN secretion to form new capillaries. The study of the chemotactic and migratory effects of APLN on glomerular endothelial cells showed that APLN accelerated wound healing in glomerular endothelial cells and increased the number of migrated cells as measured by chemotaxis. These results confirmed that APLN, as an endocrine or paracrine peptide, facilitates abnormal vessel formation in diabetic glomeruli, which helps DN progress [38].

Glomerular hyperperfusion and hyperfiltration usually occur in the early stages of DN. Afferent arterioles appear to be more dilated compared with efferent arterioles. These early hemodynamic changes alleviate albumin leakage from glomerular capillaries, overproduction of the mesangial cell matrix, thickening of the glomerular basement membrane, and injury to podocytes. Several factors, such as angiotensin II, nitric oxide, prostanoids, vascular endothelial growth factor (VEGF), and transforming growth factor-β1, have been reported to affect the irregular autoregulation in DN [38]. Japp *et al.* [39] reported that APLN causes nitric oxide-dependent arterial dilation *in vivo* in humans.

In the study by Zhang *et al*. [19], they detected an upregulation of VEGFR2 and Tie2 by APLN in glomerular endothelial cells. VEGFR2 can promote proliferation and chemotaxis, and it can induce the permeability of endothelial cells by binding to VEGF. In addition, Thurston [40] and Ward and Dumont [41] showed that Tie2 inhibits vascular permeability and tightens pre-existing vessels, and it plays a critical role in the angiogenesis of endothelial cells by binding to angiopoietin (Ang). These results suggested that APLN contributes to the glomerular hyperperfusion and hyperfiltration that often occur in the early stages of DN. The results of the present study support a role for APLN in regulating DN by modulating the permeability and proliferation of glomerular endothelial cells. APLN-mediated angiogenesis and increased permeability in diabetic glomeruli identified a crucial role of APLN in the pathogenesis of DN.

Conclusion

From this study, we can conclude that serum APLN is significantly higher in patients with DN compared with diabetic patients without nephropathy, and there is a positive correlation between serum APLN

and the degree of DN. Thus, APLN may play an important role in the development of DN, and further studies are needed to study whether the inhibition of the apelinergic system could offer new therapeutic opportunities against DN.

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Conflicts of interest
There are no conflicts of interest.

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